DEVELOPMENT OF TOOLS TO ASSESS THE ROLE OF ARYLAMIDE DEACETYLASE IN ARYLAMINE BIOTRANSFORMATION AND TOXICITY

By

Julie Green

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Pharmacology
University of Toronto

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ABSTRACT

DEVELOPMENT OF TOOLS TO ASSESS THE ROLE OF ARYLMIDE DEACETYLASE IN ARYLAMINE BIOTRANSFORMATION AND TOXICITY

Julie Green
Degree of Master of Science 2000
Graduate Department of Pharmacology
University of Toronto

The goal of the present work is to develop tools to aid in the elucidation of the role of DAC in the pharmacological, toxicological, and carcinogenic effects observed following exposure to arylamines. We devised a sensitive HPLC assay to monitor deacetylation activity, and raised a high-titre polyclonal anti-human DAC antiserum that enables the detection and quantification of immunoreactive protein with high sensitivity and specificity. Heterologous expression of DAC was also attempted with both bacterial and mammalian expression systems. Difficulties were encountered in creating the expression systems, and functional protein could not be obtained. One possibility for this difficulty in heterologous expression is that functional DAC protein has a deleterious effect on the expression host cells. Finally, DNA sequencing of these clones revealed that the published DNA sequence of DAC is incorrect.
ACKNOWLEDGEMENTS

My sincerest thanks to my supervisor, Dr. Denis Grant for his valuable scientific input, guidance, and support over the past two years, and to my advisor, Dr. David Riddick for making himself available whenever I needed him.

I would to thank Geoffrey Goodfellow, Xiaoii Lu, and Kim Sugamori for their technical expertise, advice, and assistance. To all those in the Laboratory of Drs. Grant and Harper (Hanan Abramovici, Kristi Durette, Subha Indrarajah, Christina Tunzi, Yanping Wang, and Margaret Miller) thank you for your continuous encouragement and gossip lunches. You all made coming to work fun.

I would like to thank my husband Lawrence who put up with my stress and insanity and was always there to love, support, and provide me with the perfect words of encouragement. I would like to thank my parents for their guidance, confidence, and love. Finally, I would like to thank my grandparents for their unfailing encouragement, and for calling every night for an update!
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<th>Definition</th>
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<tbody>
<tr>
<td>AAF</td>
<td>2-acetylaminofluorene</td>
</tr>
<tr>
<td>Ab.</td>
<td>antibody</td>
</tr>
<tr>
<td>Ac</td>
<td>acetyl group</td>
</tr>
<tr>
<td>AcCoA</td>
<td>acetyl coenzyme A</td>
</tr>
<tr>
<td>Ac-NAT</td>
<td>acetylated N-acetyltransferase</td>
</tr>
<tr>
<td>AF</td>
<td>2-aminofluorene</td>
</tr>
<tr>
<td>ArNH₂</td>
<td>arylamine</td>
</tr>
<tr>
<td>α-MEM</td>
<td>α-minimal essential medium</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CIP</td>
<td>calf intestinal phosphatase</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>cytochrome P450 1A2</td>
</tr>
<tr>
<td>DAC</td>
<td>deacetylase</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double stranded deoxyribonucleic acid</td>
</tr>
<tr>
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<td>dithiothreitol</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EC-PCR</td>
<td>expression cassette polymerase chain reaction</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-S-transferase</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactoside</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>KPi</td>
<td>potassium phosphate</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>NAT</td>
<td>N-acetyltransferase</td>
</tr>
<tr>
<td>NOAT</td>
<td>intramolecular N,O-acetyltransferase</td>
</tr>
<tr>
<td>OAT</td>
<td>O-acetyltransferase</td>
</tr>
</tbody>
</table>
OD  optical density
OPA  one-phor-all
PAGE  polyacrylamide gel electrophoresis
PBS  phosphate buffered saline
pDN6  random hexamers
PCR  polymerase chain reaction
PHS  prostaglandin H synthase
RNA  ribonucleic acid
RT  reverse transcriptase
SDS  sodium dodecylsulfate
ssDNA  single stranded deoxyribonucleic acid
SULT  sulfotransferase
SV  simian virus
UGT  UDP-glucuronosyl transferase
UV  ultraviolet
YT  yeast tryptone
SECTION 1 INTRODUCTION

1.1 Biotransformation and Drug Metabolizing Enzymes

All organisms are exposed to xenobiotics, which can have a multitude of effects ranging from beneficial (therapeutic drug action) to deleterious (disorders associated with exposure to toxic chemicals). The nature of these effects is dependent upon the balance of an organism's physiological and biochemical processes that interact with the foreign chemical, which include absorption, distribution, chemical-target site interaction, biotransformation, and elimination. Of these processes, biotransformation is considered to be particularly relevant to xenobiotic potency, since it has the potential to produce, prolong, maintain, or reduce the pharmacological and toxicological effects of any given chemical (Riddick 1998).

Biotransformation is an enzyme-catalyzed process by which xenobiotics are chemically altered. Enzymes of xenobiotic biotransformation exhibit both multiplicity and broad substrate specificity, and therefore a limited number of enzymes are capable of biotransforming a diversity of xenobiotics. These enzymes may be either constitutively present or they may be induced or inhibited following xenobiotic exposure (Conney 1967). Many drug-biotransforming enzymes have known endogenous substrates, whereas others have yet to be identified. As such, it is suggested that some of these enzymes may have evolved expressly for the purpose of protecting the organism from xenobiotics (Grant 1991).
Biotransformation reactions, in general, can result in an increase in hydrophilicity of chemicals, which aids in their excretion by the kidney, and/or an alteration of their chemical structure that alters pharmacologic potency (Parkinson 1996). In addition, these metabolites may be either chemically stable or unstable. In the latter case, they may form covalent macromolecular adducts within living cells, potentially leading to cytotoxic, teratogenic, mutagenic, and/or carcinogenic endpoints (Nebert et al. 1999).

The reactions catalyzed by these enzymes are divided into two classes, commonly termed Phase I and Phase II biotransformations. Phase I biotransformation reactions include oxidation, hydrolysis, and reduction, and result in the exposure or addition of a functional group (-OH, -NH2, -SH, or COOH). Phase I reactions are catalyzed by enzymes such as mixed-function monooxygenases (cytochromes P450), flavin-dependent monooxygenases, carboxylesterases, peptidases, epoxide hydrolase, dehydrogenases, and amine oxidases (Parkinson 1996). Phase II biotransformation reactions include acetylation, glucuronidation, sulfation, methylation, and conjugation with glutathione or amino acids, and are catalyzed by transferase enzymes such as the UDP-glucuronosyltransferases, sulfotransferases, methyltransferases, and acetyltransferases. Phase II reactions often occur at the functional groups exposed or added during Phase I biotransformation.
1.2 Genetic Polymorphisms of Drug Biotransforming Enzymes

Genetic mutations that occur frequently (>1% of the individuals) in the population are known as genetic polymorphisms, and when they occur in genes encoding drug-biotransforming enzymes they can potentially influence the ability to biotransform xenobiotics in affected individuals upon exposure to a variety of chemicals. These polymorphisms may influence pharmacological response by leading to increased, decreased, or no enzymatic activity. Therefore, genetic polymorphisms influence drug efficacy, drug-induced toxicity, and carcinogenesis (Kalow and Grant 1998).

Genetic polymorphisms have been discovered for the majority of biotransforming enzymes including N-acetyltransferases (NATs) and cytochrome P450s (Meyer and Zanger 1997). Genetic polymorphisms in these enzymes have been shown to have significant consequences. For example, allelic variation in the N-acetyltransferase \textit{NAT2} gene may result in either fast or slow isoniazid metabolism, leading to either subtherapeutic or supratherapeutic drug levels in the patient, respectively (Blum \textit{et al.} 1990), and genetic polymorphisms in the \textit{CYP2C19} gene result in a truncated and inactive protein, leading to poor metabolism of many clinically important drugs such as mephenytoin, diazepam, omeprazole, and propanolol (De Morais \textit{et al.} 1994).
1.3 Human Arylamine Acetyltransferases

1.3.1 Arylamine and Hydrazine Acetylation

Acetylation is one of the biotransformation reactions of xenobiotics that contain primary arylamine and hydrazine groups such as isoniazid, sulfamethazine, hydralazine, and dapsone (Vatsis and Weber 1993). Acetylation may occur through N-acetylation, O-acetylation, and/or intramolecular N,O-acetyl transfer reactions. These biotransformation reactions may lead to the metabolic activation of xenobiotics such as 2-aminofluorene (AF), 4-aminobiphenyl, and benzidine, or the metabolic inactivation of hydralazine and procainamide (Parkinson 1996).

1.3.2 The N-Acetylation Polymorphism

One of the earliest identified genetic polymorphisms was the N-acetylation polymorphism. In the 1950s Bonicke and Reif (1953) determined that following the administration of the arylamine isoniazid to treat tuberculosis in certain patients, a significant concentration of the drug was eliminated in a conjugated form in urine. In 1957, both Biehl (1957) and Mitchell & Bell (1957) observed a bimodal distribution in the elimination half-life of isoniazid, with the latter scientists categorizing the individuals as either rapid or slow isoniazid metabolizers (for example, See Evans et al. 1960: Figure 1.1). Genetic control of this polymorphism was later established through family pedigree analysis, and significant ethnic differences in the frequency of the rapid and
slow acetylator alleles have been observed, with slow acetylator status ranging from 5% in Canadian Inuit (Armstrong and Peart 1960) to 83% in Egyptians (Hashem et al. 1969) and 90% in Moroccans (Hir et al. 1964).

**Figure 1.1:** Bimodal distribution of plasma concentrations of isoniazid. The distribution shows the interindividual differences of acetylation capacity in a human population. The population is segregated into two groups of either rapid or slow acetylators (Evans et al. 1960).
Genetic analysis showed that the acetylator polymorphism was governed by two alleles at an autosomal gene locus with rapid acetylation arbitrarily classified as the wild type (Evans et al. 1960). Slow acetylators have been shown to be homozygous for the slow acetylator allele, while rapid acetylators have been shown to be either homozygous or heterozygous for the rapid acetylator allele (Hengstler et al. 1998). These discoveries have led to a large body of subsequent work which indicates that this acetylation polymorphism influences the metabolic disposition, detoxification, and bioactivation of a variety of arylamine xenobiotics (Table 1.1) (Weber and Hein 1985; Weber 1987).

Further biochemical work determined that the acetylation polymorphism was the result of variations in the N-acetylation of arylamines in the liver (Evans and White 1964). This enzymatic reaction is now known to be catalyzed by the cytosolic arylamine N-acetyltransferase (EC 2.3.1.5) (Kalow and Grant 1998). Kinetic research with partially purified NAT enzyme demonstrated that the livers of slow and rapid acetylators produce variable levels of enzymes with a comparable affinity for a given substrate such as isoniazid (Jenne 1965).

Clinically, the acetylation polymorphism has significant pharmacological and toxicological repercussions. Slow acetylators may exhibit an increased incidence of methemoglobinemia and/or drug-induced lupus erythmatosus when administered certain arylamine drugs or agents that contain the hydrazine moiety (Weber and Hein 1985). Epidemiological research has also revealed statistically significant associations between
slow acetylation and bladder cancer (Brockmoller et al. 1996) and rapid acetylation and colon cancer (Gonzalez and Idle 1994; Nebert 1991; Vineis and McMichael 1996; Roberts-Thomson et al. 1996). Other associations include a link between slow acetylator status and Gilbert’s disease (Hein 1988) and fast acetylator status and both diabetes and breast cancer (Huang et al. 1999). Finally, Figure 1.2 demonstrates that NATs play an integral role in the multitude of potential reactions undergone by arylamines and as suggested by the above examples, may influence the fate and toxicological consequences of an arylamine xenobiotic.

<table>
<thead>
<tr>
<th>Isoniazid</th>
<th>Nitrazepam</th>
</tr>
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<tbody>
<tr>
<td>Sulfamethazine</td>
<td>Caffeine</td>
</tr>
<tr>
<td>Sulfamerazine</td>
<td>Dipyone</td>
</tr>
<tr>
<td>Sulfapyridine</td>
<td>Amrinone</td>
</tr>
<tr>
<td>Hydralazine</td>
<td>Clonazepam</td>
</tr>
<tr>
<td>Procainamide</td>
<td>Benzidine</td>
</tr>
<tr>
<td>Pheneltzine</td>
<td>4-aminobiphenyl</td>
</tr>
<tr>
<td>Dapsone</td>
<td>2-aminofluorene</td>
</tr>
<tr>
<td>Aminoglutethimide</td>
<td>β-Naphthylamine</td>
</tr>
</tbody>
</table>

Table 1.1: Xenobiotics affected by the acetylation polymorphism (Grant and Spielberg 1998).
Figure 1.2: Potential competing pathways of arylamine activation (NAT, N-acetyltransferase; OAT, O-acetyltransferase; NOAT, intramolecular N.O-acetyltransferase; CYP1A2, cytochrome P4501A2; PHS, prostaglandin H synthase; UGT, UDP-glucuronyltransferase; SULT, sulfotransferase) (Grant et al. 1997).
1.4 N-Acetyltransferase and the Mechanism of Acetyl Transfer

Cloning of the NAT genes in humans has resulted in the discovery of three independent N-acetyltransferase loci, designated NAT1, NAT2, and NAT3, where NAT1 and NAT2 are functional genes and NAT3 is a nonexpressed pseudogene (Blum et al. 1990). The NAT genes each produce a 33 kDa protein (NAT1: 33,898 Da; NAT2: 33,542 Da [Blum et al. 1990]) that are 81% identical (Hickman et al. 1998; Stanley et al. 1996; Windmill et al. 1997).

NAT1 and NAT2 have been shown to be independently regulated proteins, with NAT1 expressed in most tissues of the body (Windmill et al. 1997) and NAT2 expressed predominantly in liver and gut (Ohsako and Deguchi 1990). Biochemical correlation studies led to the discovery that the NAT2 gene is responsible for the human isoniazid acetylation polymorphism (Grant et al. 1991), and that slow acetylators have a reduced level of immunoreactive NAT2 enzyme in liver cytosol (Grant et al. 1990). NAT1 has also been demonstrated to be genetically variable in humans (Grant et al. 1992).

Acetylation occurs via a two step, substituted-enzyme or a ping-pong Bi-Bi reaction mechanism (Weber 1971; Steinberg et al. 1971):

\[
\text{AcCoA + NAT } \leftrightarrow \text{ CoA + Ac-NAT (1)}
\]

\[
\text{Ac-NAT + ArNH}_2 \leftrightarrow \text{ ArNHAc + NAT (2)}
\]

In reaction 1, an acetyl group (Ac) is transferred from the donor substrate, AcCoA, to the NAT enzyme, resulting in a covalent acetyl-enzyme intermediate. Subsequently, in
reaction 2, the acetyl group is transferred from the NAT enzyme to the acceptor substrate ArNH₂, resulting in production of the final acetylated product and the regeneration of the NAT enzyme for ensuing reactions.

NAT1 and NAT2 proteins have both unique and overlapping substrate specificity. Substrates preferentially acetylated by NAT1 include p-aminobenzoic acid and p-aminosalicylic acid, and substrates preferentially acetylated by NAT2 include isoniazid, procainamide, and sulfamethazine. Substrates that are efficiently acetylated by both NAT1 and NAT2 include the xenobiotic AF (Parkinson 1996).

1.5 Human Liver Arylace Deacetylase

The N-acetylation of arylamines may be challenged by another class of enzymes known as deacetylases, which can convert the acetylated metabolite back to its primary arylamine form (Figure 1.2). Thus the direct competition between these reaction pathways could potentially affect the therapeutic and/or toxicological consequences of arylamine exposure. For example, deacetylation has been shown to be a major reaction pathway in the hepatic microsomal metabolism of the NAT substrate 2-acetylaminofluorene (AAF) into mutagenic products (Aune et al. 1985). Research has also indicated that the inhibition of deacetylase activity results in reduced mutagenicity (Holme et al. 1983) and carcinogenicity (Lai et al. 1988) of arylamines.
Deacetylation is a hydrolytic reaction catalyzed by enzymes of the esterase class
(Heymann 1982). Esterases are enzymes with relatively broad substrate specificity, and
are responsible for catalyzing the hydrolysis of esters, amides, thioesters, and
phosphoesters (Satoh 1987). These reactions have been shown to have a significant
influence on xenobiotic biotransformation and elimination. Research by Schut et al.
(1978) and Reddy et al. (1980) demonstrated that the organophosphate inhibitors
paraxon and BPNPP decreased the number of revertants in bacterial mutagenicity assays.
In addition, Aune et al. (1985) and Monteith and Strom (1990) have shown that
deacetylation is a significant reaction pathway in the metabolism of certain acetylated
arylamines into mutagenic products.

In 1991, Probst et al. purified and characterized a novel arylacetamide deacetylase
(DAC) from human liver microsomes. They determined that DAC can influence the
metabolic activation to reactive electrophiles of arylamine carcinogens. Subsequently,
Probst et al. (1994) cloned a full-length cDNA from a liver cDNA library and determined
through sequence comparisons that DAC shows significant homology to hormone
sensitive lipases and carboxylesterases but should be classified as an esterase (E.C.
3.1.1.1). This is the first known human esterase to be identified with an established role
in the metabolic activation of arylamine and heterocyclic amine carcinogens.

The cDNA of DAC was determined to have an open reading frame of 1200 base
pairs, encoding a protein of 400 amino acids with a molecular weight of 45.7 kDa.
Tissue analysis demonstrated DAC activity and immunoreactive protein in human liver and extrahepatic tissue microsomes (Figure 1.3), with liver microsomes exhibiting the greatest levels of enzyme activity. However, only liver and ileum/jejunum expressed immunoreactive protein. Other extrahepatic tissues did not exhibit immunoreactive protein. A correlation between DAC activity and the amount of immunoreactive protein was observed for liver but not for ileum/jejunum, suggesting that a portion of the observed activity in the ileum/jejunum may result from the activity of an additional deacetylase enzyme. Hepatic DAC activity was also shown to have a 7-fold variation among 28 liver samples, which could have potential consequences for variation in the toxicological consequences associated with arylamine exposure and metabolism. This variation was postulated to be the result of variations in gene product expression. Finally, Probst et al. (1994) established that although DAC is a microsomal enzyme, it does not require membrane to maintain its catalytic activity.
Figure 1.3a: The distribution of AAF-DAC enzymatic activity in various human tissues. Liver was determined to exhibit the greatest amount of enzymatic activity of all the tissues samples analyzed (Probst et al. 1994).

Figure 1.3b: Western immunoblot of DAC in various human tissues. The liver and ileum exhibited the greatest degree of DAC antibody recognition with distinct bands identified at 45 kDa (Probst et al. 1994).
The discovery of this novel deacetylase provides an important potential mechanism for the modulation of the metabolic activation or detoxification of arylamine compounds. As previously discussed, biotransformation reactions of arylamines with N-acetyltransferase have significant pharmacological and toxicological repercussions, including associations with cancer. It is possible that DAC may play a role in producing these effects, and the tools generated in the present thesis project will hopefully aid in the understanding of DAC's role and the interaction of NATs and DAC in producing mutagenic endpoints.

1.6 The DAC Substrate 2-Acetylaminofluorene

The acetylated metabolites of several NAT substrates have been investigated as potential DAC substrates (Probst et al. 1991). Of these, AAF is the only acetylarylamine that has been shown to be a substrate for DAC, although the list of tested substrates is limited to date. AAF is a commonly used model compound, which functions as a potent in vitro mutagen and in vivo liver and bladder carcinogen (Miller 1978; Miller et al. 1964; Stevens and Peraino 1983; Wilson et al. 1941), and has no other current commercial uses (Dybing et al. 1979; U.S. Department of Health and Human Services 1993; Sittig 1985). In some experimental systems, the deacetylation of AAF to AF is required for its mutagenicity (Figure 1.4). For example, Aune et al. (1985) demonstrated
that in rabbits, inhibition of AAF deacetylation by paraxon produced a marked reduction in its mutagenicity.

**Figure 1.4:** Illustration of the reaction pathway of the deacetylation of 2-acetylaminofluorene and N-acetylation of 2-aminofluorene. The products formed in this reaction may subsequently be involved in activation and detoxification pathways.
1.7 Goals and Rationale

It is likely that DAC plays a significant role in the pharmacological, toxicological, and carcinogenic effects observed upon exposure to arylamines. Through the development of experimental tools to express and to quantify the expression and functional activity of DAC, this research project will ultimately lead to a better understanding both of DAC's role and of the relative importance of NATs and DAC in producing cytotoxic, mutagenic, and carcinogenic endpoints following exposure to AAF. In addition, these systems will allow for the identification of other potential DAC substrates, and thus implicate DAC in any toxicities associated with their exposure.

In order to carefully examine the importance of NATs and DAC in producing mutagenic endpoints many objectives must be fulfilled. Firstly, a sensitive assay to quantify deacetylation enzyme activity must be established. Secondly, a polyclonal anti-human DAC antiserum must be created that will enable immunoreactive protein to be detected and quantified with both high sensitivity and specificity, so that the relationship between protein expression and catalytic function both in experimental systems and in various human tissues can be assessed. Thirdly, a suitable bacterial or mammalian heterologous expression system for functional DAC must be developed in order to obtain sufficient quantities of enzyme for detailed functional analysis.
SECTION 2 MATERIALS AND METHODS

2.1 Materials

Oligonucleotide primers for polymerase chain reaction (PCR) amplification of DAC were synthesized by ACGT and by Gibco BRL (Life Technologies Inc., Burlington, ON). PCR buffers, Mg$^{2+}$ solutions, and deoxynucleotide triphosphates (dNTPs) for PCR were purchased from Perkin Elmer (Applied Biosystems Canada Inc., Mississauga, ON). PCR amplification reactions were performed in a Perkin Elmer Cetus DNA Thermal Cycler. Restriction enzymes were purchased from Gibco BRL. T4 DNA ligase was purchased from Gibco BRL. Bacteria Lambda DNA used to generate a DNA size ladder was purchased from Amersham Pharmacia Biotech (Baie D'Urfe, PQ).

The expression vector pKEN2 and *E. coli* host strain XA90 (*FlacI*) were kindly provided by Dr. G.L. Verdine, Department of Chemistry, Harvard University, Cambridge, MA. The bacterial strain DH5α was purchased from Gibco BRL (Life Technologies Inc., Burlington, ON). The prokaryotic fusion protein expression vector pGEX-4T-2 (pGEX) was purchased from Amersham Pharmacia Biotech (Baie D'Urfe, PQ).

Protein and DNA concentrations were determined using a protein dye binding reagent from Bio-Rad Laboratories Inc. (Mississauga, ON) and the use of a Beckman Model DU-62 spectrophotometer.
SDS-polyacrylamide electrophoresis gels (PAGE) for Western immunoblots were run using a Mighty Small II SE 250 Western Blotting apparatus from Hoefer (San Francisco, CA) and transferred using a NovaBlot Multiphor II protein transfer apparatus from Amersham Pharmacia Biotech (Baie D’Urfe, PQ). Nitrocellulose paper (Hybond C+), the Enhanced Chemiluminescence (ECL) kit and the Hyperfilm ECL film used to detect the immunoreactive signal were purchased from Amersham Pharmacia Biotech.

Manual DNA sequencing involved the use of a T7 Sequencing Kit and $\alpha^{-35}$S dATP both purchased from Amersham Pharmacia Biotech. The DNA sequencing apparatus was a Base Ace™ vertical sequencing apparatus purchased from Stratagene (La Jolla, CA). Automated sequencing was performed by the DNA Sequencing Facility at the Centre for Applied Genomics at the Hospital for Sick Children, Toronto, ON.

DAC clones and a polyclonal anti-human DAC, IgG, supplemented with 0.02% azide and 5 mg BSA, were kindly provided by Dr. M. Probst of the University of California, Los Angeles, CA. Human liver microsomes were prepared by Susan Janezic, (Dr. D.M. Grant Laboratory, Hospital for Sick Children, Toronto, ON), and RNA was isolated and purified by Kristi Durette (Dr. D.M. Grant Laboratory, Hospital for Sick Children, Toronto, ON). COS-7 cells were generously donated by Dr. H.B. Niznik of the Centre for Addiction and Mental Health, Toronto, ON.
The chemicals AAF and AF were obtained from Sigma Chemical Co. (St. Louis, MO). Luria-Bertani (LB) medium and 2 x yeast tryptone (YT) medium were also obtained from Sigma Chemical Co. (St. Louis, MO).

Lysates were prepared using a Sonics and Materials Inc. Vibra Cell (Danbury, Connecticut). Dialysis cassettes and tubing were obtained through Sigma Chemical Co. (St. Louis, MO).

HPLC mobile phase reagents were obtained from the following suppliers: triethylamine, from Sigma Chemical Co. (St. Louis, MO), acetonitrile and methyl alcohol (HPLC grade) from Mallinckrodt (Mississauga, ON), and glacial acetic acid and 70% perchloric acid from BDH Chemicals (Toronto, ON). HPLC was performed using a SCL-6B System Controller, a SIL-6B autoinjector, a LC6A liquid chromatograph and a SPD-6A UV spectrophotometric detector from Shimadzu, and reversed phase C18 analytical Ultrasphere columns from Beckman Instruments Inc (Fullerton, CA). EZChrom™ from Scientific Software Inc. (San Ramon, CA) was used with an IBM-PS2 computer to control the HPLC automation system.

A GDS 7500 gel documentation apparatus from Diamed Laboratory Supplies (Mississauga, ON) was used for visualization of DNA fragments and photos of agarose gels.

All other materials were of analytical grade and were supplied by local suppliers.
2.2 Methods

2.2.1 Polymerase Chain Reaction Amplification

PCR was used to create expression cassettes for DAC expression vectors. The oligonucleotide primers used to create these expression cassettes are listed in Table 2.1, and include restriction endonuclease sites for EcoRI, SalI, NdeI, and ApaI. Subcloned DNA fragments containing DAC were used as templates for the amplification of DAC, except in the case of RT-PCR (reverse transcriptase polymerase chain reaction) where the RT reaction was used for PCR amplification (See Section 2.2.2). Final concentrations in the PCR reactions were 1 x of 10 x PCR buffer (100mM Tris (pH 8.3), 500 mM KCl, 0.1% gelatin, 15 mM $\text{MgCl}_2$), 0.1 mM dNTPs (1.25 mM of each deoxynucleotide [dATP, dCTP, dGTP, dTTP]), 0.4 $\mu$M 5' primer, 0.4 $\mu$M 3' primer, and 1.25 U AmpliTaq DNA polymerase. Reactions were started with 10 ng of plasmid template. Amplifications were performed under the following conditions: initial denaturation at 85°C for 4 minutes, 30 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. A 1 $\mu$l aliquot of the PCR reaction was loaded onto 1% TAE-agarose gel, and positive amplification was confirmed by agarose gel electrophoresis with visualization by ethidium bromide staining and UV illumination.
| Oligonucleotide primers used for EC-PCR. The primers DAC-5' and DAC-3' were prepared by ACGT and the primer DAC-3'-2 was prepared by Gibco BRL (* start amino acid; ** stop amino acid). |
|---|---|
| **Table 2.1:** | |
| **Oligonucleotide primers used for EC-PCR. The primers DAC-5' and DAC-3' were prepared by ACGT and the primer DAC-3'-2 was prepared by Gibco BRL (* start amino acid; ** stop amino acid). | |
2.2.2 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA, isolated and purified by Kristie Durette, was treated with DNase (10% v/v DNase/RNA), and reverse transcriptase was used to transcribe RNA into cDNA. Each RT reaction contained 1x of 10x RT buffer, 1 mM dNTPs, 7 μM random hexamers, 2.5 mM MgCl₂, 10 mM DTT, and 15 units of RNase Inhibitor. 2.5 μg of RNA was denatured at 90°C for 3 minutes, followed by chilling on ice. The RNA was added to the reaction, and then 200 units of M-MLV-RT and DEPC treated water in a total reaction volume of 10 μl was added. The reaction was incubated at room temperature for 10 minutes, followed by a 60 minute incubation at 42°C. PCR reactions, conditions, and verification were identical to those described.

2.2.3 Restriction Endonuclease Digestion

Restriction endonuclease enzymes were used as specified by the manufacturer to produce optimal activity at specific salt concentration. Generally, restriction enzyme equivalent to three units of enzyme activity per μg of DNA to be digested was added to reactions in order to ensure complete digestion. One unit of activity represents the volume of enzyme required to digest 1 μg of bacteriophage lambda DNA in one hour under optimal conditions. Reactions contained approximately 5 μg DNA, 15 units of one or more restriction endonucleases, and the appropriate reaction buffer. Reactions were incubated at 37°C, unless otherwise specified by the manufacturer, until complete
digestion was observed using UV illumination to visualize a 1 µl aliquot on a 1% TAE-agarose gel stained with ethidium bromide. Enzymes were inactivated either by incubation at 60°C for 20 minutes or by the addition of loading buffer dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in 1 x Tris-acetate EDTA). If necessary, digests were then purified, using a QIAEX DNA purification kit. All digestions resulting in vectors that were blunt ended were CIP (Calf Intestinal Phosphatase) treated overnight at 37°C (1 µl CIP for 2.5 ng DNA in 1 x One-Phor-All [OPA] enzyme buffer from Amersham Pharmacia Biotech [10 mM Tris-acetate, pH 7.5, 10 mM magnesium acetate, 50 mM potassium acetate]).

2.2.4 QIAEX Gel Extraction

Restriction enzyme digestions were run on 1% TAE-agarose gels with visualization via ethidium bromide staining and UV illumination. Appropriate fragments were excised from the gel and placed in 1.5 ml microcentrifuge tubes. DNA was isolated from the gel fragments using a QIAEX DNA purification kit (QIAGEN Inc., Chatsworth, CA). 300 µl of solubilization buffer (3 M NaI, 4 M NaClO₄, 10 mM Tris-HCl (pH 7.0), and 10 mM sodium thiosulfite) per gram of gel, and 10 µl of the QIAEX particle slurry were added to the excised gel fragment. The tube was placed in a 50°C heating block for 10 minutes and vigorously mixed every 2 minutes. The mixture was centrifuged at 12,000xg for 30 seconds at room temperature. The supernatant was aspirated, and the
pellet was washed twice with 500 µl of 8 M NaClO₄, 10 mM Tris-HCl (pH 7.0) and twice with 70% EtOH, 100 mM NaCl, 10 mM Tris-HCl, (pH 7.5). The pellet was air dried for a minimum of 10 minutes and resuspended in 20 µl of TE8 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to elute the bound DNA. The mixture was then centrifuged at 12,000×g for 30 seconds at room temperature, and the supernatant containing the eluted DNA was removed and retained.

2.2.5 DNA Ligations

Following gel extractions, the vector and DAC DNA digested with restriction endonucleases were ligated in microcentrifuge tubes. Each reaction contained a 1 to 4 ratio of vector to insert, 1 x ligation buffer (0.05 M Tris-HCl (pH 7.6), 1 µM MgCl₂, 1 µM DTT, 5 ng/ml BSA), and T4 ligase. The reactions were incubated at 14°C for 18 hours.

2.2.6 DNA Transformations

Ligation reactions were incubated with competent cells for 30 minutes on ice. The reaction was heat shocked at 42°C for 90 seconds in order to make the competent cells conducive to taking up the ligated DNA. The cells were rescued by being placed on ice for 2 minutes. SOC (20% w/v bacto-tryptone, 5% w/v bacto-yeast extract, 0.5% w/v NaCl, 0.01 M MgCl₂, 20 mM Glucose) to a final volume of 1 ml was then added to the
reaction, and the reaction was shaken at 250 rpm at 37°C for 1 hour. Cells were then concentrated, plated on agar plates with 100 μg/ml ampicillin, and incubated overnight at 37°C (See Figure 2.1 for a summary of the cloning methodology).

2.2.7 Bacterial Growth Conditions

Bacterial cultures were grown for protein expression experiments or to isolate plasmid DNA in either LB medium containing 100 μg/ml ampicillin (LB-amp) or 2 x YT medium (16 g/L Tryptone, 10 g/L Yeast extract, 5 g/L NaCl) containing 100 μg/ml ampicillin (2 x YT-amp). Single colonies isolated from agar plates containing ampicillin were used to inoculate 3 ml of media. The cultures were then shaken overnight at 250 rpm at 37°C.

Recombinant proteins were expressed according to Dupret and Grant’s (1992) protocol where 4 ml of media was inoculated with an aliquot (80 μl) of fresh overnight culture. The cultures were shaken at 280 rpm at 37°C or room temperature, as specified, until the OD₆₀₀ fell in the range of 0.4 to 0.5. Protein production was then induced with IPTG (isopropyl-β-D thiogalactopyranoside) from Gibco BRL (Burlington, ON), at a final concentration of 1 mM, and the bacterial cultures were shaken at 280 rpm at 37°C for 3 hours or at room temperature for 18 hours.
Figure 2.1: Summary of cloning methodology.
2.2.8 Lysate Preparation

Following IPTG induction for 3 hours at 37°C or overnight at room temperature, bacterial cultures were centrifuged at 3000xg for 10 minutes at 4°C in a swinging bucket centrifuge. The supernatant was removed, and the pellet was resuspended with 1/3 the volume of the original culture in ice cold 0.1 M KPi buffer (pH 7.4). The resuspended bacterial cells were lysed via sonication with a probe sonicator in an ice water bath at 50% full power for 2 x 15 seconds with 30 second intervals between sonications. Further sonication was performed, if necessary, to clear the solution and liberate the protein. In order to pellet the cellular debris, the lysed suspension was then spun at 18 000xg for 5 minutes at 4°C, and the supernatant was removed and transferred to a new tube, and the pellet fraction was resuspended in an equal volume of ice cold 0.1 M KPi buffer (pH 7.4). The lysate was either used immediately for enzymatic assays or Western immunoblots or was flash frozen in liquid nitrogen and stored at -80°C.

2.2.9 Isolation of Plasmid DNA

Isolation of plasmid double stranded (ds) DNA from bacterial culture was achieved using the alkaline lysis method (Sambrook et al. 1989) referred to as a miniprep. To pellet the cells, a 1.5 ml aliquot of an overnight bacterial culture was centrifuged at 12,000xg for 5 minutes at room temperature in a microcentrifuge tube. The supernatant was aspirated, and the pellet was resuspended in 200 µl of ice cold
Solution 1 (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.4 mg/ml RNaseI) by vortexing.

Bacterial cell lysis and protein and chromosomal DNA denaturation followed by adding 200 μl of a mixture of 0.2 N NaOH and 1% SDS. The mixture was inverted several times to mix it thoroughly and incubated at room temperature for 5 minutes. Protein, chromosomal DNA, and cellular DNA was precipitated using 200 μl of 3 M potassium acetate and 5 M glacial acetic acid. The mixture was then centrifuged at 17,000 rpm for 10 minutes at 4°C. The supernatant was removed and transferred to a new microcentrifuge tube where 1 ml of 100% EtOH was added to pellet the plasmid DNA. The mixture was vortexed and spun at 17,000 rpm at 4°C for 10 minutes. The EtOH was aspirated using a glass capillary, and the pellet was dried at 37°C in a heating block. The dried pellet was resuspended in 35 μl of TE8 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

If DNA of greater purity was required, DNA isolation involved the use of a Flexiprep kit from Amersham Pharmacia Biotech. The methodology outlined above was followed. However, Solution 1 contained 100 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 0.4 mg/ml RNaseI. DNA was precipitated using isopropanol, and air dried pellets were resuspended in 150 μl of Sephaglass™ FP slurry. The slurry was centrifuged at 12,000xg for 5 minutes, and the supernatant was aspirated. The Sephaglass pellet was washed once with 200 μl of 20 mM Tris-HCl (pH 7.5), 2 mM CDTA, 200 mM NaCl, and once with 300 μl of 70% EtOH. The pellet was air dried for 10 minutes, and the DNA was eluted in 50 μl of TE8 and collected.
2.2.10 Western Immunoblotting/Coomassie Staining

DAC lysates were prepared as described and mixed with either 2x or 4x polyacrylamide gel electrophoresis sample buffer. 4x sample buffer was comprised of 2% SDS, 40% glycerol, 125 mM Tris-HCl (pH 6.8), 2% β-mercaptoethanol, and 0.02% bromophenol blue. The samples were diluted in sample buffer and water to known concentrations in order to load equivalent amounts of total protein to the gel. Once prepared, the samples were either used immediately or stored at -20°C. Prior to gel loading, the samples were boiled for approximately 3 minutes and then loaded to 10% SDS-PAGE gels following Laemmli’s protocol (1970) using a Mighty Small II SE250 gel apparatus. A Molecular Weight marker was run with each gel and was comprised of rabbit muscle myosin (205 kDa), E. coli β-galactosidase (116 kDa), rabbit muscle phosphorylase b (97.4 kDa), bovine albumin (66 kDa), egg albumin (45 kDa), and bovine erythrocyte carbonic anhydrase (29 kDa). Prior to transferring, nitrocellulose membranes were soaked in dH₂O and then both the nitrocellulose and blotting paper were soaked in transfer buffer (0.04M glycine, 0.05 M Tris-HCl, 1.3 mM SDS, 20% v/v methanol). The gel was placed on top of the nitrocellulose, which was then placed between the blotting paper.

The gels were either electrophoretically transferred to nitrocellulose membrane or stained with Coomassie Blue (45% w/v Coomassie Brilliant Blue R250, 45% v/v methanol, 45% v/v H₂O, 10% v/v glacial acetic acid). Following electrophoretic transfer,
the nitrocellulose was rinsed with dH₂O to remove any traces of gel and stained with Ponceau Red for 10 minutes to confirm protein transfer and identify the location of the molecular weight markers. Nitrocellulose membranes were blocked overnight by rocking them in milk-TNT (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.2% w/v Tween 20, 0.01% thimerosal, and 4% w/v skim milk powder). The blots were rinsed with dH₂O and probed with the specified primary antibody for 3 hours. Prior to use, the anti-GST antibody (1/4000) was blocked overnight with 4 mg/ml boiled and native XA90 lysate to remove antibodies capable of recognizing endogenous *E. coli* proteins. The blots were then washed 6 x 5 minutes with 1 x TNT and then probed with a horseradish peroxidase labeled goat anti-rabbit IgG secondary antibody for 1 hour. The blots were washed 6 x 5 minutes with 1 x TNT and soaked in an ECL reagent solution containing 4 ml of Reagent 1 and Reagent 2 for 1 minute and exposed to Hyperfilm™ ECL for the specified time in order to visualize the specific protein.

Gels stained with Coomassie blue were rocked in the solution for 1 hour and then washed rocking overnight in a Coomassie destain solution (45% v/v methanol, 45% v/v H₂O, 10% v/v glacial acetic acid) in order to visualize proteins.
2.2.11 DAC HPLC Enzymatic Activity Assay

Reactions were comprised of 90 μl of lysate and 500 μM AAF in 50% DMSO and 50% dH₂O. The reactions were mixed and incubated for 30 minutes at 37°C. Reactions were stopped with 15% HClO₄, and tubes were centrifuged at 12 000xg for 3 minutes at room temperature to pellet the precipitated protein. 0.1 M KPi (pH 7.4) was substituted for DAC lysate as a negative control, and human liver microsomes were substituted for DAC lysate as a positive control for each independent assay. In addition, a negative human liver microsome control, that was not incubated, was also prepared for each independent assay. Reactions were then analyzed using HPLC.

2.2.12 HPLC Quantification of DAC Activity

The HPLC methodology, based on that outlined by Probst et al. (1991), was used to identify and quantify the deacetylated product of AAF, AF. An aliquot (50 μl) of the supernatant of each stopped reaction was injected onto a reverse phase C18 Beckman Ultrasphere® column (4.6 mm x 15 cm) at a flow rate of 2 ml/min. Peaks of the deacetylated products were detected at a UV absorbance of 260 nm. The mobile phase was comprised of 20 mM NaClO₄ (pH 2.5) and acetonitrile (70:30 w/w). EZChrom™ was used to analyze the chromatograms.
2.2.13 Manual DNA Sequencing

One to 2 μg of dsDNA in TE8, purified with the Flexiprep Kit, was denatured and made into single stranded DNA (ssDNA) by the addition of 4 μl of NaOH and incubation at room temperature for 10 minutes. The DNA was precipitated by the addition of 6.66 μl NH₄Ac and 60 μl 100% EtOH with incubation at -80°C for 15 minutes. The precipitated DNA was pelleted by centrifugation at 15 300 rpm at 4°C for 10 minutes. The pellet was washed with ice cold 70% EtOH and centrifuged at 15 300 rpm at 4°C for 3 minutes. The pellet was air dried in a 37°C heating block and resuspended in 4 μl ddH₂O.

A 2 μl aliquot of DNA sequencing primers (10 ng/μl) and 1 μl of annealing buffer (1M Tris-HCl (pH 7.5), 5 mM DTT, 100 μg BSA/ml, 5% glycerol) were added to the denatured DNA template. The mixture was vortexed and incubated at 37°C for 20 minutes and then allowed to cool to room temperature.

A Master Mix composed of 0.5 μl dH₂O, 3 μl Labeling Mix A (1.375 μM of dATP, dCTP, dGTP, dTTP), 3n/8μl (enzyme activity of T7 DNA polymerase) of T7 DNA polymerase, 2n-μl of T7 DNA polymerase of Enzyme Dilution Buffer (20 mM Tris-HCl (pH7.5), 5 mM DTT, 100 μg BSA/ml, 5% glycerol), and 0.5 μl of α-³²PdATP, was made for every 2 reactions where n equals the number of pairs of reactions. 3 μl of the Master Mix were added to the annealed template and primer to commence the labeling reactions. T7 DNA polymerase initiated primer extension and incorporation of
the radiolabeled deoxynucleotide while being incubated at 37°C for 5 minutes. 2.3 μl of the labeling reaction was then aliquoted into four 37°C tubes containing the various reaction terminating dideoxynucleotides and all four deoxynucleotides. Each reaction was incubated at 37°C for 5 minutes and then stopped with 2.5 μl of Stop Solution (0.3% bromophenol blue, 0.3% xylene cyanol FF, 10 mM EDTA (pH 7.5), and 97.5% deionized formamide). The products were then loaded onto a 0.4 mm 6% acrylamide gel and run on a Base Ace® Vertical Sequencing Apparatus. Gels were dried for one hour in a Bio-Rad Model 583 gel dryer and exposed overnight to BIOMAX autoradiographic film at room temperature.

2.2.14 Automated DNA Sequencing

Automated DNA sequences were performed using fluorescent dye labeling by the DNA Sequencing Facility at the Centre for Applied Genomics at the Hospital for Sick Children. DNA samples were prepared for plasmid purification using Qiagen™ Spin Miniprep kits (Qiagen, Chatsworth, CA).
2.2.15 Determination of DNA and Protein Concentrations

DNA concentrations were determined spectrophotometrically at an OD\textsubscript{260} (optical density) where 1 OD unit is equal to 55 \mu g/ml for double stranded DNA.

Protein concentrations were determined at an OD\textsubscript{595} using Bradford analysis. A standard curve with varying concentrations of BSA in 1 x PBS was used. Bradford reagent was diluted 1/5 in dH\textsubscript{2}O, and reactions were mixed and incubated at room temperature for 10 minutes.

2.2.16 Soluble Protein Purification

According to the manufacture's protocol, DAC was purified from the fusion protein DAC-GST using Glutathione Sepharose\textsuperscript{4B} (Amersham Pharmacia Biotech). Fresh overnight cultures were restarted in 600 ml of 2 x YT-amp, grown at room temperature, and shaken at 280 rpm until the OD\textsubscript{600} ranged between 0.4 to 0.5. The cultures were then induced with IPTG to a final concentration of 1.0 mM and grown at room temperature overnight. Overnight induced cultures were centrifuged at 7 500xg at 4\textdegree C for 10 minutes in order to pellet the cells. The supernatant was removed, and the pelleted cells were resuspended in 25 ml of 0.1M KPi (pH 7.4). The resuspended cells were then lysed in an ice water bath via sonication for 16 x 15 seconds with 30 second intervals. The sonicate was then centrifuged at 16 500 rpm at 4\textdegree C for 5 minutes. The supernatant was removed, and the pellet was resuspended in 5 ml of 0.1 M KPi (pH 7.4).
500 μl aliquots of the supernatant and pellet were removed. The balance of supernatant remaining was noted, and total protein concentration of the supernatant and pellet were determined using Bradford analysis. The bed volume of Glutathione Sepharose 4B was determined based on a binding capacity of 300 mg/ml of fusion protein. In order to bind the fusion protein to the sepharose beads, the supernatant was incubated with the Glutathione Sepharose 4B slurry for 1 hour at 4°C. The solution was then centrifuged at 1000xg at 4°C for 5 minutes. The supernatant was removed, and a 1 ml aliquot was taken and referred to as the post load supernatant. The pellet was washed with 3 x 5 bed volumes of 1 x PBS (Phosphate-Buffered Saline) (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.3) and centrifuged at 1000xg at 4°C for 5 minutes. A 1 ml aliquot of each wash was retained and referred to as wash 1, wash 2, and wash 3, respectively. For each ml of Glutathione Sepharose 4B bed volume, 0.05% v/v thrombin (500U thrombin in 0.5 ml 1 x PBS) in 1 x PBS was added, and the solution was incubated overnight with rocking at 4°C. This resulted in cleavage of the fusion protein and purification of DAC. The solution was then centrifuged at 1000xg for 5 minutes at 4°C, and the supernatant was removed and referred to as the elute. Finally, 1 bed volume of 1 x PBS was added to wash the beads, mixed, and a 200 μl aliquot was removed and centrifuged at 15 000xg at 4°C for 5 minutes. The supernatant was removed, and 100 μl of 2 x SDS-PAGE buffer was added to the beads. This permitted the detection by SDS-
PAGE of any fusion protein that had not been cleaved and remained on the beads. The samples were then run on 10% SDS-PAGE gels.

Alternately, following the three 1 x PBS washes, the beads were washed 3 x 15 minutes with 50% v/v bed volume of Glutathione Elution buffer (10 mM reduced glutathione, 50 mM Tris-HCl (pH 8.0)) at 4°C. The solution was centrifuged at 1000xg for 5 minutes at 4°C, the elute was collected, and the protein concentration was determined by Bradford analysis. The elute was then cleaved using 0.01% v/v of thrombin for each mg of protein. The vial, rocked overnight at 4°C, was then dialyzed (Slide-A-Lyzer® Dialysis Cassette). The dialyzed solution containing the fusion protein was then purified using Glutathione Sepharose 4B beads and eluted as described above.

2.2.17 Insoluble Protein Purification

Insoluble DAC-GST fusion protein was purified according to Williams et al. (1995). Fresh overnight cultures were restarted in specified volumes of 2 x YT-amp and grown at 37°C at 280 rpm until the OD$_{600}$ ranged between 0.4 to 0.5. The cultures were induced with IPTG to a final concentration of 1.0 mM and grown for 3 hours. The cultures were chilled on ice for 15 minutes to avoid protein degradation. This was followed by centrifugation at 3000xg for 10 minutes at 4°C to pellet the cells. The pellet was resuspended in inclusion body sonication buffer (25mM HEPES [pH 7.7], 100mM KCl, 12.5 mM MgCl$_2$, 20% glycerol, 0.1% v/v Nonidet P-40, 1 mM DTT), and lysozyme
was added to a final concentration of 0.5 mg/ml. After the reaction was incubated on ice for 30 minutes, it was sonicated in an ice water bath with 18 bursts of 15 seconds with 30 second intervals. An aliquot of 100 μl was removed and referred to as sonicate. The remaining sonicate was centrifuged at 8000xg for 10 minutes at 4°C to pellet the inclusion bodies and a 100 μl aliquot was removed and referred to as sonicate supernatant. The pellet was washed twice with ice cold RIPA buffer (0.1% v/v SDS, 1% v/v Triton X-100, 1% w/v sodium deoxycholate in TBS [25 mM Tris-HCl, pH 7.5, 150 mM NaCl]) and centrifuged at 8000xg for 10 minutes at 4°C. A 100 μl aliquot was taken of the wash suspension and supernatant and referred to as RIPA 1, RIPA supernatant 1, RIPA 2, and RIPA supernatant 2, respectively. The pellet was air dried to a paste, and the inclusion body paste was transferred to 1.5 ml microcentrifuge tubes such that each tube had no more than approximately 500 μl of inclusion bodies. 4 volumes of 10% SDS was then added to the inclusion body paste. The pellet was solubilized by gently pipetting with a 1ml micropipettor and further facilitated by heating the sample to 95°C for 1 hour. Once the inclusion bodies were in solution, the samples were diluted with 9 volumes of 1 x PBS. The solution was then dialyzed (Spectra/Por® Molecularporous Regenerated Cellulose Dialysis Membrane, MWCO 12-14 000, Spectrum) overnight at room temperature against a 100 fold volume of 1 x PBS containing 0.05% SDS. The following day, the dialysis buffer was changed to fresh 1 x PBS containing 0.01% SDS and dialyzed for 5 hours. The dialyzed solution was stored at 4°C, and an aliquot was
analyzed by SDS-PAGE and Bradford analysis to quantify final protein concentration, yield, and integrity.

2.2.18 Plasmid Induction

The percentage of cells in a bacterial culture that have plasmid at the time of induction and are making protein was ascertained. Three bacterial cultures were grown. The first was grown overnight in 2 x YT-amp at room temperature without shaking. The second was grown at room temperature while being shaken at 250 rpm. The third was grown at 37°C while being shaken at 250 rpm. The following day, the fresh cultures were inoculated using the three overnight cultures and grown according to the above conditions until the OD$_{600}$ ranged from 0.4 to 0.5, where 1 OD$_{600}$ is equal to 8 x 10$^4$ cells/ml. The cultures were plated on LB agar, LB agar with 100 µg/ml ampicillin, LB agar with 1 mM IPTG, and LB agar with 100 µg/ml ampicillin and 1 mM IPTG at a density of 150 cells/plate. A comparison between the number of cells on the LB plates and the number of cells on the LB-amp plates indicates the number of cells that contain plasmid, and a comparison between the number of cells on the LB plate and the number of cells on the LB-amp plate with IPTG indicates the number of plasmids that are producing protein.
2.2.19 Antibody Production

Antibody production was based on methodology outlined by Harlow and Lane (1988). A 10% SDS-PAGE gel was loaded and run with 200 µg of DAC-GST solubilized fusion protein. The gel was stained with 0.3M KCl at 4°C for 10 minutes enabling visualization of the 71 kDa band (26 kDa GST, 45 kDa DAC). The band was excised and homogenized using a syringe with 1 ml of 1x PBS. The homogenized gel slice was mixed with 1 ml of Freund’s Complete adjuvant immediately prior to injection and injected into 2 male New Zealand White rabbits with weights ranging from 2.5 to 3 kg, such that each rabbit received 100 µg of DAC-GST protein. The rabbits were identified as R8711 and R8734. Injections were performed at 4 subcutaneous sites of 0.25 ml. In an identical manner as above, boost injections were prepared and injected, except the homogenized gel slice was mixed with Freund’s Incomplete adjuvant. Injections/boosts occurred on Day 1, 21, and 48. Blood samples of 10 ml were taken on Day 1 (Prebleed), 34, and 61. Exsanguination occurred on Day 68 by premedication with acepromazine and ketalar and anesthesia with halothane and nitrous oxide. A femoral cut down was performed, and an 8 Fr umbilical catheter was introduced into the vessel. The animal was slowly exsanguinized without recovery from anesthesia. All animal handling, injections, blood sampling, and exsanguinization were performed by the Hospital for Sick Children Laboratory Animal Services (Toronto, ON).
Serum isolation from blood samples was carried out as follows: samples were kept at room temperature for 4 hours followed by 4°C overnight. The clot was loosened from the tubes and removed. The remaining sera was centrifuged at 2700xg at 4°C for 10 minutes. The sera was removed and stored at -20°C in 500 μl aliquots.

Titre experiments were performed using SDS-PAGE gels with DAC-GST protein concentrations ranging from 0.1 μg/cm to 10 μg/cm and with serum dilutions ranging from 1/50 in 1 x TNT to 1/16 000 in 1 x TNT. The protocol outlined for Western immunoblotting was followed.

2.3 Mammalian Cell Culture

2.3.1 DNA Preparation

DAC was PCR amplified, ligated into pCDNA3, using *Apa* and *EcoR1* restriction endonucleases for directional cloning, and transfected into DH5α as described. A positive clone was identified by restriction enzyme diagnostics with *EcoR1* and *Apa* and automated DNA sequencing. A 650 ml LB-amp overnight culture originating from 2 ml of fresh overnight culture was grown at 250 rpm at 37°C. The DNA from the overnight culture was purified using a Qiagen Plasmid Maxi Purification Kit. The DNA was sterilized prior to transfection using ethanol precipitation. The DNA was suspended in 1/3 volume NH₄AC, and 3 x volume of EtOH, was placed at -80°C for 15 minutes, and centrifuged at 17 000 rpm at 4°C for 10 minutes. The pellet was washed with ice cold
70% EtOH and air dried in a laminar flow hood. The dried pellet was resuspended in its original volume of ddH₂O to maintain its concentration and stored at 4°C.

2.3.2 COS-7 Growth Conditions

COS-7 cells were grown in α-MEM (α-Minimal Essential Medium) from Media Preparation Services, University of Toronto, 10% Fetal Bovine Serum (FBS) from Gibco BRL, and 0.2 μg of streptomycin per ml of media and 0.2 units of penicillin per ml of media (Gibco BRL) in an atmosphere of 5% CO₂ and 95% air at 37°C. Cells were split by trypsinization at confluency.

2.3.3 Transfection

A modification of the protocol outlined by Gibco BRL was used. COS-7 cells were split 24 hours prior to transfection in order to obtain approximately 70 to 80% confluency on day of transfection. Two polystyrene tubes were prepared for each 100 mm plate. Tube A contained 10 μg of DNA in 800 μl of Opti-MEM I (Media Preparation Services, University of Toronto). Tube B contained 48 μl of lipofectamine in 800 μl of Opti-MEM I. Then, tubes A and B were mixed together and incubated at room temperature for 45 minutes. This was followed by an addition of 6.4 ml Opti-Mem I. Plated cells were rinsed with Opti-Mem I, and the plasmid lipofectamine mixture was distributed over the cells. The cells were incubated at room temperature for 5 hours, and
then 8 ml of media with 20% FBS was added. The cells were incubated overnight. The following day, the media was changed to DMEM+10%FBS +0.2 µg of streptomycin and 0.2 units of penicillin per ml of media. Cells were collected at 48 and 72 hours after transfection by scraping, and lysates were prepared as described above for the bacterial culture experiment. However, for this experiment the cells were sonicated twice with 30 second intervals. A negative control of cells transfected without DNA was also used. Lysates were analyzed for protein content and activity using Western immunoblotting and HPLC assay as described.
SECTION 3 RESULTS

3.1 DAC HPLC Enzymatic Activity Assay

Human liver microsomes from kidney transplant donors (KDL) were used to establish a DAC enzymatic activity assay. DAC enzymatic activity was analyzed through the use of the substrate AAF, which is converted to AF by DAC. HPLC was used to detect and quantify the assay’s results.

Human liver microsomes from KDL26 were used for the enzymatic assay, and the reaction rate for the KDL26 microsomes was determined to be 0.35 nmoles of AF formed per minute per mg of total microsomal protein. The HPLC chromatographs of the control samples also indicated the formation of AF (0.001 nmoles per minute) (Figure 3.1). AAF is acid labile and experiments performed in the absence of enzyme revealed that this AF peak is attributed to the non-enzymatic hydrolysis of AAF by the acid used to terminate the enzyme reactions. Multiple experiments determined that this non-enzymatic hydrolysis peak is consistent over time and therefore both reproducible and predictable. This non-enzymatic peak in control samples was therefore subtracted from enzyme incubated samples to correct for hydrolysis. The absolute detection limit for this assay was determined to be 0.15 nmoles per mg protein per minute.
Figure 3.1: HPLC chromatographs for the DAC enzymatic activity assay. The chromatographs depict the deacetylation of AFF to AF by illustrating an increase in AF levels relative to the controls. AAF had a retention time of 5.7 minutes, and AF had a retention time of 1.65 minutes. An AAF concentration of 500 μM was used (x-axis, minutes; y-axis, volts).
3.2 Antibody Titre Experiments

In order to detect and quantify immunoreactive protein with high sensitivity and specificity, a polyclonal anti-human DAC antiserum was created. The antibody was created using solubilized DAC-GST (glutathione S-transferase) fusion protein from the DAC pGEX XA90 clone (See Section 3.4.4). The solubilized fusion protein was injected into two male New Zealand white rabbits, referred to as R8711 and R8734.

Prior to protein injection a preimmune serum sample was obtained from each rabbit to identify any bacterial proteins recognized by the rabbits’ serum. Following DAC-GST protein injection, serum samples were obtained at regular intervals to assess titre, as outlined in Section 2.2.19. The titre of the sera was determined using Western blot analysis. The preimmune and antibody sera were diluted to 1/1000, 1/2000, 1/4000, 1/8000, and 1/16 000 in 1 x TNT and used to probe a blot with a DAC-GST protein concentration of 1 μg/cm nitrocellulose. By day 68, the titre of the sera had reached sufficient levels as determined by Western blot analysis, and the rabbits were exsanguinated. The optimal serum concentration for both R8711 and R8734 was determined to be a 1/16 000 dilution for 0.1 μg/cm nitrocellulose. This concentration of serum did not enable the detection of any bacterial proteins, but enabled the DAC-GST protein to be recognized with a high degree of sensitivity and specificity. The optimal blot exposure time for a DAC-GST protein concentration of 1 μg/cm nitrocellulose and a serum concentration of 1/16 000 was also assessed and determined to be 1 minute.
Finally, a comparison between the titre of this serum and the serum donated by Probst was performed. It was determined that this new antibody had a significantly higher titre and was more sensitive (Figure 3.2).

**Figure 3.2:** Western immunoblot to assess the titre of the polyclonal anti-human DAC GST antiserum. A DAC-GST protein concentration of 1 μg/cm nitrocellulose was used with an exposure time of 1 minute. The optimal serum concentration was determined to be 1/16 000 for the above concentration and exposure time.
Legend:

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(P.I.: Preimmune serum)
3.3 DNA Sequencing Results Revealing Variations Between the Published and Cloned DNA Sequence

DNA sequencing of the DAC pKEN2 DH5α clone created by Rano Grewal revealed 5 nucleotide differences between the cloned and the published DNA sequence. In order to confirm these variations and ensure that they were not artifacts of the EC-PCR amplification, the clone was reconstructed. First, the cDNA insert of DAC obtained from Probst was again amplified using expression cassette PCR (Figure 3.3) and resubcloned into pKEN2. DNA sequencing of six different reconstructed clones revealed the same 5 nucleotide differences between the cloned and the published DNA sequence (Figure 3.4).

These nucleotide differences were further substantiated through the creation of DAC expression cassettes from two additional human liver mRNA samples. The mRNA was RT-PCR amplified to create the expression cassette for DAC and then subcloned into pKEN2 (See Sections 2.2.2 and 2.2.5). DNA sequencing of these clones again revealed the identical 5 nucleotide differences. Therefore we conclude that the published DAC cDNA sequence (Probst et al. 1994) is incorrect.
Figure 3.3: Expression cassette PCR amplification of DAC. The positive PCR signals observed in lanes 1 through 4 of 1.2 kb are consistent with the open reading frame of DAC and were used to create the DAC constructs. The DAC-5' and DAC-3' expression cassette primers were used for PCR amplification (λ BSTE II Marker; B, blank).

<table>
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<tr>
<th>GCT</th>
<th>CCA</th>
<th>TGG</th>
<th>GAG</th>
<th>TTC</th>
<th>-C-</th>
<th>ATT</th>
<th>TTT</th>
</tr>
</thead>
</table>

Figure 3.4: The 5 nucleotide differences observed between the published and RT-PCR amplified DAC DNA sequence. The upper sequence is the published DNA result, ranging from nucleotides 153 to 174, and the lower sequence is the corresponding RT-PCR amplified DAC cDNA sequence. Nucleotide numbers refer to their location in the coding region of the published DAC DNA sequence.
3.4 **Heterologous Expression Studies**

A strategy to create a heterologous expression system for DAC was pursued in order to provide sufficient quantities of DAC to allow for a detailed biochemical examination of its potential influence on the action and toxicity of aromatic amines. The following vectors were used to create the constructs for heterologous expression: pKEN2, pET-3a, pBS, and pGEX. The experiments to create the DAC pKEN2 construct were initially performed by Rano Grewal.

3.4.1 **Heterologous Expression of DAC Using the Expression Vector pKEN2**

The expression vector pKEN2 was used to create a construct capable of expressing DAC protein. DAC expression cassettes created by PCR amplification and the vector pKEN2 were restriction endonuclease digested with EcoRI and SalI and ligated together. The construct was then transformed into the bacterial strain XA90. Sequencing results from twenty positive clones revealed that the pKEN2 vector had been digested at an EcoRI restriction endonuclease star site, resulting in improper insertion of the expression cassette and failure to express functional protein (Figure 3.5).

To eliminate the problem of EcoRI star activity, various reaction conditions were altered. Restriction endonuclease digestion time was limited to 1.5 hours, and EcoRI concentrations were reduced to as little as 2 units of enzyme activity per ng of DNA.
Figure 3.5: Illustration of the restriction endonuclease digestion of pKEN2 at an EcoRI star site. Restriction endonuclease digestion at this star site resulted in the improper insertion of the expression cassette and inability to detect expressed protein.
Magnesium salt concentrations were also manipulated. Both double and serial restriction endonuclease digestions were attempted in order to ensure salt concentrations for each restriction endonuclease was optimal. However, DNA sequencing of over 40 clones created using the limited restriction enzyme concentration, digestion times, and optimal salt concentration still revealed star activity.

3.4.2 Heterologous Expression of DAC Using the Expression Vector pET-3a

Since the manipulation of restriction endonuclease reaction conditions did not alleviate the problem of star activity with EcoRI, the creation of clones using the alternative expression vector pET-3a was attempted. The PCR amplified DAC expression cassette was subcloned into pET-3a following restriction endonuclease digestion with NdeI. However, for three separate cloning experiments, the DAC fragment would not ligate to the pET-3a vector. Diagnostic agarose gels of the restriction endonuclease digestion of 16 clones with NdeI showed a single band of 5.7 kb. This was consistent with the size of the pET-3a vector, indicating that the clones were empty (Figure 3.6).
Figure 3.6:  Restriction endonuclease digestions of DAC pET-3a clones with Nde1. The diagnostic agarose gels indicate that the pET-3a vectors are empty with a single band at 5.7 kb (*, empty vector: UC, uncut vector).
3.4.3 Heterologous Expression of DAC Using the Cloning Vector pBS

The cloning vector pBS was also used to create constructs for the heterologous expression of DAC. This vector was used as an intermediate in order to improve the effective restriction endonuclease excision of the coding region of DAC. The expression cassette of DAC and the cloning vector pBS were restriction endonuclease digested with SmaI, ligated together, and transformed into DH5α.

The DAC pBS constructs were restriction endonuclease digested with EcoRI and SalI. Correct constructs should release a 1.2 kb fragment, consistent with the open reading frame of DAC. However, restriction endonuclease digestion of the constructs did not result in the excision of a DAC cDNA insert but resulted in a single band of 4.1 kb, consistent with the open reading frame of the coding region of DAC and the vector pBS. Restriction endonuclease diagnostic experiments revealed that the EcoRI restriction endonuclease site was not functional (Figure 3.7).
Figure 3.7: Restriction endonuclease digestion of DAC pBS clones with *EcoRI* and *Sall*. The diagnostic agarose gels indicate the successful ligation of the DAC expression cassette and pBS vector, with a single band at 4.1 kb (DAC 1.2 kb; pBS 2.9 kb). However, the DAC insert could not be excised from pBS. Gel b indicates that the *EcoRI* site of the clone was not functional (*uncut DAC pBS clone*).
3.4.4 Heterologous Expression of DAC Using the Expression Vector pGEX

Because of the difficulty we experienced in creating clones that produce functional native DAC protein, and the differences observed between the published and sequenced DNA results, DAC fusion protein expression cassettes from two different liver samples, KDL19 and KDL36, were created. The expression cassettes were created using the RT-PCR protocol outlined in Section 2.2.2 (Figure 3.8), and were then used to create a DAC-GST fusion protein heterologous expression system using the vector pGEX.

Positive clones were identified using restriction enzyme digestion with EcoRI and Sall, resulting in the release of a 1.2 kb fragment, consistent with the open reading frame of DAC (Figure 3.9). Two positively identified clones from each KDL sample were DNA sequenced for confirmation and were determined to have identical sequences to the published DNA results except for the nucleotides between 157 and 171 (Figure 3.10). These nucleotide differences were consistent with differences observed in Section 3.3. In addition, an enzymatic activity assay was performed on this clone, but no DAC activity was observed. However, activity was observed for the positive control of human liver microsomes, indicating that the assay worked (Figure 3.11). A Western immunoblot was also performed to identify DAC-GST fusion protein induction. The blots identified DAC-GST protein induction with a 71 kDa band, consistent with the molecular weight of DAC-GST fusion protein (Figure 3.12).
Figure 3.8: RT-PCR results for the human liver samples KDL19 and KDL36. The agarose blots identify a 1.2 kb band consistent with the open reading frame of DAC. The multiple lanes are identical RT-PCR reactions (B, blank).
(a) *EcoRI* and *SalI* digestion of DAC pGEX clones using the KDL19 DAC expression cassette

(b) *EcoRI* and *SalI* digestion of DAC pGEX clones using the KDL36 DAC expression cassette

**Figure 3.9:** Restriction endonuclease digestion of DAC pGEX clones. Positive clones release a 1.2 kb when digested with these enzymes, consistent with the open reading frame of DAC (UC, uncut pGEX vector).
Figure 3.10: DNA alignment of the sequencing results for a positive DAC pGEX clone versus the published DAC coding sequence. The upper sequence is the DAC coding sequence, and the lower sequence is the DNA sequencing result. The differences observed in the DNA sequence of the clone range from nucleotides 157 to 171. Nucleotide numbering corresponds to their location in the coding region of the published DNA sequence. The remainder of the sequence is identical to the published sequence.
Figure 3.11: HPLC chromatographs of the DAC enzymatic assay using the DAC pGEX XA90 lysate. Deacetylase enzymatic activity was not observed for the construct. However, activity was observed for the positive control of human liver microsomes, indicating that the assay worked and suggesting that the induced DAC protein was not functional. Samples were incubated for 30 minutes at 37°C. An AAF concentration of 500 μM was used. AF had a retention time of 1.6 minutes, and AAF had a retention time of 5.4 minutes (x-axis, minutes; y-axis, volts).
Figure 3.12: Western immunoblot of DAC pGEX lysate. The blot compares the degree of induced DAC-GST fusion protein for 2 different clones, A and B. A significant increase in the amount of fusion protein produced is observed for the bacterial cultures induced with 1mM IPTG. The blot is probed with the DAC antibody provided by Probst. and the values under each lane represent the mM concentration of IPTG used to induce protein expression (S/N, DAC pGEX XA90 lysate supernatant; P, DAC pGEX XA90 lysate pellet; V, empty pGEX vector).
Since fusion protein was identified, but protein activity was not observed, the percentage of cells that contained plasmid at the time of induction and the percentage of cells producing protein following induction, were determined. Both were found to be 100% (Table 3.1). These results substantiate the above findings that DAC-GST fusion protein was being produced but was not functional.

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</tr>
</tbody>
</table>

Table 3.1: The percentage of plasmid bearing cells involved in protein production. Bacterial cultures were grown under various conditions using LB media and plated on LB media with differing components. The similar number of colonies on the LB and LB-amp plates indicated that 100% of the cells contain plasmid at the time of induction. In addition, the lack of colonies on the LB-amp plates with IPTG and significant number of colonies on the LB plates indicated that 100% of the plasmid are producing protein following induction.
3.4.5 Heterologous Expression of DAC Using Mammalian Cell Culture

The difficulties encountered with the heterologous expression of DAC in bacterial expression vectors may be because DAC is a mammalian protein and therefore, may require an unknown mammalian factor in order to function. As a result, heterologous expression of DAC using the mammalian cell line of COS-7 was also attempted.

Prior to transfection in COS-7 cells, a DAC pCNDA3 construct was created and transformed into DH5α. This construct was created because the high success rate of transformation in bacterial cell lines enables easy large scale plasmid purification.

The DAC pCDNA3 DH5α construct was created according to the methodology outlined in Sections 2.2.5 and 2.2.6. A positive clone was identified using diagnostic testing with the restriction endonucleases EcoRI, ApaI, and NdeI (Figure 3.13), and DNA sequencing (Figure 3.14). A large-scale plasmid purification was performed on this positive clone, and the purified plasmid was transfected into COS-7 cells. The transfected cells were collected 48 and 72 hours post transfection and lysed.

Western immunoblotting was used to analyze the post transfection lysate for the presence of DAC protein. The blots were probed with the polyclonal anti-human DAC-GST antiserum, and did not identify any DAC protein. In addition, an HPLC assay was carried out (Figure 3.15). The reaction rates for the transfected and control COS-7 cells were determined to be 0.161 nmoles/min/mg and 0.155 nmoles/min/mg respectively, a difference of only 0.006 nmoles/min/mg. This indicates that the deacetylation observed
Figure 3.13: Restriction endonuclease digestion of the DAC pCDNA3 construct. Positive clones were identified by the release of a 1.2 kb band, consistent with the open reading frame of DAC (M. λ BSTE II marker; E/A. restriction endonuclease digestion of the DAC pCDNA3 clone with EcoRI and ApaI).
Figure 3.14: DNA alignment of the sequencing results for a DAC pCDNA3 clone versus the published DAC DNA sequence. The lower sequence is the DAC coding sequence and the upper sequence is the DNA sequencing result. The differences observed between the two sequences (nucleotide numbers 210 through 225) are identical to the 5 nucleotide differences observed in Section 3.3. Nucleotide numbering corresponds to their location in the DNA sequencing result. The remainder of the sequence is identical to the published DNA sequence.
Figure 3.15: HPLC chromatographs of the DAC enzymatic assay using DAC pCDNA3 COS-7 lysate. Deacetylase activity was observed for the positive control of human liver microsomes but not for the negative control of human liver microsomes, indicating that the assay worked. Deacetylase activity was also observed for both the experimental lysate and negative lysate control. The reaction rates for the transfected and control cells were 0.161 nmoles/min/mg and 0.155 nmoles/min/mg respectively, a difference of 0.006 nmoles/min/mg. This indicates that the deacetylation observed is not the result of DAC activity but likely the result of an alternate deacetylase present in the cell line. An AAF concentration of 500 μM was used, and the samples were incubated at 37°C for 30 minutes. AF had a retention time of 1.7 minutes, and AAF had a retention time of 6.2 minutes (x-axis, minutes; y-axis, volts).
in the enzymatic activity assay is not a result of transient expression of human DAC but likely the result of a functional AAF deacetylase present in the COS-7 cell line.

3.5 DAC Protein Purification for Creation of the Polyclonal Anti-Human DAC Antiserum

Purified DAC-GST protein was used for the production of a polyclonal anti-human DAC antiserum (See Section 3.2). The antigen of DAC-GST was isolated from the DAC pGEX construct that was found to have the correct DNA sequence and the maximum protein expression. Protein expression was analyzed using Western immunoblotting.

The DAC-GST protein used for the Western immunoblot originated from DAC pGEX DH5α bacterial cultures grown in either LB-amp or 2 x YT-amp at room temperature. The immunoblot’s results indicated that bacterial cultures grown in the nutrient rich media 2 x YT produced higher concentrations of fusion protein. All subsequent cultures were grown in this media (Figure 3.16).

The concentration of purified DAC protein from bacterial cultures grown in the two medias was also compared. Bradford analyses indicated a twofold increase in the amount of DAC protein purified from bacterial cultures grown in 2 x YT-amp. However, despite using culture sizes of 600 ml, the concentration of purified DAC protein was too low for antibody production, yielding between 33 and 36 μg of protein.
Figure 3.16: Western immunoblot comparing the growth of DAC pGEX DH5α in LB-amp and 2 x YT-amp media. The 2 x YT-amp media resulted in significantly more fusion protein production than the LB-amp sample, particularly in the insoluble pellet fraction. The immunoblot was probed with GST antibody at a concentration of 1/4000 (M, Molecular Weight Marker; S/N, DAC pGEX DH5α lysate supernatant fraction; P, DAC pGEX DH5α lysate pellet fraction; L, post load supernatant; W, 1 xPBS wash of bound fusion protein; E, elute fraction of DAC protein; B, Glutathione Sepharose 4B beads following thrombin cleavage; V, empty pGEX lysate).
In order to increase the production and concentration of DAC protein available for purification, the DAC pGEX construct was transformed into the bacterial expression strain XA90. The bacterial cultures were grown using the optimized growth conditions determined above. The amount of DAC protein purified was compared to the results obtained for the DAC pGEX DH5α clone and was determined through Bradford analysis to have doubled (Figure 3.17). However, the amount of purified DAC protein was still too low for antibody production, with a yield of only 63 µg.

The Western immunoblots for both the DAC pGEX XA90 and DAC pGEX DH5α clones indicated that the insoluble fraction of the lysate had significantly higher levels of fusion protein than the soluble fraction (Figure 3.16: Figure 3.17). Therefore, in order to increase the yield of purified DAC protein, the insoluble fraction of the DAC-GST fusion protein was solubilized. A Coomassie blot using the solubilized DAC-GST protein indicated that the experiment was successful (Figure 3.18).

Purification and isolation of the DAC protein from the solubilized DAC-GST fusion protein was then attempted, but the solubilized fusion protein would not bind to the Glutathione Sepharose 4B beads (Figure 3.19). Therefore, the insoluble DAC-GST protein was solubilized again (Figure 3.20) and was used as the antigen to generate a polyclonal anti-human DAC-GST antiserum. Finally, a Western blot of DAC pGEX XA90 lysate was performed and probed with the DAC-GST antiserum to confirm protein induction (Figure 3.21).
Figure 3.17: Western immunoblot of DAC protein purified from the DAC pGEX XA90 clone. The blot indicates that the insoluble fraction (pellet) contains a greater concentration of fusion protein than the soluble fraction (supernatant). The immunoblot was probed with GST antibody at a concentration of 1/4000 (M, Molecular Weight Marker; S/N, DAC pGEX DH5α lysate supernatant fraction; P, DAC pGEX DH5α lysate pellet fraction; L, post load supernatant; W, 1 xPBS wash of bound fusion protein; E, elute fraction of DAC protein; B, Glutathione Sepharose 4B beads once following thrombin cleavage; V, empty pGEX lysate; Blot exposure time, 2 minutes).
Figure 3.18: Coomassie stain of the solubilization of DAC-GST fusion protein. The distinct band in the Dialyzed solution lane at 71 kDa is consistent with the known molecular weight of the DAC-GST fusion protein (M. Molecular Weight Marker; S/N, DAC pGEX DH5α lysate supernatant fraction, RS. lysate suspension resuspended in RIPA; RP, pelleted lysate resuspended in RIPA; D, dialysis solution; V empty pGEX lysate).
Figure 3.19: Western immunoblot of the purification of solubilized DAC-GST fusion protein. The large amount of protein remaining in the post load supernatant lane indicates that the solubilized DAC-GST fusion protein would not bind to the Glutathione Sepharose 4B beads. As a result, DAC protein could not be purified from the fusion protein. The immunoblot is probed with Probst's DAC antibody at a concentration of 1/1000 (M, Molecular Weight Marker; L, post load supernatant; W, 1 xPBS wash of bound fusion protein; E, elute fraction of DAC protein; B, Glutathione Sepharose 4B beads following thrombin cleavage; V, empty pGEX lysate).
Figure 3.20: Coomassie stain of the solubilization of the DAC-GST fusion protein. The distinct band in the elute supernatant lane at 71 kDa is consistent with the known molecular weight of the DAC-GST fusion protein (M, Molecular Weight Marker; SUS, DAC pGEX DH5α suspension; SON, DAC pGEX DH5α sonicate; S/N, DAC pGEX DH5α lysate supernatant fraction; R, suspension of DAC-GST lysate in RIPA; RS, supernatant fraction of DAC-GST lysate suspension in RIPA; E, elute fraction of solubilized DAC-GST protein; ES/N, supernatant fraction the eluted DAC-GST fusion protein; EP, pellet fraction of the eluted DAC-GST fusion protein; V, empty pGEX lysate).
Figure 3.21: Western immunoblot depicting the induction of DAC-GST fusion protein with varying growth conditions and fusion protein concentrations. A significant increase in DAC-GST fusion protein is observed for the cultures induced with IPTG. Lysate for blots a and b were grown at 37°C. Lysate for blots c and d were grown at room temperature. Blots a and c contain 10 μg of protein. Blots b and d contain 0.05 μg of protein. The blots are probed with new DAC-GST antiserum. The values below each lane represent the mM concentration of IPTG used for DAC-GST protein induction (M, Molecular Weight Marker; D, lysate created with DAC pGEX XA90; V, lysate created with empty pGEX vector).
SECTION 4 DISCUSSION

4.1 Overview

A large body of research has established that NATs are important determinants of the biotransformation and metabolic activation of arylamines, leading to both toxicological and carcinogenic consequences. A lesser-appreciated fact, however, is that acetylation by NATs may be directly counteracted by the process of enzymatic deacetylation, catalyzed by DAC, which converts the NAT metabolite back to its primary arylamine. Therefore, the DAC pathway has the potential to markedly affect the disposition, metabolite profile, and hence the pharmacological and toxicological consequences of exposure to arylamines.

The overall goal of the research to which this thesis contributes is to better understand the relative contributions of NATs and DAC to the complex interplay of pathways that result in the formation of metabolic products with a variety of fates within the body. As a means of laying the groundwork for these investigations, this thesis presents experiments aimed at the development of sensitive enzyme assays, immunologic reagents, and heterologous expression systems that will be essential elements in this work. We have presented the successful accomplishment of the first two of these three project goals, and have encountered a variety of unexpected obstacles to devising a robust heterologous expression system for functional DAC.
4.2 DAC HPLC Enzymatic Activity Assay

HPLC is a fast, reliable, sensitive, reproducible, and automatable method by which to separate and identify compounds and examine enzymatic reactions (Ettre 1980). Literally hundreds of enzymatic reactions have been monitored using this technique, including a large number of assays that have been developed for measuring the activity of enzymes of drug biotransformation. In the present work, a sensitive HPLC enzymatic assay was developed that enables the examination of DAC based upon its known ability to catalyze the formation of AF from AAF. HPLC had been used previously to measure the NAT-dependent formation of AAF from AF (Grant et al. 1991); it is therefore logical that a similar procedure could be used to monitor the reverse reaction.

In principle, the sensitivity of HPLC-based detection of enzyme function is much greater than that of spectrophotometric methods due to the superior signal-to-noise ratio associated with sensitive in-line UV detectors. However, the AAF-DAC assay presented in this thesis is more limited in its sensitivity because of the high background of non-enzymatic AAF deacetylation that occurs due to hydrolysis in the presence of the perchloric acid used to terminate the reactions. We can partially compensate for this background by virtue of the fact that it appears to be quite highly predictable, and can therefore be subtracted from sample peaks. This process does, however, impart greater variability into assay results than would be observed in the absence of the background peak. One possible means for circumventing this problem would be to carefully titrate
the amount of perchloric acid required to terminate reactions, in order to minimize the
degree of acid hydrolysis that occurs. However, the amount of acid required varies,
depending upon the purity of the enzyme source. It should be noted that the function of
the acid is not only to stop the reaction but in doing so, to precipitate the protein, so that it
can be centrifuged out of the reaction and sample extraction can be avoided prior to
HPLC injection. Thus we have observed that efficient protein precipitation in impure
tissue extracts requires higher concentrations of acid, and results in more rapid post-
incubation acid hydrolysis of the substrate. An alternative solution would be to use
another method to terminate the enzyme reaction that does not require perchloric acid,
hence avoiding the hydrolysis issue. However, another method may require an extraction
step, which imparts greater variation into the assay than direct injection. Moreover, it
should be appreciated that an additional role of the perchloric acid in the terminated
reaction is to set up an ion-pair with the reaction substrate and product, which allows
them to elute from the HPLC column as sharp and highly resolved peaks in the
perchlorate-buffered HPLC mobile phase we employ.

An important application of the described general HPLC assay methodology is its
potential to be easily modified to accommodate the screening of other potential DAC
substrates – such as the N-acetylated metabolites of other arylamine carcinogens like 4-
aminobiphenyl, 2-naphthylamine, and benzidine. Because of the aromatic nature of all
arylamines and their metabolites, both their degrees of lipophilicity and their UV
absorbance characteristics make them ideal candidates for the use of standard reverse-phase HPLC chromatography coupled with substrate and/or product detection in the near UV range (254-280 nm). Thus future studies will involve the use of the described assay, with minor changes in mobile phase organic modifier and UV detection wavelength, to uncover additional DAC substrates.

4.3 DAC-GST Antibody Production

In order to be able to relate DAC catalytic activity with the level of its expression in tissues and in isolated expression system extracts, we created a polyclonal anti-human DAC-GST antibody using bacterially expressed fusion protein as the injected antigen. The fusion protein contained DAC coupled to the fusion partner GST. Several studies support this method of generating a highly specific and sensitive polyclonal antibody (Basu et al. 1997; Komori et al. 1997; Pakdel et al. 1994; Zhou et al. 1999), and research has shown that the GST protein is small enough not to interfere with antigen-antibody interactions (Williams et al. 1995). The titre experiments with the polyclonal rabbit antisera we raised established that it is extremely selective with high specificity for the bacterially expressed DAC-GST fusion protein on blots from SDS-PAGE gels.

The DAC antisera will allow us to quantify the amount of expressed DAC in various human tissues and in heterologous DAC expression systems as they become available to us, and to relate the amount of protein expression to the levels of enzyme
activity we observe in our HPLC-based assays as described above. The sera will also allow us to determine whether structurally related proteins are expressed in tissues from other species. Future studies will also be aimed at determining whether the antibody we have produced is able to recognize human DAC in fixed tissue sections, which would make it a valuable reagent for immunohistochemical localization of DAC expression at a cellular level in a variety of tissue. Finally, we will determine whether the sera are able to either directly inhibit or to immunoprecipitate the native functional DAC enzyme in enzyme incubations, which would give it value as a reagent for directly modulating DAC function in enzyme assays and/or in \textit{in vitro} toxicity tests such as mutagenicity or adduct formation studies.

4.4 Variations Between Published and Experimentally Determined DNA Sequences

In the course of our vector cloning experiments, we observed five nucleotide sequence differences between our clones and the literature sequence originally reported by Probst and coworkers (1994). Because such differences, if derived as artifacts of our PCR-based subcloning procedure, would adversely affect protein production from the coding-region DAC insert, we decided to perform repeat subcloning experiments from the original Probst clone and from two additional human liver mRNA isolates to determine the true identity of the nucleotides at these positions. The results of these
experiments indicate that both the Probst clone (from which the original published DAC sequence was obtained) and two other randomly chosen human DAC amplification products actually possess the altered sequence that we observed initially, and not the sequence present in GenBank. Thus the sequence originally submitted by Probst and coworkers is likely to be in error. Such errors are a relatively common occurrence, a fact that comes as no surprise when one considers the many factors that influence DNA sequence quality such as template purity and quantity, the success of template primer polymerase complex formation, nuclease contamination, priming artifacts, and GC compressions (Qiagen 1998).

4.5 Heterologous Expression of DAC

In vitro models are often used to obtain information that may predict the in vivo human situation. Although in vitro models are not perfect, they can be useful since they are readily available and the experiments are easily reproduced. In vitro methods are also advantageous for studying xenobiotic biotransformation since they enable a more detailed analysis of the biochemical characteristics and role of a particular enzyme. Many different in vitro systems for studying drug metabolism and protein expression are possible, including cellular, microsomal, and recombinant systems (Miners et al. 1994). Such systems have achieved intensive use by pharmaceutical companies, many of which
have developed automated, high-throughput *in vitro* systems for the screening of large numbers of lead compounds for their probable pathways of biotransformation.

Two types of cellular systems may be used for xenobiotic biotransformation investigation, human liver slices and human hepatocytes. These systems are advantageous because they contain the majority of the microsomal and cytosolic enzymes involved in biotransformation and enable the analysis of two endpoints, enzyme induction and hepatocellular toxicity. However, the systems also have disadvantages. It is difficult to isolate the effects of a particular enzyme, and the cell cultures generally must be primary and cannot be passaged. As well, the cell cultures are not easily frozen or thawed for assays, and usually, the cellular systems must be prepared from fresh tissue. Finally, only a restricted sampling of the variability in human biotransformation can be realized (Guengerich 2000).

An alternate approach is the use of human liver microsomes, which are derived from the subcellular fractionation of liver homogenates so as to enrich for the smooth endoplasmic reticulum fraction. The advantages of this system are that microsomes are easily prepared from either fresh or frozen liver tissue, are more readily available than fresh tissue slices, and can be frozen indefinitely without loss of enzymatic activity. In addition, the ease of storage and use of microsomes enables enzymatic variability within larger populations to be identified. A disadvantage is that in some cases microsomes do not facilitate the investigation of a protein independently of other competing enzyme
systems, and in other cases a desired interaction with components of other subcellular compartments (such as the cytoplasm) is prevented (Friedberg et al. 1999). In our case, because human liver microsomes may contain other deacetylases aside from DAC and this system does not enable us to examine DAC independently of other enzyme systems, they were only used as positive controls for the DAC HPLC enzymatic assay. In addition, supplies of human liver tissue (usually derived from life-supported organ donors at the time of cessation of circulation) are a relatively scarce resource and are becoming even more difficult to obtain as liver transplantation becomes more widespread.

Recombinant expression systems are an increasingly valuable tool for xenobiotic biotransformation research. Early in the field of enzymatic research, the purification of enzymes from human liver were limited by both tissue availability and technical difficulties due to the amount of protein purified. Heterologous expression alleviates these problems, enabling large amounts of protein to be easily purified, and this is why we chose to attempt development of a heterologous expression system as a preferred method for providing us with supplies of human DAC (Friedberg et al. 1999).

Many different types of heterologous expression systems exist, including bacterial and mammalian systems. Bacterial expression systems are advantageous for heterologous expression since they are well defined, stable, and readily available because of the ease of growth of bacterial cultures. They have the ability to produce large concentrations of protein that can be easily purified. In addition, the purified protein can
be used as an antigen for the preparation of polyclonal antibodies (Soucek et al. 1995).

However, this system also has its disadvantages. It is often difficult to obtain the correct molecular structure and substrate specificity of the human protein in bacterial expression systems, because of the requirement for post-translational modifications that may not occur in bacterial hosts, or the need for specific insertion into intracellular membranes that may not exist in bacteria (such as the endoplasmic reticulum) in order to achieve function (Wiebel et al. 1996). As a result, the cDNAs often have to be modified before expression is possible (Gold 1990). Because of the previous observation that human DAC, although a microsomal enzyme, does not appear to require insertion into membranes to be functionally active, we chose to attempt its expression in E. coli.

Mammalian cell lines are also advantageous for heterologous expression since they too are well defined, stable, and are readily available. In addition, they possess many of the proteins produced in vivo, as well as intact protein folding and post-translational protein modification machineries, if required. This could be important since proper folding, processing, and other protein-protein interactions may be required for protein functionality. Mammalian cell lines are disadvantageous because they generally have lower levels of protein expression, requiring the development of more sensitive assays and requiring larger batch volumes of more expensive reagents if the goal is high-level protein production and isolation. Also, transient expression systems such as African green monkey (COS-7) cells require transfection each time expression studies are done, a
costly and tedious procedure, and low transfection efficiencies generally lead to quite modest levels of protein production. However, the latter can also be advantageous for research since it enables selection for positive clones, and there is little variability between the transfected cells (Wiebel et al. 1996). Because of the possibility that failure to detect DAC activity in bacteria could be due to improper protein folding, processing or membrane insertion in the prokaryotic host, we chose to investigate the use of a transient mammalian expression system to detect DAC activity.

4.5.1 Heterologous Expression of DAC Using the Expression Vector pKEN2

pKEN2 is a bacterial expression vector that has a tac promoter, a derivative of the lac operon. Protein expression is regulated by lactose levels and is induced when IPTG is added to the bacterial culture. Therefore, this system enables tight regulation of protein production, and we chose to use it for the heterologous expression of DAC.

DNA sequencing of the DAC pKEN2 clones revealed that the vector had been digested at a star site, resulting in disruption of the promoter region and rendering DAC protein production impossible. We were unable to optimize reaction conditions to minimize star activity, since all clones were shown to be ligated at the EcoRI star site depicted in Figure 3.5. For this restriction endonuclease, star activity is an uncommon occurrence when reaction conditions are optimized and therefore, it is believed that the difficulty experienced creating a correct construct is the result of an alternate factor such
as the death of cells containing correctly ligated clones or possibly, that DAC is unclonable.

It is possible that correct constructs may have been created and did not survive since, as described. restriction endonucleases strictly recognize specific nucleotide sequences within DNA under optimal reaction conditions. Research by Lesser et al. (1990) has shown that under optimal conditions, the difference in cleavage rates between the cognitive site and the star site is very high. For example, a single nucleotide change in the recognition site can result in differential cleavage rates in the order of $10^5$.

The death of cells containing correct constructs may be the result of the target protein interfering with gene expression or with the integrity of the cell, hindering both protein synthesis and cell survival (Baneyx 1999). In addition, large amounts of DAC production may have overwhelmed the cell. This would lead to severe metabolic stress and depletion of the cellular energy and metabolic precursors, resulting in cell death (Borth et al. 1998). This is not an uncommon result with heterologous expression and is supported by other published research. For example, Shuster et al. (1989) has shown that heterologous expression of extracellular human IGF-I in yeast is toxic to the host cell, and Miroux and Walker (1996) have shown that heterologous expression of seven different membrane and ten different globular proteins in E. coli also results in host cell death.
An alternate explanation for these difficulties is that DAC may be an inherently unclonable sequence. The DNA structure of DAC may render it unable to ligate to a correctly digested cloning vector. Unclonable DNA fragments have been identified. For example, fragments of human mitochondrial DNA have been determined to be unclonable (Drouin 1980) and recombinant clones were able to be created and identified only by DNA mutation (Mita et al. 1988).

4.5.2 Heterologous Expression of DAC Using the Expression Vector pET-3a

PET-3a is a bacterial expression vector that contains a prophage which encodes an enzyme under the control of an IPTG-inducible lac promoter. This enzyme induces the production of T7 RNA polymerase. The T7 RNA polymerase leads to the synthesis of large amounts of mRNA and subsequently, the production of large amounts of heterologous protein. Heterologous protein concentrations often comprise as much as 40 to 50% of total cellular protein (Baneyx 1999).

Experiments revealed that DAC could not be successfully ligated into the pET-3a vector, and only self-ligating constructs were identified. We again believe that correct constructs may have been created, but that cells containing correct constructs did not survive. There are several possible explanations for this, including those addressed in Section 4.5.1. Another possible explanation is that high levels of mRNA can cause
ribosome destruction and leaky expression of T7 RNA polymerase, leading to plasmid or expression instability, both of which can lead to cell death (Baneyx 1999).

4.5.3 Heterologous Expression of DAC Using the Cloning Vector pBS

The cloning vector pBS was used as an intermediary for subcloning the DAC expression cassette into pKEN2. DAC expression cassettes were restriction endonuclease digested with Smal and therefore, blunt ended. Once ligated into pBS, the expression cassettes were excised using the restriction endonuclease EcoRI and SalI which would result in an expression cassette with cohesive ends. This second digestion was performed to ensure the expression cassettes were digested prior to ligation with pKEN2 since restriction endonuclease digestion is not optimal at the ends of small pieces of DNA. In addition, ligation efficiency increases with cohesive fragments (Nath and Azzolina 1981).

Diagnostic agarose gels indicated that the DAC expression cassette could not be excised from DAC pBS clones with restriction endonucleases EcoRI and SalI. Restriction endonuclease experiments revealed that the EcoRI restriction site was not functional. The destruction of the EcoRI site may be the result of methylation. When DNA is cleaved by a restriction endonuclease the amino groups are methylated (Woodbury and Von Hippel 1981). This methylation has been shown to block site-specific binding and therefore cleavage by the endonuclease (Jack et al. 1981).
4.5.4 Heterologous Expression of DAC Using the Expression Vector pGEX

Fusion proteins were originally created to aid in protein purification and immobilization. However, research determined that certain fusion proteins could improve the solubility of passenger proteins that otherwise accumulated in the inclusion bodies of the cell. It is believed that fusion proteins reduce the formation of inclusion bodies by helping the heterologous protein achieve its native conformation. However, this is not always the case, and not all fusion systems are capable of eliminating the inclusion body difficulty (Baneyx 1999).

The pGEX vector was chosen for heterologous expression of DAC for the aforementioned reasons and because it also enables the creation of an integrated system for the expression, purification, and detection of proteins produced in E. coli. Furthermore, it is an ideal expression system for antibody production (Smith and Johnson 1988).

Constructs created using the pGEX vector result in a protein fused to GST. GST is a 27 kDa protein and has been shown to have complete enzymatic activity in E. coli. Fusion proteins that contain the complete GST amino acid sequence have also been shown to exhibit enzymatic activity (Parker et al. 1990; Ji et al. 1992; Maru et al. 1996).

Fusion proteins are easily purified from bacterial lysate by affinity chromatography using Glutathione Sepharose 4B. The GST fusion protein is cleaved
using the site-specific protease thrombin whose recognition sequence is located upstream of the multiple cloning site of the pGEX vector. The fusion protein contains a lacP repressor gene that tightly controls a tac promoter. The addition of IPTG results in induction of the tac promoter and leads to high-level protein expression (Pharmacia 1997).

Western immunoblot analysis of DAC pGEX XA90 lysate identified the presence of DAC-GST fusion protein. These results suggest that the GST moiety influenced the three dimensional structure of the DAC protein so that it was no longer toxic to the host cell nor was it expressed at excessive levels that lead to host cell death (Wiebel et al. 1993). However, the protein was determined to be in both the soluble and insoluble fractions of DAC pGEX lysate, despite the fact that the pGEX system was designed to generate soluble fusion protein. Since GST is water soluble, the fusion protein generated is usually in the soluble fraction of lysate. However, in this case, the DAC-GST fusion generated was determined to be in both fractions, with the insoluble fraction containing the greatest amounts of fusion protein.

Insoluble fusion proteins are a common occurrence when generating fusion proteins (Komori et al. 1997; Soler et al. 1995; Sundaram and Brandsma 1996; Yuan et al. 1997) and may be the result of protein aggregation of an incompletely folded fusion polypeptide. In addition, highly regulated promoters such as the tac promoter, can lead to the formation of insoluble protein and inclusion bodies since large amounts of protein
amass quickly in the bacteria and the polypeptides readily aggregate together
(Grisshammer and Nagai 1995).

Growth conditions can also affect the degree of solubilized protein production
(Zanette et al. 1998). Culture media, growth temperature, and bacterial strain were
manipulated to reduce inclusion body formation. However, the study leads us to
conclude that it was necessary to solubilize the fusion protein from the inclusion bodies
in order to purify significant concentrations of DAC.

Solubilization followed the methodology outlined by William et al. (1995). This
method uses 10% SDS and high heat for solubilization and has been shown to be an
extremely reliable method for solubilizing GST fusion proteins to be used for antibody
production. In addition, proteins solubilized in this manner tend to remain in solution
following the removal of the solubilizing agents (Williams et al. 1995), which is ideal
for both expression studies and antibody production.

Solubilization and purification of the DAC protein from the fusion protein was
unsuccessful since the solubilized fusion protein would not bind to the Glutathione
Sepharose 4B beads. This suggests that either the fusion protein had been denatured
during sonication or that it may have altered the conformation of the GST protein,
reducing its affinity to the beads (Pharmacia 1997). It is also possible that the SDS and
high heat used to solubilize the fusion protein may have resulted in denaturation of the
GST moiety (Grisshammer and Nagai 1995). Since solubilization and purification of
unfused DAC was not possible, solubilized DAC-GST fusion protein was used for the generation of the antiserum.

An enzymatic assay using lysate containing induced fusion protein did not exhibit DAC activity. We determined that 100% of the cells contained plasmid and made protein at the time of induction. Therefore, the lack of DAC activity could be attributed to many factors. For instance, the GST moiety may have influenced the three dimensional configuration of the protein which is required for its biological function (Wiebel et al. 1993). On the other hand, it may be the result of limitations of bacterial expression systems whereby the cDNA often has to be modified in order to obtain protein expression (Gold 1990). Finally, the protein’s insolubility could have influenced functionality since insoluble proteins tend to be inactive (Geisse et al. 1996).

4.5.5 Heterologous Expression of DAC Using Mammalian Cell Culture

Since functional protein was not observed in the bacterial heterologous expression systems, we hypothesized that DAC may require proper membrane insertion, eukaryotic post-translational modification systems, or possibly interaction with other endogenous proteins to be functional.

We selected the transient COS-7 expression system for heterologous expression of DAC. This system is usually selected for the production of smaller amounts of bioactive protein (Edwards and Aruffo 1993; Trill et al. 1995) and for the rapid

The COS-7 system is derived from the African Green Monkey cell line CV-1 and is driven by SV40 large T-antigen (Asselbergs 1992; Boast et al. 1983; Gerard and Gluzman 1985). Once transfected with an expression plasmid that contains a SV40 origin of replication, as is the case with expression vector pCDNA3, the T-antigen interacts with the origin of replication, which leads to protein production (Mellon et al. 1981).

Plasmid replication in COS-7 cells peaks at approximately 48 hours following transfection, and recombinant protein expression reaches its maximum at 72 hours following transfection (Edwards and Aruffo 1993). Therefore, the time line of 48 and 72 hours following transfection was selected for cell harvesting. After this period, the cells begin to loose plasmid copies and eventually die as a result of intolerance of the high levels of extrachromosomally replicating DNA (Gerard and Gluzman 1985). Because of this, the COS-7 system is not suitable for the production of large amounts of protein over a prolonged period of time.

A Western immunoblot and enzymatic activity assay was performed on the COS-7 cells transfected with the DAC pCDNA3 construct. The results indicated that DAC protein was not produced. Although deacetylase activity was detected in the assay, it is concluded that the activity is the result of an alternate deacetylase present in the COS-7
cell line since the same level of deacetylase activity was observed in empty COS-7 cells as well. Therefore, this system was unable to solve the cloning difficulties encountered with DAC. This also suggests that the toxicity of DAC protein production in heterologous systems extends over both prokaryotic and eukaryotic hosts. We are unaware of any other examples of this phenomenon, and the possible mechanisms by which this could occur are unclear to us.

4.5.6 Discussion of Heterologous Expression Studies

Although initial heterologous expression studies using pKEN2 and pET-3a suggested that the DAC DNA sequence could be inherently unclonable, it is more likely that DAC is difficult to clone because its expressed product is toxic to the host when heterologously expressed. This theory is substantiated by the fact that DAC protein was identified with the pGEX fusion system where the GST moiety likely influenced the DAC protein so that it was no longer deleterious. Mutational studies could alleviate this cloning problem and enable both heterologous expression and protein function. For instance, the active site of DAC could be mutated using site-directed mutagenesis. This would enable us to perform structure-function analyses and identify the amino acids that are responsible for the toxicity observed with heterologous expression.
4.6 Conclusions

The biological role of DAC is not completely understood. Its only known function is the deacetylation of N-acetylated arylamines. Research by Probst et al. (1994) determined that the DNA sequence of DAC shows significant homology to hormone sensitive lipases and carboxylesterases and should be classified as an esterase. Therefore, DAC may have biologically significant roles that are related to lipases and esterases. Future research could include immunohistological analysis of various mice tissue to identify the presence of DAC and the subsequent creation of DAC knock-out or knock-in mice. Since lipases are response for the mobilization of lipids, variations in the animal’s lipid levels could then be examined. The loss or introduction of DAC may influence these levels and the health of the animal. In addition, esterases have been identified in many cells and tissues and are responsible for the biotransformation of both endogenous and exogenous esters, amides, thioesters, and phosphoesters (Satoh 1987). DAC knock-out or knock-in mice could also be used to determine if DAC influences the biotransformation of these compounds. This research may determine that DAC is a critical factor in xenobiotic metabolism and detoxification and could influence the results of both epidemiological and risk assessment research.

Probst et al. (1994) has also identified DAC in fetal liver samples, suggesting that it may have an important endogenous role even prior to birth. For example, DAC may have endogenous substrates that require deacetylation for cellular survival. Determining
the role of DAC may also provide the reasons why it is so difficult to clone and why it appears to be toxic to host cells in heterologous expression systems.

Further research is required in order to answer the above questions and to fully elucidate the role of DAC as it pertains to its endogenous functions, NATs, and the metabolic activation of arylamines. The development of a sensitive HPLC enzymatic assay and a DAC-GST antiserum, together with the knowledge that DAC is a difficult to clone sequence, provide the preliminary work on which to base this research.
SECTION 5 SUMMARY

In order to provide tools to enable the elucidation of the role of DAC in the pharmacological, toxicological, and carcinogenic effects observed upon exposure to arylamines and their metabolites, a sensitive HPLC assay was developed to monitor enzyme activity, and a polyclonal anti-human DAC antiserum was created that enables the detection and quantification of immunoreactive DAC protein with high sensitivity and specificity. Heterologous expression of DAC was also attempted with both bacterial and mammalian expression systems. Numerous difficulties were encountered in creating the expression systems, such that functional protein could not be detected in any of the tested systems. Our results suggest that DAC may be difficult to clone in heterologous expression systems because it is toxic to host cells. Finally, DNA sequencing of these clones revealed that the published DNA sequence of DAC is incorrect.

Further research is required to fully elucidate the role of DAC as it pertains to its endogenous functions, NATs, and the metabolic activation of arylamines. The development of the HPLC assay and DAC-GST antiserum, together with the knowledge that DAC is a difficult to clone sequence, provide the preliminary work on which to base this research.
SECTION 6 REFERENCES


