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ANTISENSE AND GENE THERAPY APPROACHES AS AN ALTERNATIVE TO DISULFIRAM FOR THE TREATMENT OF ALCOHOL ABUSE

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A Dissertation Submitted In Partial

Fulfillment of the Requirements for the Degree

Doctor of Philosophy

Thomas Jefferson University

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Eric M. Garver

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ABSTRACT

A mutation in the gene encoding for mitochondrial aldehyde dehydrogenase (ALDH2) present in some Asians populations, lowers or abolishes the activity of this enzyme and results in marked elevations in blood acetaldehyde upon ethanol consumption. The resulting phenotype greatly protects against alcohol abuse and alcoholism. The protective Asian phenotype can be elicited in alcoholics who are administered the aversive medication disulfiram (Antabuse®), affording these individuals the same protection from alcohol abuse. However, disulfiram is a non-specific drug that binds to sulfhydryl groups and not only inhibits ALDH, but also dopamine-beta hydroxylase and other enzymes. In addition, disulfiram is associated with many side effects including sensory and motor neuropathies, optic neuritis, orthostatic hypotension and hypersensitivity reactions. Hence, a new aversive medication without the side effects of disulfiram would be a welcome development in the treatment of alcoholism. Antisense oligonucleotides (ASO's) provide a possible means by which ALDH2 gene expression can be specifically inhibited to yield the low-ALDH2 Asian phenotype, and opens the possibility for a new aversive medication. This thesis describes the discovery of an ASO that inhibits ALDH2 gene expression and provides strong proof that such an inhibition mimics the naturally-occuring ALDH2-2 Asian phenotype, evidenced by: (i) a low liver mitochondrial ALDH2-1 activity, (ii) elevated blood acetaldehyde levels following the administration of ethanol and (iii) an aversion to ethanol, shown as a reduction in alcohol consumption in a rat aversion model. In addition, a gene

transduction approach was investigated to assess the possibility of establishing a long-term inhibition of ALDH2 gene expression. In these studies, the negative dominant human mutant ALDH2-2 cDNA was transduced into a rat hepatoma cell line with a retrovirus, known to integrate its genetic load into the mammalian genome. Transduction of the mutated human ALDH2-2 gene significantly reduced the endogenous rat ALDH2 activity. These data suggest that gene therapy with the dominant negative mutant of ALDH2 may also be an effective alternative to develop a long-term aversion to ethanol. Overall, the studies presented here represent new in-roads into the development of an alternative aversive medication for the treatment of alcoholism.

DEDICATION

I wish to dedicate this thesis to

my loving wife Deanne Dulik Garver

for believing in me, for her unfaltering devotion, support, trust, faith, and above all else, for sticking with me through some rough times during this work.

I owe the success and completion of this work to you honey.

All my love to you always!

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TABLE OF CONTENTS

	PAGE
ABSTRACT	iv
ACKNOWLEDGEMENTS	vii
TABLE OF CONTENTS	viii
LIST OF FIGURES	xiii
LIST OF TABLES	xv
LIST OF SYMBOLS AND ABBREVIATIONS	xvi
Section I **INTRODUCTION**	1
Alcoholism	1
Treatments	2
Anticraving Medications	3
Aversive Medication	6
Disulfiram	8
Genetic Protection	9
Antisense	10
Occupancy Mechanisms	13
Occupancy of Translation Initiation Codon	13
Occupancy at Exon/Intron Boarders	13
Occupancy of Necessary RNA Structures	13
Occupancy at 5'-UTR, 3'-UTR or Polyadenylation Sites	14
Confirmation of Occupancy Mechanism	14
RNase Mediated Mechanism	15
Gene Therapy	17
Pre-Clinical Animal Model	18
Disadvantages of Available Animal Models	19
Retrovirus Opens Possibility of Gene Therapy	23
Normal Retrovirus Life-Cycle	25
Replication-Incompetent Retrovirus for Gene Therapy	26

Section II **MATERIALS AND METHODS (Supplement to manuscripts)..... 29**

Chemicals.......... 29

Cell Culture and Oligonucleotide Delivery.......... 29

Mitochondrial Isolations.......... 31

Determination of Mitochondrial ALDH and GDH Activity.......... 32

ALDH Assay Reagents.......... 32

GDH Assay Reagents.......... 34

Determination of Mitochondrial ALDH Half-Life by Cycloheximide.......... 34

Determination of mRNA Expression by Competitive RT-PCR.......... 36

ALDH mRNA.......... 36

GDH mRNA.......... 38

Cannula Implantation.......... 40

Osmotic Pump Implantation.......... 41

Acetaldehyde Determination by HPLC.......... 41

Section III **PARADIGM TO TEST A DRUG-INDUCED AVERSION TO ETHANOL.......... 44**

SUMMARY.......... 45

INTRODUCTION.......... 46

MATERIAL AND METHODS.......... 47

Plasma Acetaldehyde Levels.......... 48

Determination of Mitochondrial ALDH2 Activity.......... 49

RESULTS.......... 49

ALDH2 Activity and Acetaldehyde Levels.......... 50

DISCUSSION.......... 51

LEGEND TO FIGURES.......... 54

FOOTNOTES.......... 55

REFERENCES.......... 56

Section IV **ELICITING THE LOW-ACTIVITY ALDEHYDE DEHYDROGENASE ASIAN PHENOTYPE BY AN ANTISENSE MECHANISM RESULTS IN AN AVERSION TO ETHANOL** **62**

ABSTRACT 63

INTRODUCTION 64

MATERIALS AND METHODS 66

Chemicals 66

Antisense Oligonucleotides 66

Cell Culture 66

In vitro Oligonucleotide Delivery 66

Mitochondrial Isolations 67

Determination of ALDH and GDH Activity 68

Determination of ALDH2 Half-Life by Cycloheximide (CHX) 69

Sequencing of H4-II-E-C3 ALDH2-1 cDNA by Reverse Trancriptase-Polymerase Chain Reaction (RT-PCR) 70

Determination of mRNA expression by Competitive RT-PCR 70

Animals and Treatment 73

Intravenous Administration of ASO-9 73

Intraperitoneal Administration of ASO-9 74

Acetaldehyde Determination by HPLC 74

Statistics 74

RESULTS 75

Low-Km Mitochondrial Aldehyde Dehydrogenase (ALDH2) in Rat Hepatoma Cells and the Liver of the Lewis Rat 75

Reduction in ALDH2-1 Activity of H4 Cells Incubated in the Presence of ASO-9 75

Effect of ASO-9 76

Reduction in ALDH2 mRNA Levels Induced by ASO-9 on H4 Cells 77

Specificity 77

Sequence 77

mRNA Levels 77

In vivo Inhibition of ALDH2 Activity and mRNA by Antisense Oligonucleotide ASO-9 77

Inhibition of Ethanol Consumption Following Treatment with ASO-9...... 78
DISCUSSION.......... 80
ACKNOWLEDGMENTS.......... 85
REFERENCES.......... 86
FIGURE LEGEND.......... 92

Section V **TRANSDUCTION OF RAT HEPATOMA H4-II-E-C3 CELLS WITH HUMAN ALDH2*1 OR ALDH2*2 cDNA**.......... **101**
ABSTRACT.......... 102
INTRODUCTION.......... 103
MATERIAL AND METHODS.......... 106
Vector Preparation.......... 106
Retroviral Vectors.......... 106
Control Vector Preparation.......... 108
Cell Culture.......... 109
Transfection of PA317 Cells.......... 109
Glycerol Shock.......... 110
Virus Collection.......... 111
Infection and Selection.......... 111
Assay Reagents.......... 112
Quantitation of Transgene by Competitive RT-PCR.......... 112
RESULTS.......... 116
Confirmation of Human ALDH2 cDNA in Vectors.......... 116
Control Vector Preparation.......... 117
ALDH Activity in Transduced H4-II-E-C3 Cells.......... 117
pLNCE (Wild-Type ALDH2*1).......... 118
pLHCK3'UT (Mutant ALDH2*2).......... 119
Trends in Transduced Clone Groups.......... 121
Glutamate Dehydrogenase Activity.......... 123
DISCUSSION.......... 125
REFERENCES.......... 129

Section VI **DISCUSSION.. 132**
Pre-Clinical Animal Model.. 132
Gene Regulation.. 135
Future Direction for ALDH2 Antisense... 141
Gene Therapy.. 142
Further Research Suggestions... 142
Considerations and risk assessment of aversive therapy that results in Acetaldehyde accumulations due to ALDH2*1 activity reduction........... 143
REFERENCES... 149

LIST OF FIGURES

Section I **INTRODUCTION**

FIGURE		PAGE
1.1	Ethanol Metabolism	6
1.2	Antisense Design	11
1.3	Translation Arrest by Antisense	12
1.4	DNA with Phosphate Backbone or Phosphorothioate Modification	16
1.5	Antisense Delivery to the Cell and RNase H Activation	17
1.6	Normal Retrovirus Life-Cycle	24
1.7	Retroviral Genome Orientation	25
1.8	PA317 Packaging Cell Used with Replication-Defective Retroviral Vectors Containing Human ALDH2*1 (pLNCE) or ALDH2*2 (pLHCK3'UT) cDNA (Transgenes)	27

Section II **MATERIAL AND METHODS (Supplement to Manuscripts)**

FIGURE		PAGE
2.1	Construction of Rat ALDH2 Internal Standard for Competitive RT-PCR	37
2.2	Construction of Rat GDH Internal Standard for Competitive RT-PCR	39
2.3	Alzet® Osmotic minipump (2ML1)	41

Section III **PARADIGM TO TEST A DRUG-INDUCED AVERSION TO ETHANOL**

FIGURE		PAGE
3.1	Aversion to Ethanol Induced by Disulfiram Administration	60
3.2	Alcohol Consumption and Acetaldehyde Levels in Disulfiram-Treated Rats	61

Section IV **ELICITING THE LOW-ACTIVITY ALDEHYDE DEHYDROGENASE ASIAN PHENOTYPE BY AN ANTISENSE MECHANISM RESULTS IN AN AVERSION TO ETHANOL**

FIGURE PAGE

4.1 Quantitation of ALDH2-1 and GDH Steady-State mRNA by Competitive RT-PCR.......... 98
4.2 Effect of ASO-9 on Ethanol Consumption Following an 18-hour Water Deprivation Period.......... 99
4.3 Comparison of the Inhibitory Effect on Ethanol Consumption of ASO-9 (24 mg/kg/day) for 4 Days (Data from present study) and of Disulfiram Administration (100 mg/kg/day) for 4 Days (Data from Garver et al 2000).......... 100

Section V **TRANSDUCTION OF RAT HEPATOMA H4-II-E-C3 CELLS WITH HUMAN ALDH2*1 OR ALDH2*2 cDNA**

FIGURE PAGE

5.1 Construction of Human ALDH2 Internal Standard for Competitive RT-PCR.......... 114
5.2 Confirmation of Human ALDH2 cDNA in Vectors.......... 116
5.3 Control Vector Preparation.......... 117
5.4 ALDH2 Activity in Vector Clone 14.......... 118
5.5 ALDH2 Activity in Wild-Type Clone 2.......... 119
5.6 ALDH2 Activity in Mutant Clone 9.......... 121
5.7 Trends in Transduced Clone Groups.......... 122
5.8 GDH Activity Comparison in Control H4-II-E-C3 Cells and Mutant Clone 7.......... 124

LIST OF TABLES

Section IV **ELICITING THE LOW-ACTIVITY ALDEHYDE DEHYDROGENASE ASIAN PHENOTYPE BY AN ANTISENSE MECHANISM RESULTS IN AN AVERSION TO**

TABLE PAGE

4.1 Effect of ASO-9 on the Activity of the Low-Km Aldehyde Dehydrogenase (ALDH2) in Mitochondria of H4-II-E-C3 Rat Hepatoma Cells.......... 94

4.2 Reduction of ALDH2 mRNA Levels by ASO-9.......... 95

4.3 Reduction of the Steady-State Levels of ALDH2-1 and GDH .... mRNA Following Treatment with PBS or ASO-9.......... 96

4.4 In Vivo Effect of ASO-9 Administration on the Activity of Liver ALDH2-1, GDH and Plasma Acetaldehyde Levels Following Administration of Ethanol (1 g/kg).......... 97

Section V **TRANSDUCTION OF RAT HEPATOMA H4-II-E-C3 CELLS WITH HUMAN ALDH2*1 OR ALDH2*2 cDNA**

TABLE PAGE

5.1 Summarization of Mitochondrial Aldehyde Dehydrogenase Specific Activities for Transduced H4-II-E-C3 Cells.......... 123

Section VI **DISCUSSION**

TABLE

6.1 Risk of Alcoholic Cirrhosis or Pancreatitis According to Genotype of Asian Alcoholics.......... 147

6.2 Risk of Cancer According to Genotype of Asians.......... 148

LIST OF SYMBOLS AND ABBREVIATIONS

AA	Alcoholics Anonymous
AA Rats	ALKO Alcohol Rats
ADH	Alcohol Dehydrogenase
ADP	Adenosine Diphosphate
ALDH	Aldehyde Dehydrogenase
ALDH2	Mitochondrial Aldehyde Dehydrogenase
ALDH2*1	Wild-Type or Normal Aldehyde Dehydrogenase
ALDH2*2	Mutant form of Aldehyde Dehydrogenase (Alcoholism Protection)
ASO	Antisense Oligonucleotide
ASO-9	Antisense Oligonucleotide Nine (Used as Lead Drug in Studies)
AUG	Translation Initiation Codon
AWS	Alcohol Withdrawl Syndrome
bp	Base-Pair
BSA	Bovine Serum Albumin
B.W.	Body Weight
CBT	Cognitive-Behavioral Therapy
CHX	Cycloheximide
CMV	Cytomegalovirus
DMEM	Delbecco's Modified Eagles Medium
DNP	2,4-Dinitrophenylhydrazine
DS	Disulfiram
DTT	Dithiothreitol
EDTA	Ethylene Diamine-Tetraacetic Acid
env	Envelope Glycoprotein of Retrovirus
gag	Group-Specific Antigen
GDH	Glutamate Dehydrogenase
Glu487	Glutamine at Amino Acid Position 487
H4	Rat Hepatoma Cell Line (H4-II-E-C3)
HAD	High Alcohol Drinking Rat Strain
HeBS	Hydroxyethylpiperazine-Ethanesulfonic Acid Buffered Saline
HEPES	Hydroxyethylpiperazine-Ethanesulfonic Acid
HIV	Human Immunodeficiency Virus
hnRNA	Heteronuclear Ribose Nucleic Acid
HPLC	High Pressure Liquid Chromatography
IN	Integrase
i.p.	Intraperitoneal
i.v.	Intravenous
K_{cat}	Maximum Catalysis Rate
K_m	Michaelis Constant
L	Leader Region Before Start of Retroviral Group-Specific Antigen
LB	Lauria Broth
LTR	Long-Terminal Repeat
Lys487	Lysine at Amino Acid Position 487
MBO	Modified Backbone Oligonucleotide
MET	Motivational Enhancement Therapy
MEOS	Microsomal Ethanol-Oxidizing System

mRNA	Messenger Ribose Nucleic Acid
NAD	Nicotinamide Adenine Dinucleotide (oxidized form)
NADH	Nicotinamide Adenine Dinucleotide (reduced form)
NADP	Nicotinamide Adenine Dinucleotide Phosphate (oxidized form)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
NIAAA	National Institute on Alcohol Abuse and Alcoholism
NMDA	N-Methy-D-Aspartate
Oligo	Oligonucleotide
PS-Oligo	Phosphorothioate Oligonucleotide
P	Alcohol-Preferring Rat Strain
PA317	Retrovirus Packaging Cell Line
PB^-	Primer Site for Retrovirus Minus-Strand DNA Synthesis
PBS	Phosphate Buffered Saline
PB^+	Primer Site for Retrovirus Plus-Strand DNA Synthesis
PCR	Polymerase Chain Reaction
pLHCK3'UT	Retroviral Plasmid Containing Human Mutant ALDH2*2 cDNA
pLNCE	Retroviral Plasmid Containing Human Wild-Type ALDH2*1 cDNA
pol	RNA Dependent DNA Polymerase
prt	Protease
ψ	Retrovirus Packaging Signal
rAAV	Recombinant Adeno-Associated Virus
rcRNA	Recombinant Ribose Nucleic Acid
RT	Reverse Transcriptase
SA	Specific Activity of Isotope
SEM	Standard Error of the Mean
SIP	Schedule-Induced Polydipsia
SP	Sardinion Alcohol-Preferring Rat Strain
SPP	Sodium Pyrophosphate Buffer
SSRIs	Selective Serotonin Reuptake Inhibitors
T_7	Promoter Sequence for Bacterial Transcription
TAR	Trans-Activating Responsive Sequence
TCA	Trichloroacetic Acid
TE	Tris- Ethylene Diamine-Tetraacetic Acid Buffer
TEA	Triethanolamine
TNF	Tumor Necrosis Factor
TNFRI	Tumor Necrosis Factor Receptor Type I
U3	Unique Sequence Element at 5'-End of Retrovirus Genome
U5	Unique Sequence Element at 3'-End of Retrovirus Genome
UchB	University of Chile Alcohol-Preferring Rat Strain
UTR	Untranslated Region
Xgal	Beta-Galactosidase Substrate

Section I INTRODUCTION

Alcoholism. Alcoholism, as defined in Webster's dictionary, is the continued excessive or compulsive use of alcoholic drinks. Medically, alcoholism is considered a disease that is initiated by the interaction of alcohol and the brain which is thought to affect neurotransmitters in key areas of the brain to sustain addictive behavior (Tabakoff and Hoffman, 1980). According to a recent overview by Fuller and Hiller-Sturmhofel, approximatey 7.5% of the U.S. population (14 million Americans) abuse and/or are dependent on alcohol. Furthermore, a survey in 1993 from a National Drug and Alcoholism Treatment Unit found that more than 700,000 Americans receive alcoholism treatment on any given day (Fuller and Hiller-Sturmhofel, 1999).

Alcohol consumption can impair judgement, coordination, and ability to perceive and respond to hazards, with the risk of injury increasing exponentially with increasing blood alcohol levels.

A current report from the National Institute on Alcohol Abuse and Alcoholism (NIAAA) published in 1999 on epidemiology provides staggering insight into problems associated with alcohol consumption or abuse. Alcohol involvement is generally more common in severe injuries than in more minor injuries, e.g., 41% involvement in fatal automobile crashes, 9% in injury crashes, and 5% in property damage crashes (Fell, 1999). Smith et al. (1999) estimated that 21-42% of fatal unintentional injuries involved alcohol, and Hingson and Howland (1993) estimated that 17% of nonfatal unintentional burns and 24% of nonfatal unintentional fall injuries involved alcohol. Alcohol use is also implicated in fatal pedestrian injuries (36%); non-commercial aviation pilot fatalities (10%,

Holdener, 1993); bicycle fatalities (25%; Abel *et al.*, 1984); and drowning (21-42%; Hingson and Howland, 1993).

In addition, alcohol involvement in perpetrators of violence has been estimated to be 28-86% of homicide offenders, 24-37% of assault offenders, 13-60% of sexual offenders, and 6-57% of male domestic offenders (Roizen, 1997). Alcohol involvement in victims of violent events is as prevalent as that for the perpetrator. Alcoholism and alcohol abuse are second to depression and other affective disorders as major risk factors identified for suicide (Blumenthal, 1988).

Although the above mentioned statistics include individuals that are under the influence of alcohol and are not just limited to individuals with alcoholism, it becomes obvious that the disease of alcoholism can place an individual at risk for any of the above mentioned dangerous situations. Hence, treatment of alcoholism and the intervention and prevention of alcohol abuse are necessities given the magnitude of the problems associated with excessive alcohol consumption.

Treatments. Many treatment programs for alcoholism include three components, which include 1) detoxification, 2) behavioral therapy, and 3) pharmacotherapy. Detoxification is a period in which the alcoholic becomes alcohol free under controlled conditions due to "alcohol withdrawal syndrome" (AWS). Alcohol withdrawal syndrome includes a variety of symptoms and signs which include irritability, insomnia, tremors, seizures, hallucinations and delirium tremors depending on the degree of withdrawal (Fuller and Hiller-Sturmhofel, 1999). The treatment of choice for AWS is the use of benzodiazepine which is a

class of sedative that affects some of the same molecules in the brain as does alcohol and prevent delirium tremens (Kaim *et al.*, 1969). Behavioral therapy includes treatment which is nonpharmacological with the objective being to change behavior (reducing ethanol consumption). One type of behavioral therapy used is called Cognitive-behavioral therapy (CBT). CBT is designed to help the patient identify high-risk situations for relapse, learn and rehearse strategies for coping with those situations, as well as to cope with their cravings. Another type of behavioral therapy that has been used is motivational enhancement therapy (MET) which motivates a patient through their recovery by having the patient use his or her resources to change their behavior. In addition, behavioral therapy has been provided through Alcoholics Anonymous (AA) meetings which has a 12-step philosophy to help maintain abstinence. Pharmacotherapy can be implemented shortly after detoxification to assist with the risk of relapse, and includes both anticraving and aversive medications.

Anticraving Medications. The anticraving medications target various brain chemicals called neurotransmitters which are responsible for the pleasant effects that alcohol can provide, including euphoria and reduced anxiety. The neurotransmitter systems include opioids, glutamate, serotonin (5HT) and dopamine. Opioid peptides are neurotransmitters that produce similar effects to those of morphine and heroin, and alcohol consumption affects the production, release and activity of these opioid peptides (Herz, 1997). Naltrexone and naloxone are anticraving medications which are antagonists of the mu receptor, which is one of the subtypes of the opioid receptor in the brain (Froehlich, 1997). Studies by Volpicelli et al. (1992) showed that abstinent male patients undergoing psychosocial alcoholism treatment who received a 50 mg/day dose

of naltrexone for 12 weeks had less craving for alcohol than subjects who only received psychosocial treatment alone (Vopicelli *et al.*, 1992). Recently, Sinclair reported naltrexone's safety and efficacy as an adjunct in alcoholism treatment in eight double-blind placebo-controlled clinical trials from five countries. The patients in these trials all found benefits from naltrexone with the coping therapy, however, none of them found any significant benefit of naltrexone over placebo for abstinence in the presence of support therapy (Sinclair, 2001).

Glutamate is another type of neurotransmitter which can act at the N-methyl-D-aspartate (NMDA) receptor and may contribute to some of alcohol's effects such as intoxication, cognitive impairment, and some withdrawal symptoms. The drug acamprosate is a NMDA-receptor antagonist that appears to reduce the intensity of craving after drinking cessation, particularly when the patient is exposed to situations or enviroments associated with previous alcohol use, where the risk of relapse is greatest (Spanagel and Zieglganberger, 1997). An analysis of pooled data from a series of randomized, double-blind, placebo-controlled trials in Europe showed that twice as many patients remained abstinent from alcohol for up to 1 year after treatment as compared to the placebo group (Swift, 1999). In addition, a study over a 2 year period was done to compare the costs of acamprosate in maintaining abstinence in weaned alcoholic patients, to patients without pharmacotherapy. A controlled trial involving a 12-month treatment with acamprosate and a 12-month follow-up showed a statistically significant advantage of treated versus placebo groups in the prevention of relapse (Annemans, *et al.*, 2000).

Another area of pharmacotherapy is in the neurotransmitter serotonin. Serotonin plays a role in regulating diverse physiological functions, including body rhythms, sleep, and mood states, as well as appetitive and consummatory behaviors (Lovinger, 1997). At least 15 subtypes of the serotonin receptor exist, some of which have potential roles in the development of alcohol-use disorders through multiple interactions with other neuotransmitter systems (Johnson and Ait-Daoud, 1999). Selective serotonin reuptake inhibitors (SSRIs) interfere with removal of serotonin from the synapse after release of the neurotrasmitters. SSRIs are used to treat psychiatric conditions, including mood disorders such as depression and anxiety and are not limited to specific serotonin-receptor subtypes. It appears as though SSRIs such as fluoxetine (Prozac®) are not effective in treating early alcoholics and actually increased alcohol consumption in early onset alcoholics (Kranzler *et al.*, 1996), but are effective in treating alcoholics with co-occuring depression (Cornelius *et al.*, 1997). Hence, the relationship between serotonerigic dysfunction and the onset of alcoholism is neither simple nor direct. There are also medications that are specific to serotonin-receptor subtypes. Buspirone (Buspar®) has partial agonist activity at the $5HT_1$ receptor and has been reported to have limited use in alcoholics with independent co-occuring anxiety disorders (Malec *et al.*, 1996). Ritanserin is a $5HT_2$ receptor antagonist which has been shown to reduce alcohol intake by modulating the effect of dopamine in the nucleus accumbens of rats (Lovinger, 1997). A multicenter, placebo-controlled clinical trial with 5 mg/day of ritanserin showed that participants had significantly reduced their alcohol consumption, but failed to reduce their drinking frequency in treated versus placebo groups (Johnson *et al.*, 1996). The most promising serotonergic medication is ondansetron, which is a $5HT_3$ receptor antagonist. Antagonists at the $5HT_3$ receptor have been shown to

reduce the rewarding effects of alcohol due to suppression of dopamine release in the nucleus accumbens (Lovinger, 1997). In humans, ondansetron reduced some of alcohol's positive subjective effects including self-reported desire to drink (Johnson and Cowen, 1993 and Swift *et al.*, 1996). However, it should be noted that although ondansetron has been found to reduce alcohol drinking and increase abstinence rates among early onset patients, it did not assist late onset alcoholics (Johnson, 1999).

Aversive Medication. In order to understand how an aversive medication elicits its effects, one must understand the general metabolism of ethanol in the liver. The metabolism of ethanol includes two key steps the first step is the oxidation of ethanol to acetaldehyde. The three liver enzymes that are responsible for initial metabolism of ethanol are the cytosolic alcohol dehydrogenase (ADH), the microsomal ethanol-oxidizing system (MEOS) located in the endoplasmic reticulum and catalse located in the peroxisomes. Figure 1.1 depicts the three pathways for the initial steps in ethanol metabolism.

Figure 1.1

All three ethanol metabolism pathways lead to the formation of acetaldehyde with the majority of ethanol being metabolized by ADH into acetaldehyde. The generated acetaldehyde is further oxidized by the ALDH enzymes to acetate, and is the rate-limiting step in acetaldehyde metabolism (Svanas and Weiner, 1985,

Farres *et al.*, 1989, Smolen *et al.*, 1990, Huang and Lindahl, 1990, Mitchell and Petersen, 1991, Tsai and Senior, 1991). Acetate is subsequently converted to carbon dioxide in the Kreb's cycle. There are many aldehyde dehydrogenase isozymes, and thus far, 16 ALDH genes with distinct chromosomal locations have been identified in the human genome (Vasiliou and Pappa, 2000).

However, the action of the mitochondrial (low-Km) ALDH (ALDH2) is widely believed to be the major contributor to the clearance of ethanol-derived acetalehyde in both man and rat. Furthermore, Klysosov et al. have reported the Km for acetaldehyde at pH 7.5 to be 0.2 μM for both human and rat ALDH2; while the Km of the cytosolic isozyme (ALDH1) for acetaldehyde was 180 and 17 μM, respectively, demonstrating ALDH2's lead role in the metabolism of ethanol-derived acetalehyde (Klyosov *et al.*, 1996).

The ALDH2 enzyme from most species is isolated as a tetramer of molecular mass 220-250 kDa, resulting in a subunit molecular mass of 55-60 kDa (Weiner, 1979). The amino acid sequence in the mature rat mitochondrial aldehyde dehydrogenase is 96% identical to that of humans out of 500 amino acids (Farres *et al.*, 1989). In contrast, the cytosolic isozyme shares only 66% amino acid identity with the mitochondrial isozyme (Farres *et al.*, 1989). Acetaldehyde by itself can become toxic when blood levels are elevated if its metabolism to acetate is hindered. Accumulations of acetaldehyde can result from: (1) treatment with an aversive medication such as Disulfiram (Antabuse®) which inhibits the mitochondrial enzyme aldehyde dehydrogenase (ALDH, see figure above) or (2) a genetic mutation in the ALDH2 gene (ALDH2*2). Elevated blood acetaldehyde due to presence of an aversive medication or genetic mutation in

the ALDH2 gene results in the dysphoric effects that include dizziness, nausea, hypotension and heart palpitations (Mizoi *et al.*, 1983).

Disulfiram. Disulfiram was approved in 1948 and is currently the only U.S. approved aversive medication available on the market (Assmusen, 1948). This drug was initially used as an anti-helminth to treat workers in rubber plants and many of the workers became ill due to the presence of aldehydes in their environment. Hence, the drug was discovered to be of potential benefit for the treatment of alcoholism due to its ability to inhibit aldehyde dehydrogenase needed for acetaldehyde metabolism following ethanol consumption.

Disulfiram is a non-specific drug which inhibits all aldehyde dehydrogenases by reacting with sulfhydryl groups in the enzyme (Weiner, 1979). Disulfiram must be metabolized in the liver to its active form (S-methyl N,N-dithiocarbamate sulfoxide) in order to inhibit ALDH2-1 (Madan *et al.*, 1995; Mays *et al.*,1996), which results in marked interindividual variation. In addition, disulfiram produces drug-drug interactions, including those with phenytoin (epilepsy), oral anticoagulants and isoniazid (acts against tubercle bacilli).

A major problem with disulfiram is the lack of patient compliance, mainly due to side effects including sensory and motor neuropathies, optic neuritis, orthostatic hypotension and hypersensitivity reactions (Gallant, 1987, Peachey and Annis, 1989, Hugues *et al.*, 1992, Dupuy *et al.*, 1995, Chick, 1999). Because of the side effects, doses which are fully therapeutic can rarely be achieved. At normally tolerated doses (250-300 mg per day), less than 50% of patients achieve the desired therapeutic effect (Brewer, 1984; Christensen *et al.*, 1991). At therapeutic

doses, disulfiram inhibits dopamine-beta hydroxylase (Musacchio *et al.*, 1966; Goldstein and Nakajima, 1967; Major *et al.*, 1979), the enzyme responsible for the conversion of dopamine into norepinephrine, which produces orthostatic hypotension (Hugues *et al.*, 1992). Nevertheless, in 10 studies in which disulfiram was administered under supervision, all studies demonstrated its effectiveness in reducing alcohol consumption (Brewer, 1993).

Genetic Protection. The most compelling evidence for any role of genetics in the protection from the development of alcoholism is that a single gene can determine who will have an innate protection against development of alcoholism (Harada *et al.*,1982; Thomasson *et al.*, 1991, Tu and Israel, 1995). The high-affinity (low Km) aldehyde dehydrogenase (ALDH2-1) (Yoshida, 1985) is such a gene. The ALDH2 enzyme is a homotetramer of equal subunits (Feldman and Weiner, 1972; Greenfiend and Pietruszko, 1977; Guan *et al.*, 1988). Human ALDH2 mRNA has been found in heart, brain, lung, liver, skeletal muscle, kidney and pancreas with the highest levels being located in liver, kidney, skeletal and cardiac muscle, and lung (Stewart *et al.*, 1996). The liver by far had the highest mRNA levels which is consistent with the role of the liver in alcohol metabolism (Stewart *et al.*, 1996). A point mutation in an ALDH2 allele (ALDH2-2) renders the gene product inactive as an enzyme (Yoshida *et al.*, 1984), and the mutation is partially dominant, as ALDH2-1/ALDH2-2 heterozygotes present an activity that is only 15% that of ALDH2-1 homozygotes (Enomoto *et al* .,1991, Xiao *et al* .,1995).

The prevalence of alcoholism in subjects who carry the ALDH2-2 allele is 75-90% lower than that of subjects who only carry the active ALDH2-1 form, indicating marked protection of the ALDH2-2 allele against alcoholism (Harada *et al.*,1982; Thomasson *et al.*, 1991, Higuchi, 1994). Other studies show that ALDH2-1/ALDH2-2 heterozygotes of Asian origin-born in Canada or the United States consume two thirds less alcohol than their ALDH2-1 homozygote counterparts, while the respective ALDH2-2 homozygotes are virtual abstainers (Tu and Israel 1995). Individuals carrying the ALDH2-2 allele who consume moderate amounts of alcohol (0.4 g/kg) display elevated blood acetaldehyde levels and dysphoric effects that include dizziness, nausea, hypotension and palpitations (Mizoi *et al.*, 1983). Many of the intoxicating effects of ethanol are accentuated in subjects carrying the ALDH2-2 allele (Wall and Ehlers 1995, Wall *et al.*, 1999), affording these individuals a marked protection against alcoholism (Harada *et al.*, 1982, Goedde *et al.*, 1983, Thomasson *et al.*, 1991, Higuchi, 1994, Tu and Israel, 1995).

There is the obvious need to develop alternatives to disulfiram to mimic the strong protective effects of a low ALDH2 activity seen in Asians, without the side effects of disulfiram. With the advent of gene regulation and gene therapy, new approaches to design an aversive medication are conceivable which could offer a drug without the side effects or other disadvantages mentioned above associated with Disulfiram.

Antisense. One type of *gene regulation* that has gained much support is the use of antisense oligonucleotides (ASO's) as effective therapeutics. ASO's of 17-21 bases with a sequence complementary to the primary RNA transcripts (pre-

mRNA) or the mRNA target sequence can inhibit gene expression in a specific manner because a sequence of this length is unlikely to repeat more than once in the human genome. Figure 1.2 below depicts how an effective antisense oligonucleotide (ASO-9) was designed based on the mRNA target sequence of rat ALDH2 that is presented in section II of this thesis.

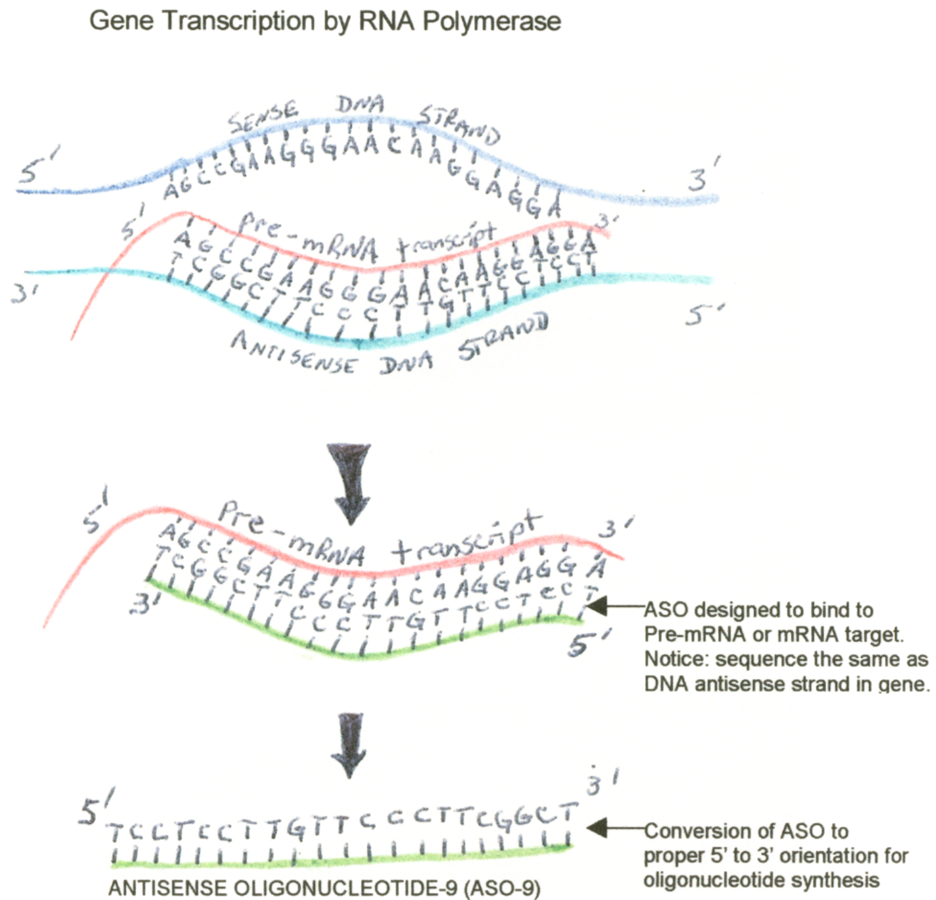


Figure 1.2

In principle, an antisense oligonucleotide prevents protein production from a targeted RNA of a gene. Many mechanisms have been proposed by which an ASO can mediate its effects of reducing the presence of a particular protein.

These mechanisms can be placed into two categories, which include (1) *occupancy* and (2) *RNase-mediated* mechanisms.

Figure 1.3. depicts the general concept of the occupancy mechanism.

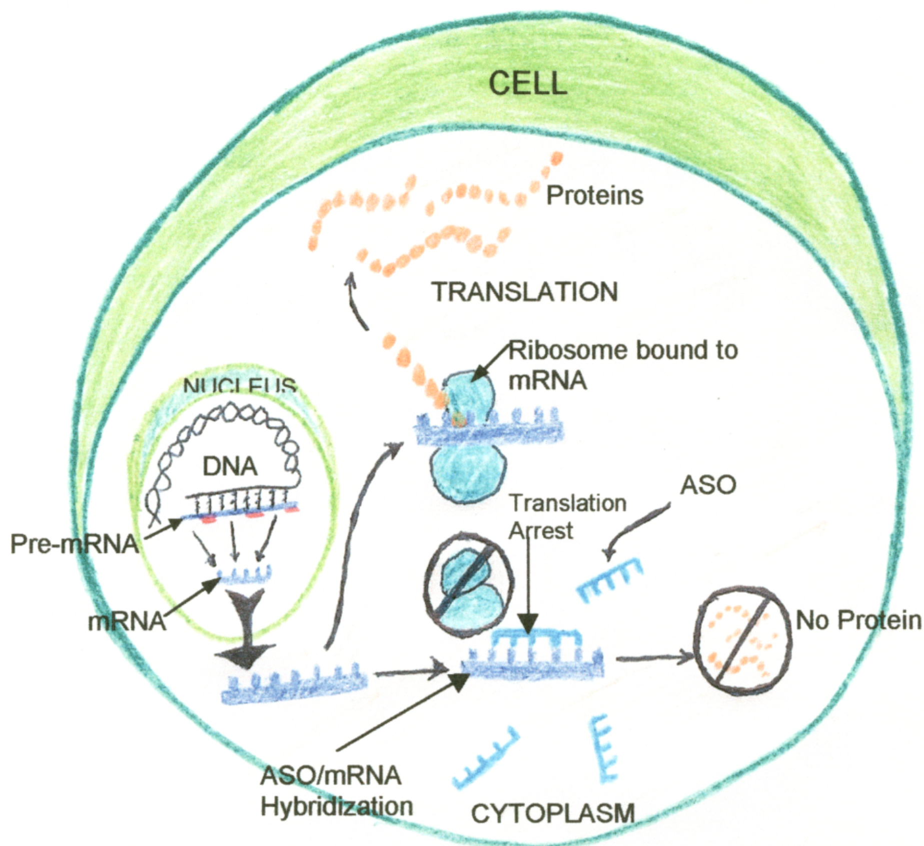


Figure 1.3 Occupancy mechanism (*Translation Arrest*). An ASO hybridized to the mRNA in the cytosol of the cell preventing translation of the mRNA into the protein.

Occupancy mechanisms. *Occupancy of the translation initiation codon (AUG).* Translation arrest is the process by which the ASO binds to the translation initiation codon (AUG) and results in the inability of translation machinery (ribosomes) to bind to the target mRNA to assemble a protein. An example of translation arrest was recently shown with ASO's targeted to the Huntington gene in the –25 to 35 nucleotide region in which the most effective ASO, targeted to the methionine initiation codon, resulted in a marked reduction in the *in-vitro* incorporation of ^{3}H leucine (Boado *et al.*, 2000).

Occupancy at exon/intron boarders. The primary RNA transcript is subjected to "splicing", a process in which introns are removed from the pre-mRNA to form the mature RNA by spliceosomes in the nucleus. In 1993, Dominski and Kole were able to confirm that an ASO could *inhibit splicing* in a cell-free splicing system where an oligonucleotide was found to induce alternative splicing (Dominski and Kole, 1993).

Occupancy of necessary RNA structures. RNA adopts a variety of three-dimensional structures induced by intramolecular hybridization. These structures provide additional stability for RNA and function as recognition motifs for a number of proteins, nucleic acids, and ribonucleoproteins that participate in the metabolism and activities of RNA species (Crooke, 1998). Disruption of these necessary RNA structures by an ASO can lead to inhibition of the sythesis of a specific protein. In 1991, Vickers et al showed that they were able to take advantage of disrupting the RNA stem-loop structure of a trans-activating responsive sequence (TAR) which binds to the HIV trans-activator protein which mediates increased viral gene expresssion of HIV (Vickers *et al.*, 1991).

Occupancy at 5'-UTR, 3'-UTR or Polyadenylation sites. RNA has a number of structural features which can regulate stability, processing, subcellular distribution and transport. The process of 5' capping of the pre-mRNA is important to the stability of mature mRNA, in binding to the nuclear matrix, and transport of the mRNA after splicing out of the nucleus to the cytosol. ASO's targeted to this region have been effective in stopping translation or destabilizing the RNA structure. Likewise, the post transcriptional addition of polyadenylate at the 3' end of the mRNA stabilizes the message and is a region in which polyadenylation could be inhibited by an ASO such that the mRNA is destabilized. An example of this was demonstrated in which ASO's designed to hybridize to the 3'-polyadenylation signal region of the p53 tumor necrosis factor receptor type I (TNFRI) were effective in reducing TNF-alpha binding to this receptor (Ojwang *et al.*, 1997). In addition to targeting the 3'-polyadenylation region, others have found that the occupancy of an oligonucleotide in the 3'-UTR to be effective, as well as, in the destabilization of the mRNA structure (Bennett, *et al.*, 1994; Tu *et al.*, 1998).

Confirmation of Translation Arrest Occupancy Mechanism. If an antisense oligonucleotide is reducing protein synthesis by translation arrest alone, then the steady-state levels of mRNA will remain constant. Other occupancy mechanisms can lead to destabilization of the pre-mRNA or mRNA therefore, it is difficult to distinquish between RNase H degradation and destabilization by an occupancy mechanism.

RNase Mediated Mechanism. RNase H is an enzyme that degrades the RNA strand of an RNA-DNA duplex. Mammalian RNase H participates in DNA replication, in removal of the Okazaki fragments during the synthesis of the lagging strand at the DNA replication fork (together with flap endonuclease 1), and possibly in DNA repair (Turchi *et al.*, 1994). According to Crum, RNase H concentration in the nucleus is greater than in the cytoplasm, and although RNase H was found in the cytoplasm, some of the enzyme found in the cytoplasmic preparations may have been the result of leakage from nuclei during subcellular fractionation (Crum *et al.*, 1988). The backbone of an antisense oligonucleotide can greatly influence it ability to act as a substrate for RNase. For instance, a methylphosphonate oligonucleotide does not act as a substrate for RNase H (Maher and Dolnick, 1988), while an oligonucleotide with a deoxy-phosphate or phosphorothioate backbone serves as excellent substrate for RNase H (Cazenave *et al.*, 1989, Stein and Chen, 1993). In addition, a phosphorothioate oligonucleotide is nuclease-resistant. Initially, it was found that delivery of an oligonucleotide with a natural phosphate backbone was subjected to nuclease degradation which dramatically reduced the oligonucleotide's efficacy (see Figure 1.4).

NATURAL vs PHOSPHOROTHIOATE DNA

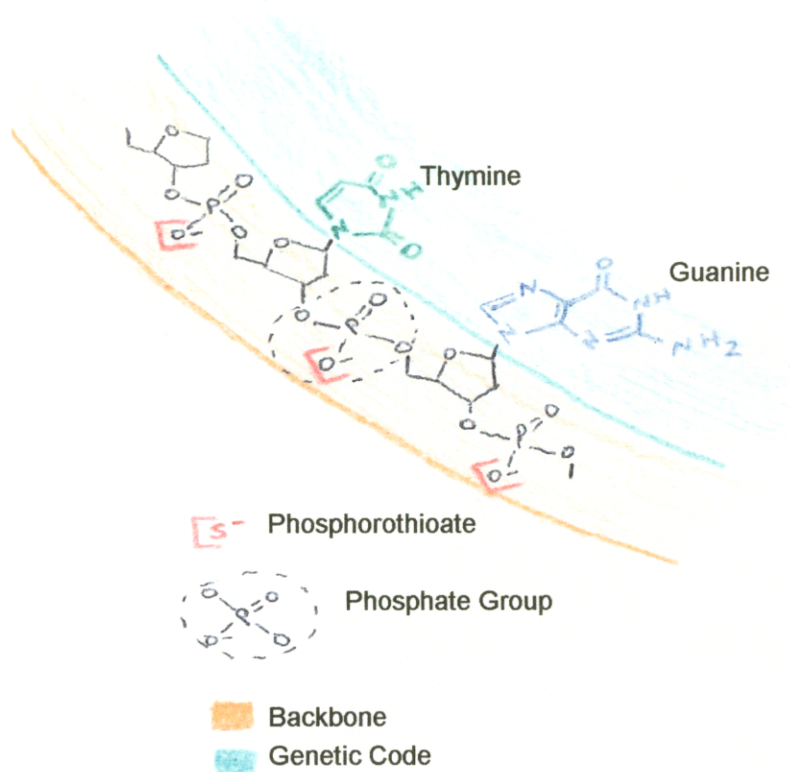


Figure 1.4. Natural DNA configuration with phosphate backbone that is subject to nuclease attack. A modified phosphorothioate backbone is nuclease resistant (note the substitution of ^{-}S for O^{-} in the phosphate group; see page 6 of Discussion section for methylphosphonate which is very resistant to nucleases or 2'-O-alkyloligoribonucleotides which improves target affinity as well as stability in the presence of nucleases).

The way in which a phosphorothioate oligonucleotide binds to its RNA target and activates RNase H degradation in the cell is depicted in Figure 1.5 below.

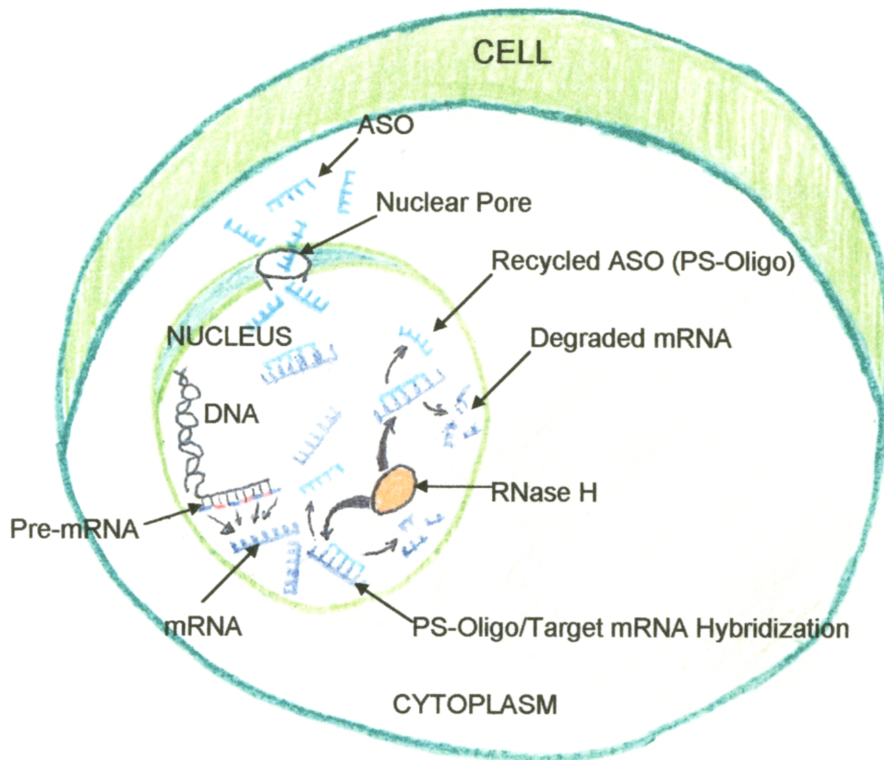


Figure 1.5. Antisense in the Cell and RNase H Degradation of Target mRNA.

Although phosphorotioate modified oligonucleotides can be effective ASO's, they are not without their disadvantages in that they can have non-antisense effects on other proteins within the cell and in extracellular milieu (Crooke, 1998). Advances in modified backbone oligonucleotides (MBO's) from that of the phosphorothioates will be discussed later in the discussion section of this thesis.

Gene Therapy. Since no single gene can be attributed to alcoholism and given the current knowledge of the protection that is afforded to some of the Asian population from alcohol abuse that have a mutation in at least one ALDH2 allele

(see above), introduction of the mutant ALDH2*2 gene by a gene therapy approach might provide a means by which the ALDH2*2 transgene can be expressed for a protracted period of time (1 month to a lifetime) from a single administration (Morral *et al.*, 1999). This approach could simplify patient compliance in the treatment of alcoholism.

Gene transfer using recombinant adeno-associated virus (rAAV) vectors shows great promise for human gene therapy. The broad host range, low level of immune response and longevity of gene expression observed with these vectors in numerous disease paradigms has enabled the initiation of a number of clinical trials using this gene delivery system (Smith-Arica and Bartlett, 2001). However, to warrant the introduction of the human ALDH2 mutant gene into humans, it is imperative to demonstrate the effectiveness of gene therapy in a pre-clinical animal model.

Pre-Clinical Animal Model. In order to test an antisense-mediated gene regulation or gene therapy approach, we were first faced with the need for a pre-clinical model in which a new aversive medication could be tested. In literature searches, we found no record of animal models specifically designed to test an aversive medication that were not time-consuming in order to get animals to consume a large amount of ethanol in a short period of time to mimic an alcoholic binge.

Selective breeding has been successfully used to establish rat lines (ALKO alcohol AA, alcohol-preferring P, University of Chile B UchB, high alcohol

drinking HAD, and Sardinian alcohol-preferring sP) that consume greater than 5 g ethanol/kg/day (Lumeng *et al.*, 1995). However, the P line which is perceived to meet all the criteria for an animal model of alcoholism (Cicero, 1979), does not binge, but rather consumes the 5 g/kg ethanol over a 24 hour period in numerous bouts (Murphy *et al.*, 1986). This P line can be trained to binge (1.1 g/kg/hour) by restricting ethanol availability to 4 hours or less per day (Murphy *et al.*, 1986). Similarly, Sinclair demonstrated that Wistar (outbred strain commercially available) and AA (selectively bred) rats could be trained to consume 0.5-1.0 g ethanol/kg/hour in a one hour limited-access paradigm after approximately 20 to 30 days in the described paradigm (Sinclair, 1992). In addition, Samson has described a procedure that uses a "fade-out" of sucrose from a sucrose-ethanol solution (Samson, 1986). In this model, a 20% sucrose solution is presented in an operant chamber with a lever to dispense the solution for drinking. Once lever pressing is maintain by rats for the 20% sucrose solution, ethanol is then added gradually while the sucrose content of the solution is reduced or faded-out. Finally, the rats will consume a 10% solution of just ethanol without sucrose present and ethanol solutions can be increased up to 40% while maintaining responding in the operant chamber. Rats can be trained to consume between 0.9 and 0.95 g ethanol/kg in a 30 minute session in the operant chamber.

Disadvantages of Available Animal Models. While Samson's fade-out procedure and the limited access paradigm described by Sinclair present valid methods for training commercially available stock rats to binge rather than using selective breeding to obtain ethanol-preferring rats, these procedures require at least 20-30 days of training prior to study initiation. Furthermore, an aversive medication such as disulfiram relies on recognition of the substance (ethanol)

that is causing the dysphoria in order to achieve the desired efficacy (abstinence). Thus, it is unlikely that animals trained to consume ethanol would readily associate the dysphoria caused by ethanol in the presence of an aversive medication to the very same ethanol that they have been consuming without any ill effects. Unlike humans, rats cannot be informed of the dysphoria they will experience if they consume ethanol in the presence of an aversive drug. In fact, a literature search failed to reveal the use of any of the above mentioned rat models to test the efficacy of disulfiram to establish an aversion to ethanol consumption most likely because these models are more suited to testing of anti-craving medications.

Consequently, the aim of the studies in section I were to develop an animal model which would consume large amounts of ethanol in a short period of time and mimic the dysphoria experienced by ALDH2-2 homozygotes or heterozygotes when consuming alcohol. We chose the rat to develop this model, since in this animal the acetaldehyde generated from the oxidation of ethanol is metabolized by the high affinity mitochondrial aldehyde dehydrogenase, as it is in man. Further, the rat has been shown to have a very limited cytosolic ALDH activity (Chen *et al.*, 1996), which parallels the low involvement of cytosolic aldehyde dehydrogenase in man (Klyosov *et al.*, 1996). Since the cytosolic ALDH plays a major role in the oxidation of the acetaldehyde generated in the metabolism of ethanol in the mouse (Little and Petterson, 1983), this animal was not appropriate for the development of the aversion model.

Hence, the inbred Lewis rat, derived from an original Wistar rat was chosen for the development of our pre-clinical model because this breed had been shown to

consume more ethanol than the inbred Fisher rat (Suziki *et al.*, 1988). Furthermore, the selection of an inbred rat strain allows others to reproduce the aversive effects using Disulfiram as a model drug for comparison of any novel aversive medication.

In order to induce naive rats to consume a large amount of ethanol in a very short period of time, we used a modification of a procedure first described in 1961 by Falk, called schedule-Induced polydipsia (SIP) (Falk, 1961). In these studies, rats were placed on an intermittent schedule of food and water access which created a state of polydipsia (excessive thirst), and it was shown that rats would drink up to one-half of their body weight in water over a 3.17 hour period. Subsequently, Lester determined, using Falk's SIP, that rats would consume 5.6% (W/V) ethanol and intoxication was produced in 3-hr test sessions with blood alcohol levels reaching 0.2% (Lester, 1961). By using an overnight 18 hour SIP with food freely available, we were able to induce naïve rats to consume as much as 1 g ethanol/kg within a period of 1 hour (Garver *et al.*, 2000).

Once a "Paradigm to Test A Drug-Induced Aversion to Ethanol" (Garver *et al.*, 2000) was completed and a pre-clinical aversion model was established in the rat, efforts were focused on the antisense approach as a means by which specific inhibition of ALDH2 gene expression could be obtained (Garver *et al.*, 2000).

About one half of the most active antisense deoxyoligonucleotides described in the literature contain a motif 5'-TCCC-3' in the molecule (Tu *et al.*, 1998). In

addition, he also found that antisense molecules designed with this motif in the 3' untranslated region and intron regions were most effective in reducing mRNA levels.

The nucleic acid sequence of the 5' untranslated region (UTR), 3' UTR and the open reading frame of the Sprague-Dawley rat was previously published by Farres in 1989 (Farres *et al.*, 1989). Although our lab had success in finding active ASO's targeted to the 3' UTR or intronic RNA regions using the motif 5'-TCCC-3' in the molecule design, very little was known about design of ASO's with this motif in the exonic regions. Using the TCCC motif for antisense design, two ASO's were designed in the 3' UTR and seven ASO's were designed in the open reading frame to target the rat mitochondrial aldehyde dehydrogenase (ALDH2).

In order to test the designed ASO's, we chose a rat hepatoma cell line H4-II-E-C3, because the majority of acetaldehyde oxidation is performed in the liver hepatocytes by ALDH2. In addition, Huang and Lindahl reported that this cell line contains a protein with the immunological characteristics of the low Km mitochondrial ALDH2-1 and a similar protein distribution pattern to primary rat hepatocytes (Huang and Lindahl, 1990). Thus, these cells provided an ideal *in-vitro* model in which to test the ASO's. The results of these *in-vitro* and *in-vivo* studies are contained within section II "Eliciting the Low-Activity Aldehyde Dehydrogenase Asian Phenotype by an Antisense Mechanism" (Garver *et al.*, 2001, submitted). In this section, we present the specific effects of our most effective ASO-9 for rat ALDH2

Retrovirus Opens Possibility of Gene Therapy. The retrovirus is comprised of a diverse group of metazoan viruses that have a replication step whereby DNA is synthesized from double-stranded virion RNA in a process of reverse transcription using the viral-coded RNA-dependent DNA polymerase (reverse transcriptase). The retroviral replication cycle is divided into early and late phases (see Figure 1.6). Once the viral particle's envelope glycoprotein attaches to the host cell's receptor, the virion is uncoated and the viral DNA is synthesized from two identical single-stranded virion RNA molecules that are associated with reverse transcriptase and integrase in the cytoplasm. The viral DNA is then transported into the nucleus of the host cell that has been infected and becomes incorporated by integrase into the host cell's genome as a provirus with long-terminal-repeats (LTR) at either end of the incorporated provirus. The proviral DNA can then be transcribed in the late phase by the host cell's DNA-dependent RNA polymerase to produce the viral mRNA encoding the virion polyproteins necessary for assembly of infectious virions that bud through the host cell's lipid bilayer.

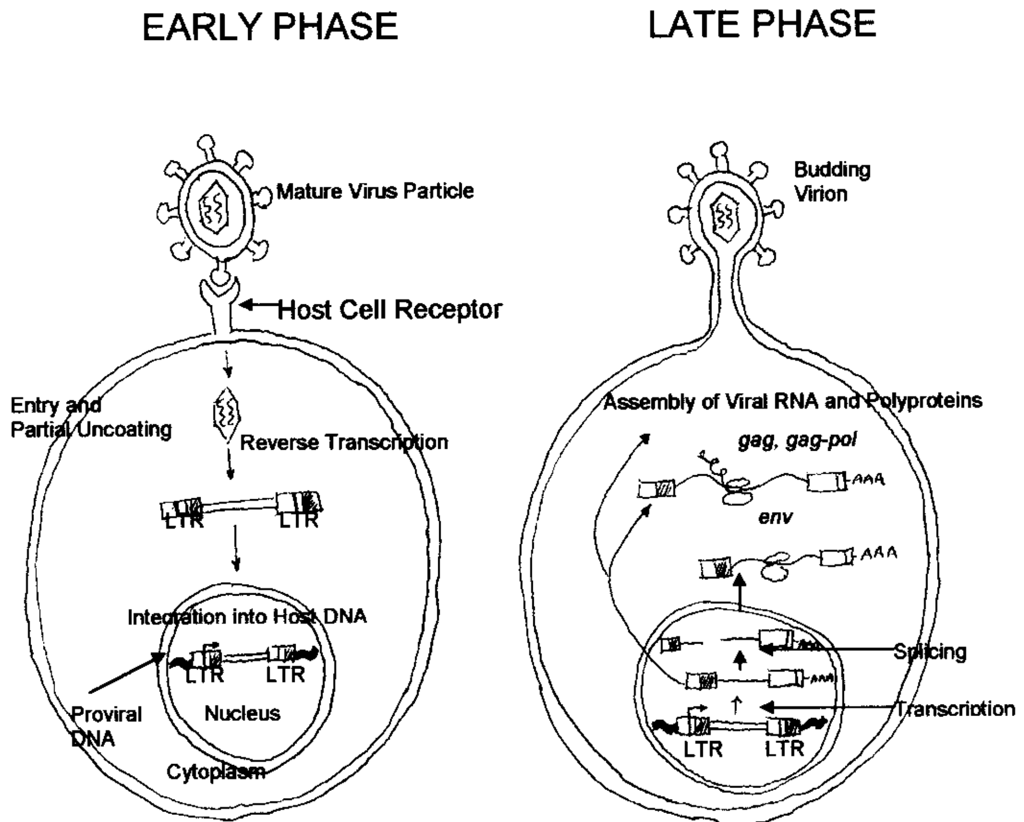


Figure 1.6. Retrovirus replication cycle. In early phase, virion attaches to host cell receptor on surface of cell, enters the cell and is uncoated so viral DNA can be reverse transcribed by reverse transcriptase. The viral DNA is then incorporated into the host cell's genome via integrase leading to proviral formation. The late phase of the cycle begins when the proviral DNA is transcribed into viral transcripts (RNA) which are then translated by polysomes to produce of *gag, prt,* and *pol* polyproteins. The *env* protein is translated after the full-length viral transcript has been spliced. These proteins are then processed during viral budding to form mature virions for release and infection of other host cells.

Normal Retrovirus Life-Cycle. Elements required for retrovirus replication can be divided into *cis*- and *trans*-acting factors. The *trans*-acting factors include proteins encoded by the viral genome [see Figure 1.7 for *gag* (group-specific antigen, *prt* (protease), *pol* (RNA-dependent DNA polymerase), and *env* (envelope glycoprotein) genomic orientation], which are required for encapsidation of the viral RNA, entry of virions into cells, reverse transcription of the viral genome, and integration of the viral DNA into the host cell's genome.

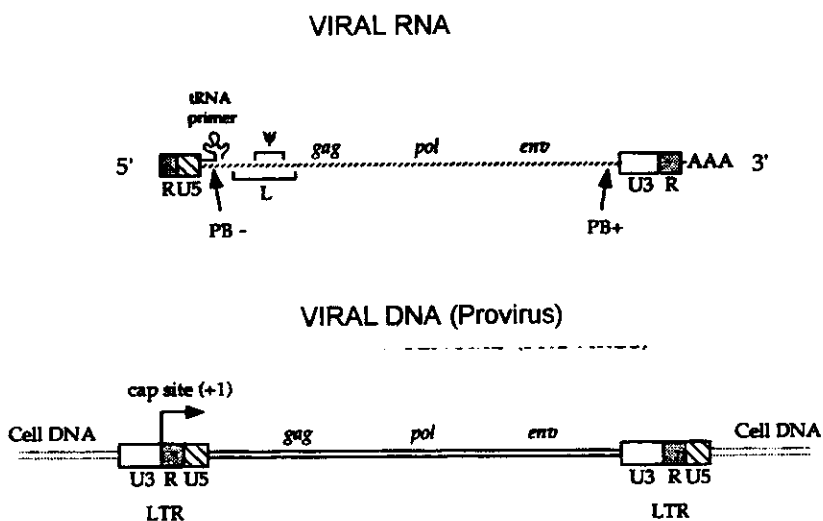


Figure 1.7. Retroviral Genome Orientation

Attachment to a host cell's receptor is mediated by the the *env* glycoprotein. The *pol* gene encodes for the reverse transcriptase (RT), RNase-H and integrase (IN) proteins. The RT is used to reverse transcribe the genomic viral RNA into double-stranded linear DNA in the host cell's cytoplasm, and then the double-stranded linear DNA flanked by long terminal repeats (LTRs) is transported into the nucleus where it is integrated into the host-cell's genomic DNA by IN,

representing the provirus stage. The retroviral protease (PR) encoded by *prt* mediates the cleavage of *gag* and *pol* polyproteins during virion assembly.

The cis-acting factors include signals present in the viral RNA which interact with these proteins and other factors during virus replication. (Refer to Figure 1.7 above) The 5' end of viral RNA has a cap and the 3' end has a poly-A tail. R is the short repeat at each end of the genome, U5 is a unique sequence element immediately after the 5'R sequence, PB- is the primer site for minus-strand DNA synthesis (tRNA binding site), L is the leader region before the start of gag, ψ is the element required for assembly of viral RNA into virions, PB+ is the primer site for plus-stand DNA synthesis, and U3 is a unique sequence at the 3' end of the genome.

Replication-Incompetent Retrovirus for Gene Therapy. In 1986, Miller and Buttimore made a packaging cell line that did not produce replication-competent virus (Miller and Buttimore, 1986). Alterations in the viral genome were made to interfere with cis-acting elements while trans-activating factors were preserved, and the retroviral packaging cell line PA317 that did not permit helper virus production was established (Miller and Buttimore, 1986) (see Figure 1.8).

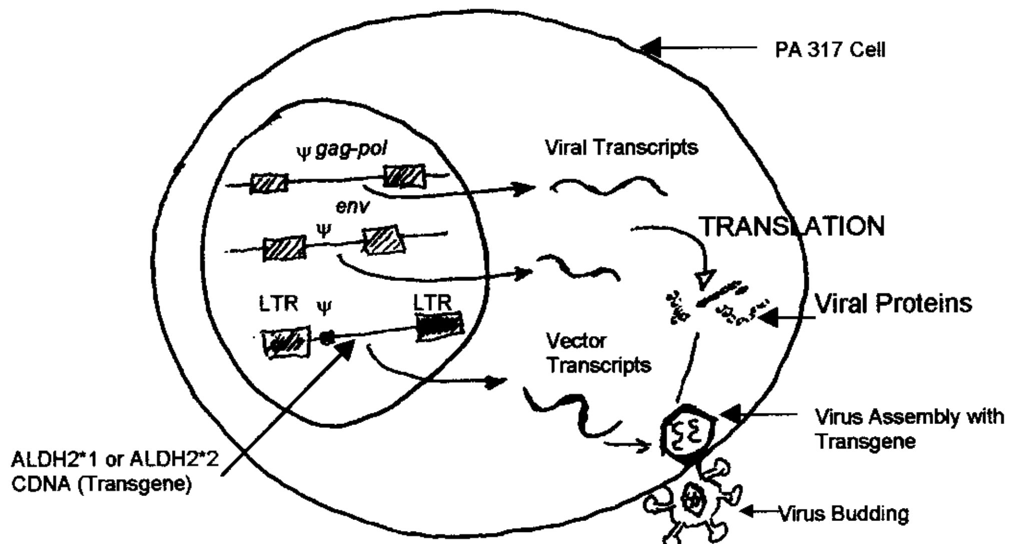


Figure 8.I. PA317 packaging cell used with replication-defective retroviral Vectors containing human ALDH2*1 (pLNCE) or ALDH2*2 (pLHCK3'UT) cDNA (transgenes). Viral transcripts encoding for the *trans*-activating factors (*gag, pol and env*) in the PA317 cells are translated into proteins, while the viral transcripts for *gag, pol and env* are not packaged into the assembling virion. However, the transgene (cDNA) is packaged into the assembling virion due to the presence of the packaging signal ψ in the retroviral vector at the 5' end of the transgene.

This cell line made it possible to form infectious viruses that were replication incompetent after the transfection of a retroviral vector (plasmid) designed to carry a transgene. The virus that is generated can infect and integrate the desired transgene into the mammalian host cell's genome, but cannot replicate, thus avoiding viral disease. The PA317 cell line places an amphotropic envelope protein on the viral particles, which allows infection of other eukaryotic host cells including those from mice, rats, chickens, cats, dogs, monkeys and humans.

These modifications produced a cell line that could be transfected with a viral vector containing a gene of interest (i.e. ALDH2 cDNA transgene) and in-turn package non-competent virus with the capability of infecting a new host-cell only once.

Our laboratory did not have the rat mutant ALDH2*2 cDNA to insert into a retroviral vector; however, the human ALDH2*1 and 2*2 cDNA's were kindly given to us in retroviral vectors from Dr. David Crabb's Laboratory at Indiana University. At the time, it was unknown whether the human mutant ALDH2*2 monomer expressed from a human cDNA encoding for the same point mutation that is found in the Asian population could combine with rat monomers. Since the rat and the human amino acid sequence were 95% identical, perhaps it was possible to produce the combined species monomers to form the mature tetrameric enzyme structure. If the incorporation of the human mutant ALDH2*2 monomer could form a mature combined rat-human tetramer and reduce the enzyme activity, it would provide future possibilities for delivery of the human transgene via a viral vector such as the (rAAV) for long-term suppression of mitochondrial aldehyde dehydrogenase activity. Non-competent viral particles were formed by transfecting the PA317 cell line with the retroviral vectors (LTR-ψ-ALDH2*1 or ALDH2*2 cDNA-LTR). The results of incorporation of a human monomer with and without a point mutation in the H4-II-E-C3 cell line is included in section III "Combination of Human ALDH2*1 or ALDH2*2 Monomers with Endogenous Rat Monomers in the H4-II-E-C3 Cell Line Results in Increased or Decreased Enzyme Activity", respectively.

Section II **MATERIALS AND METHODS** (Supplement to manuscripts)

Chemicals. Acetaldehyde and propionaldehyde (99.8% by assay), pyrazole, sodium acetate, 2,4-dinitrophenylhydrazine, anhydrous monobasic sodium phosphate, β-NAD$^+$, β-NADH, dithiothreitol, triethanolamine, ammonium acetate, EDTA, α-ketoglutaric acid, Tris-HCl, trypan blue and Triton X-100 were purchased from Sigma Chemical Co (St Louis, MO). All other chemicals: perchloric acid, hydrochloric acid, HPLC grade water, acetonitrile, isooctane, sucrose, magnesium chloride, and sodium pyrophosphate were purchased from Fisher Scientific (Pittsburgh, PA)

Cell culture and Oligonucleotide Delivery. H4-II-E-C3 cells were seeded 18-24 hours prior to the delivery of phosphorotioate oligonucleotides at a density of 2.5 X 10^6 cells/100 mm surface modified polystyrene tissue culture dish (Becton Dickinson Company, Franklin Lakes, NJ). On the morning of study, oligonucleotides were prepared using cationic liposomes (Lipofectamine Plus®, GIBCO BRL Life Technologies). Oligonucleotides were maintained throughout most studies at 1 μM, however, in some studies lower concentrations of 0.25 or 0.5 μM were used and the volume of the stock oligonucleotide solution (0.8 mM) and DMEM were adjusted to obtain the final desired concentration. Likewise, the treatment volumes for cells treated with Lipofectamine alone were adjusted by adding DMEM instead of oligonucleotide. The procedure used to deliver oligonucleotide at 1μM for a 24 hour incubation is as follows. For each 100 mm culture dish to be treated, 400 μl of serum-free Dulbecco's modified Eagle's medium containing 4.5 g/L of L-glutamine (DMEM) was added to two 1.5 ml

microcentifuge tubes. In one tube, oligonucleotide (10 μl of a 0.8 mM solution) was mixed with the medium followed by the gentle addition and mixing of the PLUS reagent® (27 μl) to complex with the oligonucleotide for 15 minutes at room temperature. Immediately following the additions to the first microcentrifuge tube, 40 μl of Lipofectamine alone (2 mg/ml) was gently mixed with DMEM in the second microcentrifuge tube and also incubated at room temperature for the remaining time period. At the end of the initial 15 minute incubation, the DMEM containing the oligo-PLUS complex was added to microcentrifuge tube containing the DMEM-Lipofectamine solution, gently mixed by tapping the bottom of the microcentrifuge tube and incubated for an additional 15 minutes at room temperature. The final Lipofectamine Plus-oligo complex (800 μl) was added dropwise to 7.2 ml of DMEM at 37°C and pippeted up and down once. Prior to addition of the prepared Lipofectamine Plus-oligo complex, the medium with serum was removed from the cells and the final 8 ml of prepared of Lipofectamine Plus-oligo complex was added slowly to the side of the culture dish and incubated for 6 hours in the absence of serum.

Thirty minutes prior to the end of the 6 hour delivery, serum (20% horse serum, 2.2 ml and 5% fetal bovine serum, 0.6 ml) was added back to the cells for overnight culture. Since the oligonucleotide concentration had been reduced by addition of serum, in order to maintain the concentration of oligonucleotide at 1μM, a second addition of Lipofectamine Plus-oligo complex (280 μl) was prepared. The second addition was prepared as described above where microcentrifuge tube 1 contained 127.05 μl DMEM, 3.5 μl oligonucleotide, 9.45 μl PLUS reagent and tube 2 contained 126 μl DMEM and 14 μl Lipofectamine. The

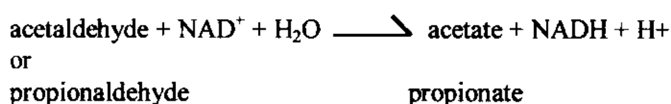
final Lipofectamine Plus-oligo complex (280 μl) was added the culture dish at 6 hours. Immediately after the second addition of Lipofectamine Plus-oligo complex, a mixture of horse (2.2 ml) and fetal bovine serum (0.6 ml) was added to the cells and incubated for an additional 18 hours.

Mitochondrial Isolations. Upon completion of incubations, H4-II-E-C3 cells were washed, detached mechanically from the culture dish and homogenized in 0.25 mM sucrose containing 10 mM Tris-HCl, pH 7.4 in a Wheaton Potter-Elvehjem glass tissue grinder with a teflon pestle (Fisher, PA). Cell lysis was confirmed by microscopy. The lysate was centrifuged at 1250 x g for 10 minutes at 4°C to remove the cellular debris and the centrifuged at 12,500 x g for 20 minutes at 4°C to collect the crude mitochondrial pellet. The mitochondrial pellet was washed by centrifugation at 12,500 x g for 5 minutes. The mitochondrial pellet was resuspended in 250 μl of 50 mM sodium pyrophosphate pH = 9.0, frozen-thawed in liquid nitrogen and stored at -70°C until the time of analysis. Prior to analysis, the mitochondria were thawed on ice, 1% Trition X-100 was added and the mitochondria were incubated for 15 minutes on ice to ensure maximum solubilization of the mitochondrial membranes. In some studies (Study III, Table 1), the Triton X-treated mitochondria were transferred to a 7x20 mm ultracentrifuge tube (Beckman,CA) and centrifuged at 4°C for 30 minutes at [illegible] 500 x [illegible] ɔ remove insoluble particulates.

Mitochondria from rat livers collected from control (PBS) and ASO-9-treated animals were isolated in a similar fashion as desribed above for the H4-II-E-C3 cells with a few modifications. Liver (2 g) was removed from each animal,

washed 1X in 10 ml isolation buffer and placed into 5 ml of isolation buffer for homogenization, transferred in 50 ml Wheaton Potter-Elvehjem tissue grinding chamber and homogenized as described above until cell rupture was confirmed. Then 20 ml of isolation buffer was added to the 5 ml of lysis suspension and mixed to bring the volume up to 25 ml. The centrifugation steps, washes, mitochondrial resuspension and the ultracentrifugation of the Triton X-100 lysate were as described above. Soluble mitochondrial fraction protein content isolated from H4-II-E-C3 cells or liver was determined by the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL) as described by the manufacturer with bovine serum albumin as the standard.

Determination of mitochondrial ALDH and GDH Activity. The low Km ALDH activity in the isolated mitochondria was assayed as described by Tank et al (1981) with minor modifications. The assay was performed at 37°C in a 400 μl reaction mixture containing 150 μg of soluble mitochondrial protein, and was initiated by the addition of by either acetaldehyde (10 μM) or propionaldehyde (14 μM), 80 μM NAD^+ and 0.25 mM $MgCl_2$ (Farres *et al.*, 1994, Takahashi and Weiner, 1980).



ALDH Assay reagents were prepared as follows:

1. Sodium Pyrophosphate buffer(SPP) (50 mM, pH 8.5-9.0)
   Dissolve 2.23 g in 95 ml ddH_2O, adjust pH with HCl to 9.0 and QS to 100 ml

2. Dithiothreitol (DTT, 200 mM)
   Dissolve 30.84 mg in 1 ml ddH_2O

3. Magnesium ($MgCl_2 \cdot 6H_2O$, 125 μM)
   Dissolve 25.41 mg in 0.5 ml ddH_2O

4. Pyrazole (1 M)
   Dissolve 68.1 mg in 1 ml ddH_2O

5. β-NAD^+ (80 mM)
   Dissolve 53.07 mg in 1 ml ddH_2O

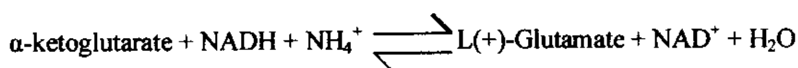
ALDH Reactions (400 μl) were performed in microcuvettes at 37°C

The ***blank*** contained 372 μl of sodium pyrophosphate buffer, 8 μl DTT, 8 μl Mg^{+2}, 4 μl pyrazole, 4 μl NAD^{+} and 4 μl acetaldehyde (1 mM) or propionaldehyde (1.4 mM) stocks

The ***sample*** (solubilized mitochondrial protein) contained 372 μl SPP – sample volume, sample (150 μg-volume (μl) depended on mitochondrial protein concentration), 8 μl DTT, 8 μl Mg^{+2}, 4 μl pyrazole, 4 μl NAD^{+} and the reaction was initiated with 4 μl acetaldehyde (1 mM) or propionaldehyde (1.4 mM) from stock solutions.

ALDH2 activity was determined by recording the change in absorption at 340 nm, due to NADH formation, with a Beckman DU®640 spectrophotometer. The change in OD was linear with time until nearly 100 % of the aldehyde or propionaldehyde was depleted after which any changes in OD stopped fully, indicating a high affinity for the ALDH system in liver and H4-II-E-C3 mitocondria. ALDH activity was expressed as nmoles NADH/min/mg protein.

Glutamate dehydrogenase (GDH) was assayed as described by E. Schmidt in 1983 with minor modifications (Schmidt, 1983). The assay was performed at 37°C in a 400 μl reaction mixture containing 25 μg of soluble mitochondrial protein, and was initiated by the addition of α-ketoglutarate to a final concentration of 6.9 mM. The reaction proceeds as shown.



$$\alpha\text{-ketoglutarate} + NADH + NH_4^{+} \rightleftharpoons L(+)\text{-Glutamate} + NAD^{+} + H_2O$$

GDH Assay reagents were prepared as follows:

1. Triethanolamine (TEA) buffer (100 mM; pH 8)
   Dissolve 1.857 g TEA into 100 ml water, add NaOH to adjust pH to 8.
2. NADH (0.16 mM)
   Dissolve 5.675 mg β-NADH into 500 μl of the above TEA buffer
3. α-ketoglutarate (6.9 mM)
   Dissolve 116 mg into 1 ml water
4. Ammonium Acetate (100 mM)
   Dissolve 770.8 mg in 1 ml water
5. EDTA (2.5 mM)
   Used 0.5 M stock solution
6. ADP (1 mM)
   Dissolve 21.36 mg in 500 μl of the above TEA buffer

GDH Reactions (400 μl) were performed in 1 ml microcuvettes at 37°C

The ***blank*** contained 386 μl of TEA buffer, 4 μl ammonium acetate, 4 μl ADP, 2 μl EDTA and 4 μl α-ketoglutarate

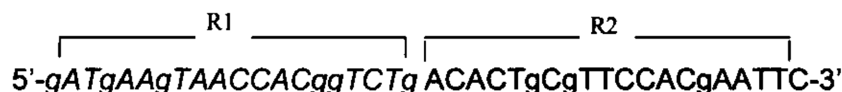
The ***sample*** (solubilized mitochondrial protein) contained 382 μl TEA buffer – sample volume, sample (25 μg -volume (μl) depended on mitochondrial protein concentration), 4 μl ammonium acetate, 4 μl ADP, 4 μl NADH, 2 μl EDTA and the reaction was initiated with 4 μl α-ketoglutarate.

GDH activity was determined by recording the change in absorption at 340 nm, due to NADH oxidation, with a Beckman DU®640 spectrophotometer. The reaction was linear with time. GDH activity was expressed as μmoles NADH oxidized/min/mg protein.

Determination of mitochondrial ALDH half life by Cycloheximide. Initial studies were conducted to determine the optimal concentration of cycloheximide required

to maximally inhibit (>90%) protein synthesis in the H4-II-E-C3 cells. Cell were seeded at a density of 16X10^6 cells/T75 flask 24 hours prior to the treatment with cycloheximide to ensure that they were in a stationary phase. The incubation medium containing serum was removed from the cells and the cells were washed twice with 5 ml of methionine-free DMEM (L-glutamine was added) (GibcoBRL Life Technologies, NY). Cells were then cultured for 1 hour in 8 ml of methionine-free DMEM containing 1, 3, 5, 7 or 10 µg/ml cycloheximide after which a 15-minute pulse of ^{35}S-methionine (1.5 µCi, SA=1 CI/µM) was added to the flask. After the pulse, ^{35}S-methionine was removed from the flask and the cells were washed 2 x with 5 ml phosphate buffered saline. Cells were then lysed in 10 ml solution of 0.15 M KCl containing 1 mM EDTA (pH = 8.0). The cellular suspension was then put through three freeze-thaw cycles (1 cycle = liquid-nitrogen to 42°C) and then centrifuged at 1,200Xg to remove cellular debris. A 100 µl aliquot of the supernate was then placed on a filter paper disk and dried. The filter paper disk was then placed in 10% trichloroacetic acid(TCA). Washed 2X in 5% TCA, heated to 90° C for 15 min in 5% TCA, washed 1X in 5% TCA, washed 2X in ethanol ether (1:1 at 37° C , washed 2X in diethyl ether and then air dried (Mans and Novelli, 1961). This procedure was used to ensure that all unincorporated ^{35}S-methionine associated with the cellular proteins was removed prior to radioactivity determination. The filter disk was then placed into a 20 ml vial with 15 ml EcoLite® Liquid Scintillation fluid (ICN, CA) for radioactivity determination on a Beckman LS 6500 liquid scintillation counter. The total cellular protein content of cells treated with or without cycloheximide was determined as described above.

Determination of mRNA expression by Competitive RT-PCR. **ALDH mRNA.** Competitive RT-PCR for ALDH was developed by first designing PCR primers to amplify a 584 base pair fragment from the Sprague-Dawley rat cDNA, kindly donated by Dr. Henry Weiner of Purdue University. The primers (specific to mitochodrial ALDH) used to generate the 584 base pair fragment (refered to as a parent fragment) were as follows: forward (**F**) 5'-ACgTggTggTgATgAAAgTg-3' and reverse (**R1**) 5'-gATgAAgTAACCACggTCTg-3'. The parent fragment was amplified from the cDNA template under the following conditions: denaturation at 95° C for 3 min followed by 30 cycles at 95°C –1 min, 63 °C –30 sec, 72 ° C 1.2 min and a final extension at 72 ° C for 10 min and the resulting band was purified from a 1% agarose gel. An internal standard, which served as a competitor for primers of the parent fragment during reverse transcription (as recombinant RNA, rcRNA) and PCR (as first strand cDNA) reactions, was constructed using a similar technique to that described previously (Wang *et al.*, 1989) (see Figure 2.1). The same forward primer mentioned above and a new reverse primer (NRP), comprised of the previous reverse primer sequence (R1) and an added reverse primer sequence (R2), was used to amplify a smaller internal standard fragment of 429 base pairs.



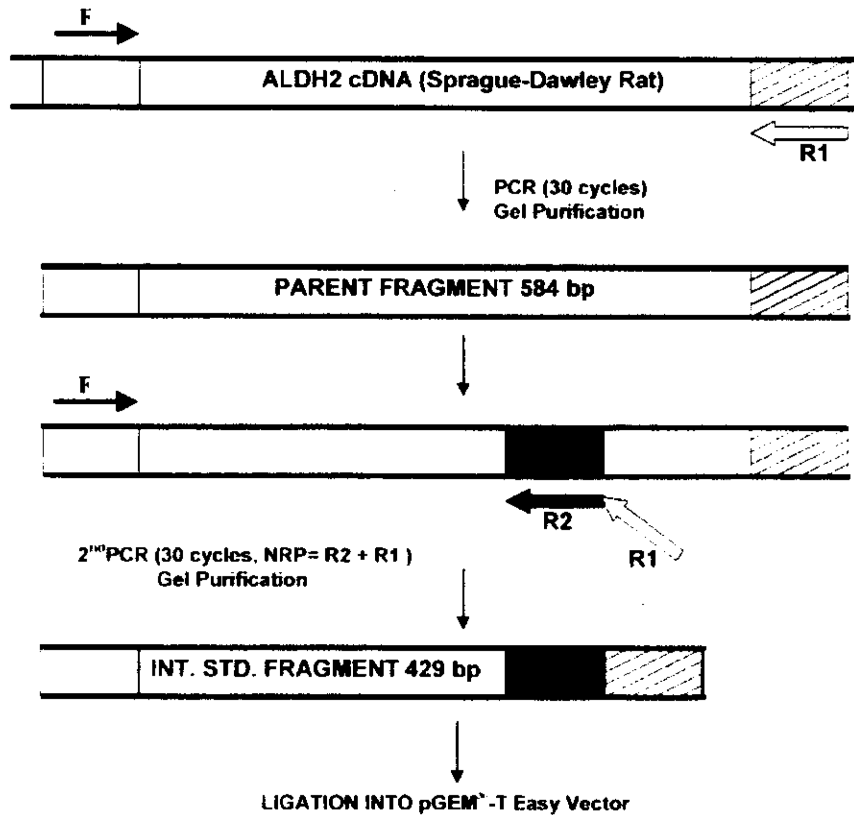
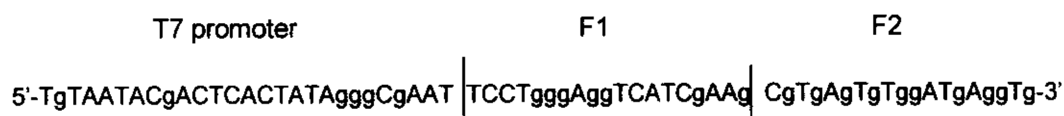


Figure 2.1.

The pGEM®-T Easy Vector (Promega, WI) containing the 429 bp recombinant DNA fragment (rcDNA) was transformed into DH5α Competent E. coli cells according to the manufacturers protocol (Life Technologies, NY). The plasmid DNA was linearized using Not I restriction enzyme and then purified before use as template in the *in vitro* transcription reaction. The *in vitro* transcription reaction for generating RNA to be used as competitor in the RT-PCR reaction was carried out using the RiboProbe® In Vitro Transcription System (Promega, WI). The *in vitro* transcription and RNA purification was performed as recommended in the Promega technical manual.

GDH mRNA. A streamlined procedure similar to that reported in 1993 by Vanden Heuvel et al for design of a recombinant RNA (rcRNA) was used to make the GDH internal standard which eliminated any subcloning and relied only on PCR to obtain the rcDNA template for in-vitro transcription (Vanden Heuvel *et al.*, 1993) (see Figure 2.2). Primers were designed to amplify a 782 base-pair parent fragment (forward (**F1**) 5'-TCCTgggAggTCATCgAAg-3', reverse (**R**) 5'-AACTgCTTCTCgCTggCTg-3'). The cDNA template for GDH was obtained by reverse transcription using random hexamers (pd(N)6 20 ng/20 μl RT reaction) (Amersham Pharmacia Biotech, NJ) and Omniscript RT (Qiagen, CA) from RNA isolated from H4-II-EC-3 cells. The GDH parent fragment primers were then used to amplify the 782 bp fragment by PCR using similar conditions to those described above for ALDH using 55° C for primer annealing. The PCR product was run on a 1% agarose gel and the 782 bp parent fragment was purified from the gel. The purified fragment was then amplified with a newly designed forward primer and the same reverse primer as mentioned above. The new forward primer was constructed as shown below. F1 = previous forward primer sequence and F2 = new forward primer sequence.



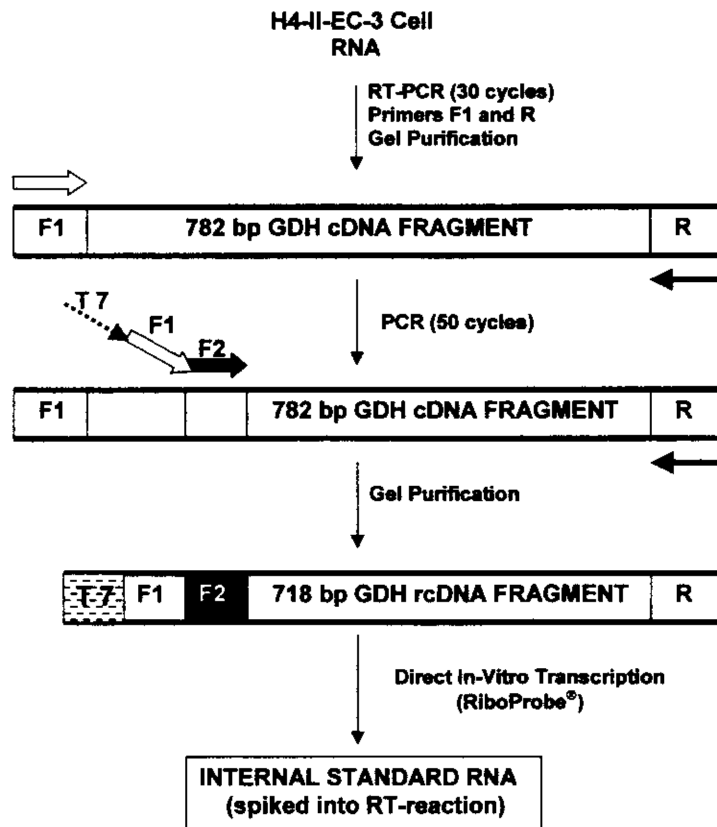


Figure 2.2

The smaller fragment was then used as the DNA template for *in vitro* transcription to make RNA which served as the GDH internal standard for competitive RT-PCR reactions with sample RNA.

Sample analysis for ALDH or GDH mRNA expression was performed using 2 μg of total RNA spiked with a range of internal standard RNA (ALDH 0-100 pg and GDH 0-32 ng) depending on the anticipated gene expression. Omniscript RT was used for all the reverse transcription reactions according to the Qiagen protocol using random heximers (Amersham Pharmacia Biotech, NJ) to prime

first strand cDNA synthesis. The parent fragment and internal standard bands were analyzed on a Kodak Digital Science Image Station 440CF (Eastman Kodak Co, NY) by analyzing the net intensity of the UV/fluorescence emitted by the ethidium bromide in the bands. The actual amount of internal standard that was needed to compete for amplification with the parent fragment was determined by plotting the amount of internal standard against its corresponding net intensity ratio (internal standard band / parent fragment band). Assuming a linear relationship, the exact amount of internal standard needed to equally compete with the amount of sample mRNA present was determined from the linear regression equation when y=1.

Cannula Implantation. Rats were placed under general anesthesia using isoflurane delivered by rodent anesthesia inhalation unit. Once under anesthesia, the animals were catheterized by placing Tygon flexible plastic tubing (0.015 I.D X 0.03 O.D) into the femoral artery, and a small rodent trocar was used to pass the tubing from the femoral region to the animal's neck region. A specially designed protective jacket was used to protect the cannula during a five day recovery period. Patency of catheters was maintained by using a lock solution containing heparin (444 units/ml), dextrose (22%) and streptokinase (16,667 units/ml), and a small piece of monofilament which was inserted at the exposed end of the cannula to retain the lock solution. The patency of the catheter was ensured by flushing every day with 10 units/ml heparin followed by lock replacement to fill the tubing dead volume (approx. 75 μl) between flushing sessions.

Osmotic pump implatation. Rats were placed under general anesthesia using isoflurane delivered by rodent anesthesia inhalation unit. Once under anesthesia, the hair in the animal's abdominal area was shaved and scrubbed with betadine, and a midline inscision was made approximately 2-3 cm in length. An Alzet® minipump 2ML1 (cat. No. 0000323, DURECT Corp., Cupertino, CA, www.alzet.com) was filled with 2 ml of the desired solution (PBS or ASO-9, 25 mg/ml) using the supplied blunt end needle and a syringe. The pump cap (see Figure 2.3) was placed into the pump body for sustained delivery rate of 10 μl/hour which equated to the animals receiving 24/ mg/kg/day.

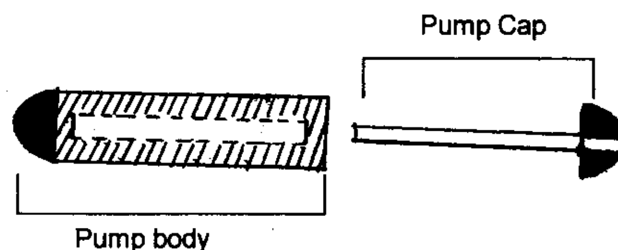


Figure 2.3.

The assembled pump filled with the desired solution for delivery was then placed through the abdominal incision and the muscle layers apposed with 4-0 Nylon suture (Ethilon®, Johnson and Johnson, Somerville, N.J). The skin was then apposed with stainless-steel suture clips. Buprenorphine (0.3 mg/ml, Reckitt & Colman Pharmaceutical, Inc. Ridgemont, VA) was administered as the analgesic at 0.1 ml/ 300 g B.W.

Acetaldehyde Determination by HPLC. Blood was collected in heparin, placed immediately on ice and centrifuged at 10,000 g for 5 minutes to harvest plasma for subsequent acetaldehyde determinations. The plasma was then keep at

-70°C until analysis. Acetaldehyde was determined by HPLC using a modification of the method described by Lucas (Lucas *et al.*, 1986). Plasma (250 µl) was mixed with 4 ml of ice cold perchloric acid (0.6 M in 0.15 M sodium chloride) and centrifuged at 10,000 g for 10 minutes at 4°C. Acetaldehyde present in 4 ml of the supernatant was derivatized with the addition of 100 µl of a solution of 2.5 mg/ml 2,4-dinitrophenylhydrazine (DNP) in 6M HCl solution. The reaction was stopped by adjusting the pH to 4.5-5.0 by addition of 2 ml ice cold sodium acetate (3 M).

The samples were extracted with the addition of 2 ml isooctane, and mixed by vortexing and orbital rotation on a Orbitron Rotator I (Boekel Industries Inc., Feasterville, PA) for 20 minutes at room temperature. The emulsion was then separated by centrifugation at 10,000 g for 10 minutes at 4°C. The upper isooctane layer (1.2 ml) containing the acetaldehyde-DNP derivative was evaporated to dryness in a Centrivap concentrator (Labconco, MO). The samples were reconstituted in 100 µl of water:acetonitrile (70:30). Reconstituted samples (20 µl) were separated on a Beckman System Gold Programmable Solvent Module 125 equipped with a Rheodyne Model 7725 injection valve (20 µl loop), a System Gold Diode Array detector module 168 and an Ultrasphere ODS column (5 µm, 4.6 mm x 4.5 cm, Beckman, CA). Separations were carried out using gradient elution with a two solvent system consisting of water (solution A) and acetonitrile (solution B). The flow rate was 1 ml/minute and the gradient went from 30 to 70% B in the first 10 minutes, was sustained at 70% B for 5 minutes, followed by an increase to 90% B over the next 3 minutes, and returned to 30% B over the remaining 3 minutes. The absorbance of the eluted

derivatives was monitored at 365 nm and the peak height was electronically integrated with System Gold software version 8.0. A five point standard curve with an acetaldehyde concentration range of 0-20 μM was prepared from a pool of rat plasma with the samples that were extracted for HPLC analysis. All samples were quantified from a standard curve prepared on the same day the samples were extracted into isooctane. The linear correlation of the standard curve was ≥ 0.95.

Section III

PARADIGM TO TEST A DRUG-INDUCED AVERSION TO ETHANOL

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SUMMARY

The screening of new agents for aversive therapy of alcoholism requires a simple animal model. Animals trained to ingest ethanol solutions which are subsequently administered a drug known to produce an aversion to ethanol in humans, do not readily make the association between the malaise induced by the aversive drug-ethanol reaction and the consumption of the same ethanol-containing solution that has been consumed previously without ill effects. We report an experimental paradigm in which the malaise of the drug-ethanol reaction is quickly recognized by rats as derived from ethanol. Disulfiram was used as the model drug. Lewis rats were deprived of water for 18 hours after which 6% ethanol was offered as the only fluid. On the first hour of ethanol access, both controls (vehicle) and disulfiram (100 mg/kg) treated animals consumed intoxicating amounts of ethanol (0.7-0.9 g ethanol/kg). Plasma acetaldehyde levels developed were 3-5 μM and 40-50 μM respectively. After such time, disulfiram-treated animals virtually cease consuming alcohol (90% inhibition), indicating that the disulfiram-ethanol reaction is associated with alcohol ingestion. Control animals continue consuming the alcohoi solution for the additional 4-5 hours tested. This model should be of value in the testing of new agents that reduce aldehyde dehydrogenase levels for prolonged periods for their potential as an aversive treatment in alcoholism.

INTRODUCTION.

Disulfiram is an "anti-alcohol" drug that non-specifically inhibits ALDH2-1 by reacting with sulfhydryl groups in the enzyme (Weiner,1979; Vallari and Pietruszko, 1982). Supervised disulfiram administration is effective in reducing alcohol consumption in alcoholics (Brewer,1993). However, the effectiveness of non-supervised disulfiram has been criticized (Swift, 1999). In a well controlled study of non-supervised administration of disulfiram of 202 subjects, a therapeutic dose of the drug significantly reduced the number of drinks consumed daily (Fuller et al, 1986). However, the time to the first drink was not reduced nor was absolute abstinence. The latter are not unexpected; an aversion to ethanol would only occur after the patient challenges the effect of the drug.

Disulfiram has serious disadvantages, including a number of side effects such as severe hypotension, parasthesias and motor neuropathies (Gallant,1987; Peachey and Annis,1989; Dupuy, et al.,1995) which markedly reduced compliance with self-administration. In addition, Brewer (1984) and Christensen et al (1991) showed that when disulfiram is administered in tolerable doses, only 50% of patients develop the disulfiram-ethanol reaction.

In humans a single gene mutation protects 70-90% against alcohol abuse or

alcoholism (Harada, et al.,1982; Thomasson, et al.,1991; Higuchi, 1994 ; Tu and Israel,1995). Such a gene encodes a high-affinity aldehyde dehydrogenase (ALDH2) (Yoshida, et al.,1985), a mitochondrial enzyme that metabolizes acetaldehyde, the first product of ethanol oxidation (Svanas and Weiner,1985; Cao, Tu and Weiner,1988; Klyosov, et al.,1996). Means to specifically inhibit the transcription or translation of this gene on a long-term basis could be used as potential agents for the treatment of alcoholism.

The development of new aversive means to reduce alcohol consumption requires an experimental paradigm to test the drug-induced aversion to ethanol. We describe studies designed to test the effect of an aversive medication.

MATERIALS AND METHODS.

Male Lewis rats (Harlan, Indianapolis, IN) weighing 200-300g, were used. Prior to the ethanol consumption studies all rats were acclimated for at least 3 days in either a plastic shoebox cage or a specially designed plexiglass cage with stainless steel wire bottom. All animals were maintained on a 12 hr light/dark cycle and had free access to laboratory rodent diet 5001(PMI Feeds, Inc., St. Louis, MO) and tap water. Finely powdered disulfiram was suspended in 1% carboxymethylcellulose (CMC) in saline to yield a final concentration of 15 mg/ml and was administered orally.

Animals were administered disulfiram (100 mg/kg) or vehicle (at 9:00 and 10:00 AM) for 4 days. On the evening of day 3, water was removed for 18 hr while food was provided ad-libitum. On the morning of day 4, animals were dosed as described above, and two hours later the animals were allowed access to a 6% ethanol solution (v/v) as the only fluid. Consumption of the ethanol solution was measured at 1, 2, 3, 4 and 5 hours. In some experiments plasma acetaldehyde was determined at 0, 1, 3, 5 and 24 hours after animals were given access to 6% ethanol. For the latter studies, blood samples were removed from femoral catheters while the animals were freely consuming the ethanol solution.

Plasma acetaldehyde levels

Blood was collected in heparin, placed immediately on ice and centrifuged at 10,000 g for 5 minutes to harvest plasma for subsequent acetaldehyde determinations. Acetaldehyde was determined by HPLC using a modification of the method described by Lucas et al (1986). Plasma (250 μl) was mixed with 4 ml of ice cold perchloric acid (0.6 M in 0.15 M sodium chloride) and centrifuged at 10,000 g for 10 minutes at 4°C. Acetaldehyde present in the supernatant was derivatized by the addition 2,4-dinitrophenylhydrazine (DNP). The absorbance of the eluted derivatives was monitored at 365 nm. A five point standard curve with an acetaldehyde concentration range of 0-20 μM was prepared for HPLC analysis. All samples were quantified from a standard curve prepared on the same day the samples were extracted into isooctane. The linear correlation of each of the standard curves was ≥ 0.991.

Determination of Mitochondrial ALDH2 Activity

Mitochondria from the livers collected from control and disulfiram-treated animals were isolated as described by Tank, Weiner and Thurman, (1981). Mitochondria were resuspended in 0.1 M sodium phosphate buffer, pH 7.4 and stored at -70°C until protein content and aldehyde dehydrogenase activities were determined. Prior to enzyme activity determinations, the thawed mitochondria samples were incubated with Triton X-100 (2% v/v) at 37°C for 15 minutes to ensure complete lysis and release of aldehyde dehydrogenase.

The low Km ALDH activity in the isolated mitochondria was assayed as previously described by Tank et al (1981) with minor modifications. The assay was performed at 37°C in 1.0 ml of reaction mixture, and was initiated by the addition of acetaldehyde to a final concentration of 10 μM. ALDH2 activity was determined by recording the change in absorption at 340 nm, due to NADH formation, with a Beckman DU®640 spectrophotometer. Protein concentration in the resuspended mitochondria was measured by the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL) as described by the manufacturer with bovine serum albumin as the standard.

RESULTS.

The consumption of 6% ethanol was determined at intervals over a 5 hour period in 18-hour water-deprived rats pre-treated with disulfiram (100 mg/kg) or vehicle (Fig. 1). Cumulative intake is shown at hourly intervals throughout the first five hours.

During the first hour of 6% ethanol presentation, ethanol consumption was similar in the disulfiram and vehicle control groups, amounting to 0.87 ± 0.12 g ethanol /kg (disulfiram) and 0.69 ± 0.12 g ethanol/kg (controls). However, after the first hour, control animals continued to drink the ethanol solution while the disulfiram-treated animals virtually ceased consuming it. During the subsequent 4-hour interval (hours 1 to 5), disulfiram-treated animals consumed 88.1% less ethanol than controls ($p<0.002$).

In a separate group of disulfiram-treated animals, alcohol consumption and plasma acetaldehyde concentrations were measured simultaneously in animals with implanted femoral catheters (Figure 2). Data show that disulfiram animals consumed the 6% ethanol solution during the first hour of access (0.8 g/kg), as previously observed, then stopped consuming ethanol until 4-5 hours when minor ethanol consumption was resumed. Alcohol consumption in the first hour led to marked elevations in plasma acetaldehyde averaging 59.6 ± 11.1 μM. Acetaldehyde levels were reduced to 24.4 ± 10.8 μM by the third hour and 4.0± 1.1 μM at 5 hours.

ALDH2 activity and acetaldehyde levels

Three hours after the administration of 100 mg/kg disulfiram (given daily for 4 days), liver mitochondrial aldehyde dehydrogenase activity was found to be inhibited by 87% ($p< 0.0001$) when compared to that in control animals given the vehicle. Under the same conditions, the administration of ethanol (1g/kg orally) increased plasma acetaldehyde from 5.0 ± 0.2 μM (before ethanol) to 41.9 ± 4.9 μM, one hour after

administration of ethanol ($p < 0.001$). In line with studies by Eriksson and co-workers (1985), endogenous acetaldehyde levels prior to any exposure to ethanol, were increased in disulfiram-treated animals (5.03 ± 0.21 μM) versus those in control animals (1.28 ± 0.43 μM) ($p < 0.001$).

DISCUSSION.

Alcoholics given disulfiram are normally informed of the dysphoria that may result from the disulfiram-ethanol reaction, and thus a *guided association* can be made between ethanol consumption and the malaise that ensues. This is less feasible with animals. Earlier work, which led to the present study, suggests that animals which have learned to consume ethanol do not quickly associate that the same ethanol solution which has been consumed over a prolonged learning period without ill effects has (after drug administration) become the cause of malaise. In one study (Schlesinger, Kakihana and Bennett,1966) in which mice were offered the choice of water or 10% ethanol for 14 days, the subsequent administration of disulfiram (100 mg/kg/day) for 3 days led to only small reductions (20%) in ethanol consumption. In another study (Amit, Levitan and Lindros, 1976), rats were trained for 50 days to drink solutions containing increasing concentrations of ethanol up to 10% on the two bottle choice condition. Subsequent administration of disulfiram or calcium carbimide every second day for 10 days led to only minor reductions (10%) in ethanol consumption, despite the fact that disulfiram (50 mg/kg/day) and calcium carbimide

(25 mg/kg/day) treatment markedly elevated blood acetaldehyde levels following the intraperitoneal administration of 1.5 g/kg ethanol.

The use of the limited access paradigm to assess the effect of aversive medication has resulted in conflicting data which also limits its use. In this paradigm rats were trained for about 60 days to consume alcohol solutions which were available to the animal for 10 minutes per day. Cyanamide, an inhibitor of aldehyde dehydrogenase, increased, rather than decreased-ethanol consumption in this model (Aragon, et al.,1993).

The method used in the present study was to pair the putative dysphoric effects of the disulfiram-ethanol reaction with the taste of ethanol, thus a variant of the conditioned taste aversion paradigm in which a novel taste is paired with the administration of a noxious substance or a noxious condition (the ethanol-disulfiram reaction). It is well established (Barber, et al.,1998; Nolan, et al.,1997; Scalera, Grigson and Norgren, 1997; Yasoshima and Yamamoto,1998) that a robust conditioned taste aversion quickly develops after the first pairing of the new taste with a noxious agent. In our studies the effect was fully demonstrable in as short as 5 hours following ethanol presentation, which allows fluid deprivation of the animals to slightly less than 24 hours.

Such a paradigm requires (i) that ethanol be consumed for the first time by animals that have been pretreated with disulfiram, (ii) that enough ethanol be consumed by the animal in a short time to generate high blood acetaldehyde levels which leads to the malaise of the disulfiram-ethanol reaction, and (iii) ideally, that an inbred strain be used

to reduce variability and increase inter-laboratory reproducibility

Data obtained after the disulfiram dosing regimen described indicates that a strong aversion to ethanol is observed when plasma acetaldehyde levels are in the range of 40-60 μM resulting from a reduction in mitochondrial aldehyde dehydrogenase activity of 87%. This reduction in activity is in line with the reduction of aldehyde dehydrogenase activity observed in human ALDH2-2/ALDH2-1 heterozygotes (Enomoto, et al.,1991; Xiao, et al.,1995) who are markedly protected against alcohol abuse and alcoholism (Harada, et al.,1982; Higuchi,1994; Thomasson, et al.,1991; Tu and Israel,1995). In ALDH2 deficient individuals the oral consumption of 0.5 g/kg ethanol yielded plasma acetaldehyde concentrations of 35.4 μM as compared to 2.1 μM in controls (Harada, Agarwal and Goedde, 1981).

Overall, studies presented show that a simple animal model can be used to determine the aversion against ethanol elicited by drugs that reduce aldehyde dehydrogenase activity and which result in increases in acetaldehyde levels comparable to those seen in humans carrying an inactive aldehyde dehydrogenase allele who consume ethanol.

LEGENDS TO FIGURES.

Figure 3.1. Aversion to ethanol induced by disulfiram administration. Cumulative ethanol consumption (g/kg) of 6% ethanol (v/v) in control (vehicle) and disulfiram-treated animals at hourly intervals for the first 5 hours of fluid re-access. Closed circles represent animals that received disulfiram (100 mg/kg, i.p., n=6). Open circles represent animals that received vehicle (n=6). Values given as means (± SEM). Ethanol consumption in the first hour was not different between the two groups. However, ethanol consumption in the following 1 to 5 hours was 88% ($p<0.002$) lower in disulfiram-treated animals than in controls.

Figure 3.2. Alcohol consumption and acetaldehyde levels in disulfiram-treated rats. Cumulative consumption of 6% ethanol (v/v) at 1, 2, 3, 4 and 5 hours and plasma acetaldehyde concentrations at 1, 3 and 5 hours in disulfiram-treated (100 mg/kg, i.p.) unrestrained animals with exteriorized femoral cannulas. Values are given as mean (± SEM). Open circles represent ethanol consumption (g/kg) and filled squares represents plasma acetaldehyde concentrations (μM) in the same animals (n=3). Standard error bars for acetaldehyde at 5 hours are encompassed by the symbol.

Footnotes to the title page:

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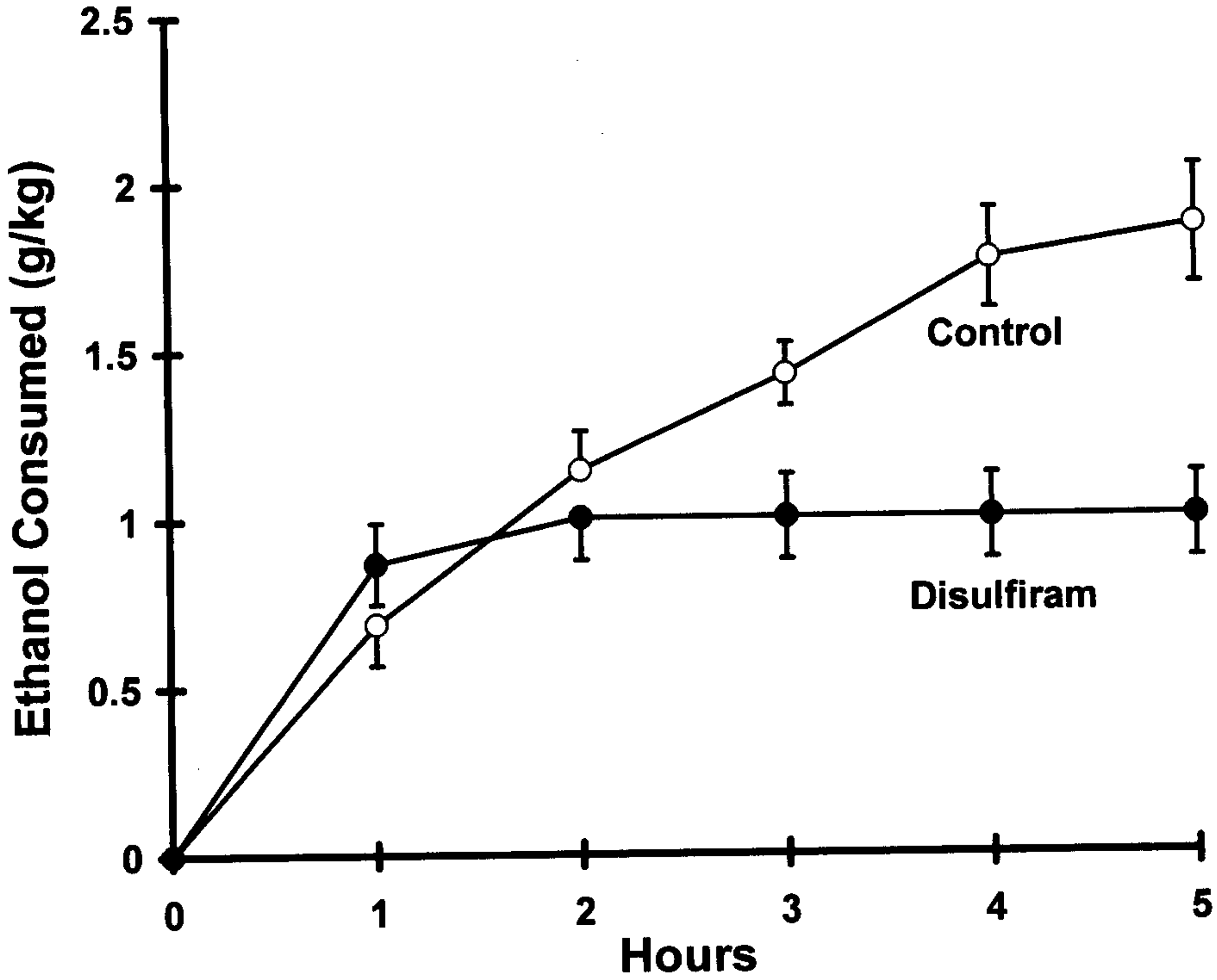
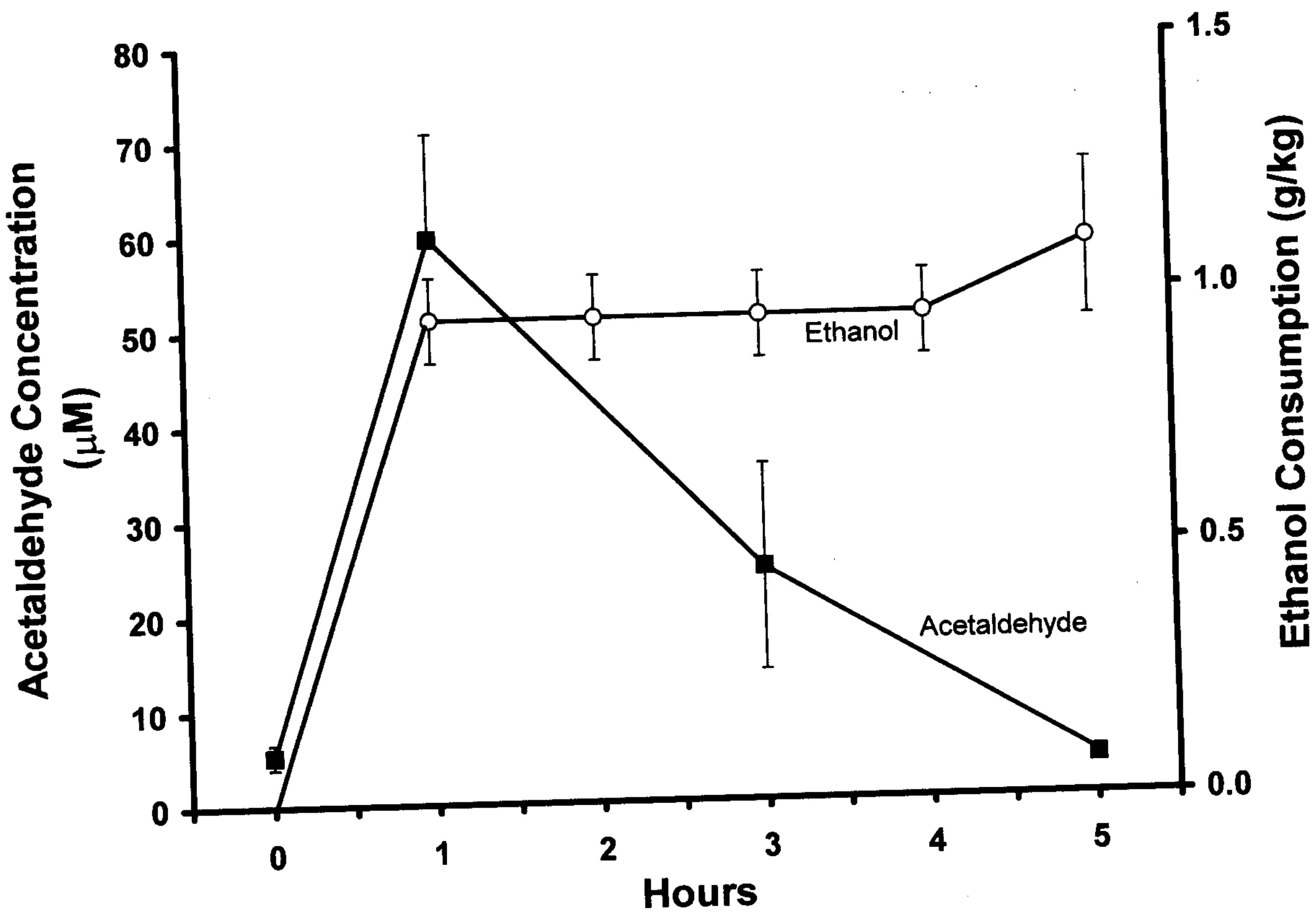

Ethanol Consumed (g/kg)
2.5
2
1.5
1
0.5
0
Control
Disulfiram
0
1
2
3
4
5
Hours


Acetaldehyde Concentration (μM)
80
70
60
50
40
30
20
10
0
Ethanol Consumption (g/kg)
1.5
1.0
0.5
0.0
0
1
2
3
4
5
Hours
Ethanol
Acetaldehyde

Section IV

ELICITING THE LOW-ACTIVITY ALDEHYDE DEHYDROGENASE ASIAN PHENOTYPE BY AN ANTISENSE MECHANISM RESULTS IN AN AVERSION TO ETHANOL

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ABSTRACT

A mutation in the gene encoding for the liver mitochondrial aldehyde dehydrogenase (ALDH2-2), present in some Asian populations, lowers or abolishes the activity of this enzyme and results in elevations in blood acetaldehyde upon ethanol consumption, a phenotype that greatly protects against alcohol abuse and alcoholism. We have determined whether antisense oligonucleotides (ASOs) administration can mimic the low-activity ALDH2-2 Asian phenotype. Rat hepatoma cells incubated for 24 hours with an antisense oligonucleotide (ASO-9) showed reductions in ALDH2 mRNA levels of 85% and ALDH2 (half-life of 22 hours) activity of 55% equivalent to an >90% inhibition in ALDH2 synthesis. Glutamic dehydrogenase mRNA and activity remained unchanged. Base mismatches in the oligonucleotide rendered ASO-9 virtually ineffective, confirming an antisense effect. Administration of ASO-9 (20 mg/kg/day for 4 days) to rats resulted in a 50% reduction in liver ALDH2 mRNA, a 40% inhibition in ALDH2 activity and a four-fold ($p<0.001$) increase in circulating plasma acetaldehyde levels following ethanol (1 g/kg) administration. Administration of ASO-9 to rats by osmotic pumps led to an aversion (-61% $p<0.02$) to ethanol. These studies provide a proof of principle that specific inhibition of gene expression can be used to mimic the protective effects afforded by the ALDH2-2 phenotype.

INTRODUCTION

Research on the genetics of alcoholism indicates that there are (i) predisposing and (ii) protective factors in the development of alcoholism. The heritability of predisposing factors has been estimated to be 40-60% (1), while the protective genetic influence can approach 100% (2). The protective genetic influence is associated with a low activity of liver mitochondrial aldehyde dehydrogenase (ALDH2)[1], an enzyme that plays a major role in the detoxification of acetaldehyde generated in the metabolism of ethanol. In some Asian populations, a dominant mutation in the gene that codes for this enzyme (ALDH2-2) renders the enzyme largely inactive (3, 4). Individuals carrying the ALDH2-2 allele who consume small amounts of alcohol display elevated blood acetaldehyde levels and dysphoric effects that include dizziness, nausea, hypotension and palpitations (5). It has been postulated that many of the intoxicating effects of ethanol are accentuated in subjects carrying the ALDH2-2 allele (6, 7). Overall, the protective effect of the ALDH2-2 allele against alcohol abuse and alcoholism ranges from 66 and 90% for heterozygotes to 100% for homozygotes (2, 8-12).

With the advent of gene pharmacology, new approaches now exist by which the expression of a single gene can be inhibited. Antisense phosphorothioate oligonucleotides (ASO's) with a sequence complementary to the primary RNA transcripts or to mRNA can inhibit gene expression in a specific manner (13). The clinical applications of antisense are becoming accepted. An antisense phosphorothioate oligonucleotide (Vitravene™) designed to inhibit human cytomegalovirus (CMV) replication was approved for commercialization by the

FDA. In addition, over 20 other antisense phosphorothioate oligonucleotides are undergoing clinical trials (*www.recap.com*).

In 1997, a consensus conference on the design of antisense concluded that a large number of ASO's (>30) had to be designed and tested empirically to find one effective antisense molecule (14). However, recent work in our laboratory led to the finding that about one half of the most active antisense deoxyoligonucleotides reported in the literature contains a 5'-TCCC-3' motif in the molecule (15). Antisense deoxyoligonucleotides containing the TCCC motif and designed prospectively were shown to act by greatly reducing mRNA levels (15). This is in line with recent postulates that the RNA moiety of DNA-RNA hybrids is hydrolyzed by RNase H (16).

Here, we report studies on a phosphorothioate-modified deoxyoligonucleotide containing the 5'-TCCC-3' motif directed against ALDH2 mRNA which was shown to be most effective in: (i) reducing ALDH2 mRNA levels and mitochondrial ALDH2 activity in rat hepatoma cells *in vitro*, (ii) reducing liver ALDH2 mRNA and mitochondrial ALDH2 activity in rats *in vivo*, (iii) increasing four-fold the plasma acetaldehyde levels following an oral dose of ethanol and (iv) eliciting a marked reduction in ethanol consumption. Overall, we present studies in which an antisense molecule designed to anneal to ALDH2 mRNA in the rat results in a phenotype that mimics the elevated plasma acetadehyde levels observed in Asians who have a low ALDH2 activity (ALDH2-2) and consume ethanol. Such an antisense molecule led to a marked reduction in voluntary ethanol consumption in animals.

MATERIALS AND METHODS

Chemicals. All chemicals used were purchased from Sigma (St Louis MO), except for sucrose, sodium pyrophosphate, sodium phosphate, magnesium chloride, perchloric acid, hydrochloric acid, acetonitrile, and isooctane which were purchased from Fisher Scientific (Pittsburgh, PA).

Antisense oligonucleotides. Phosphorothioated oligonucleotides with specific base sequences used for *in vitro* studies were manufactured by Genset Corp (La Jolla, CA). Purified ASO-9 for *in vivo* work and for the last two *in vitro* studies was purchased from Hybridon (Milford, MA). For the *in vivo* study, HPLC purified ASO-9 was dissolved in phosphate buffered saline (PBS) at a concentration of 20 mg/ml. A large stock solution was prepared a day ahead of the initiation of the *in vivo* study and aliquoted into separate tubes which were stored at -70° C until the day it was needed for dosing to prevent any freeze-thaw cycles.

Cell Culture. Rat hepatoma cell line H4-II-E-C3 (H4) (ATCC CRL-1600) was purchased from American Type Culture Collection (Rockville, MD). Cells were grown in Dulbecco's modified Eagle's medium containing 4.5 g/L of L-glutamine (Mediatech,Cellgro®,VA), 20% horse serum and 5% fetal bovine serum (Life Technologies, NY) in 10% CO_2 at 37° C. All delivery of oligonucleotides and incubations were conducted in the presence of 10% CO_2 at 37° C.

In vitro Oligonucleotide Delivery. H4 cells were seeded 18-24 hours prior to the delivery of phosphorothioate oligonucleotides at a density of 2.5 X 10^6 cells/100 mm

culture dish. Oligonucleotides were prepared using cationic liposomes, Lipofectamine Plus™ (Life Technologies, NY). The procedure used to deliver oligos for a 24 hour incubation was essentially the same as those recommended by the manufacturer. For each 100 mm culture dish to be treated, oligo and PLUS reagent® (27 μl) were complexed with 40 μl of Lipofectamine (2 mg/ml) to form the Lipofectamine Plus-oligo complex in serum-free Dulbecco's modified Eagle's medium containing 4.5 g/L of L-glutamine (DMEM). The final Lipofectamine Plus-oligo complex (800 μl) was added to 7.2 ml of DMEM at 37° C. Prior to addition of the prepared Lipofectamine Plus-oligo complex, the medium with serum was removed from the cells and the final 8 ml of prepared of Lipofectamine Plus-oligo complex was added to the culture dish and incubated for 6 hours in the absence of serum.

Thirty minutes prior to the end of the 6 hour delivery serum were added back to the cells for overnight culture. Since the oligo concentration was reduced by addition of serum, a second addition of Lipofectamine Plus-oligo complex (280 μl) was prepared to maintain the desired concentration. A second addition was prepared using oligo, 9.45 μl PLUS reagent and 14 μl Lipofectamine. The final Lipofectamine Plus-oligo complex was added to the culture dish at 6 hours with a mixture of 20% horse (2.2 ml) and 5% fetal bovine serum (0.6 ml) and incubated for an additional 18 hours.

Mitochondrial Isolations. Upon completion of incubations, H4 cells were collected by removing the culture medium containing the Lipofectamine Plus-oligo complex and washing (1 x 5 ml) with ice-cold mitochondrial isolation buffer comprised of 0.25

mM sucrose in 10 mM Tris-HCl, pH 7.4. Cells were collected and transferred to an ice-cold conical tube for centrifugation at 152 x g for 5 minutes at 4° C to concentrate the cells into a pellet which was then resuspended in 200 μl of the isolation buffer. The concentrated cellular suspension was then homogenized and fractionated as previously described (17). The final purified mitochondrial pellet was resuspended in 250 μl of 50 mM sodium pyrophosphate pH = 9.0, immediately frozen in liquid nitrogen, and stored at -70° C until the time of analysis. Prior to analysis, the mitochondria were thawed on ice, solubilized by adding 1% Trition X-100 and incubated for 15 minutes on ice. In some studies (Study III, Table 1 and Table 3), the Triton X-treated mitochondria were transferred to an ultracentrifuge tube and centrifuged at 4° C for 30 minutes at 69,500 x g to remove insoluble particulates.

Mitochondria from rat livers collected from control (PBS) and ASO-9-treated animals were isolated in a similar fashion as desribed previously described (17). Soluble mitochondrial protein content isolated from H4 cells or liver was determined by the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL) as described by the manufacturer with bovine serum albumin as the standard.

Determination of ALDH and GDH Activity. The low-Km ALDH activity in the isolated mitochondria was assayed as previously described (18) with minor modifications. The assay was initiated by the addition of propionaldehyde to a final concentration of 14 μM, 80 μM NAD and 2.5 mM $MgCl_2$ (19). The reaction was linear with time, and ALDH activity was expressed as nmoles NADH/min/mg protein. Glutamate dehydrogenase (GDH) was assayed as described with minor

modifications (20). The assay was performed at 37° C in a 400 μl reaction mixture containing 25 μg of soluble mitochondrial protein. The reaction was linear with time, and GDH activity was expressed as the oxidation of NADH to NAD^+ in μmoles NAD/min/mg protein.

Determination of ALDH2 half-life by Cycloheximide (CHX). Initial studies were conducted to determine the optimal concentration of CHX required to maximally inhibit (>90%) protein synthesis in the H4 cells. Cell were seeded at a density of 16 X 10^6 cells/T75 flask 24 hours prior to the treatment with CHX to ensure that they were in a stationary phase. The incubation medium containing serum was removed from the cells and the cells were washed twice with 5 ml of methionine-free DMEM (L-glutamine was added) (Life Technologies, NY). Cells were then cultured for 1 hour in 8 ml of methionine-free DMEM containing 1, 3, 5, 7 or 10 μg/ml CHX after which a 15-minute pulse of ^{35}S-methionine (1.5 μCi, SA=1 CI/μM) was added to the flask. After the pulse, ^{35}S-methionine was removed from the flask and the cells were washed twice with 5 ml PBS. Cells were then lysed in 10 ml solution of 0.15 M KCl containing 1 mM EDTA (pH = 8.0) and processed for radioactivity determination essentially as described earlier (21). The total cellular protein content of cells treated with or without CHX was determined as described above.

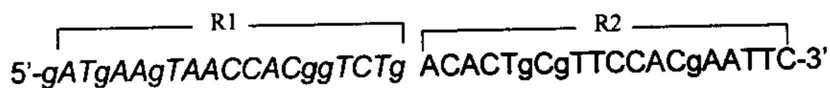
The half-life of ALDH2 (time to reduce the activity by 50%) in the H4 cells was determined in the presence of 5 μg/ml CHX. H4 cells (3 replicates/time per point) were treated for 1, 5, 7, 12, or 24 hours with CHX in DMEM without serum present.

The mitochondrial aldehyde dehydrogenase activity remaining at the different times of incubation with CHX was then determined.

Sequencing of H4-II-E-C3 ALDH2-1 cDNA by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). The published cDNA sequence of the mitochondrial aldehyde dehydrogenase in the Sprague-Dawley rat (22) was used to design PCR primers to amplify the cDNA obtained from the mRNA isolated from the H4 cell line. Total cellular RNA was isolated using Tri-Reagent® (Molecular Research Center, Inc. Cinvinnati, OH) and the first strand cDNA was made using a First Strand cDNA Synthesis Kit using random hexamer primers (Amersham Pharmacia Biotech Inc., NJ) according to the manufacturer's protocols. Three sets of PCR primers were designed to amplify 3 fragments of 476, 753, and 598 base pairs which combined constituted 96.7% of the published Sprague-Dawley rat sequence. The PCR primers were as follows: (476 bp, Forward 5'-CACgCCTgAgCCgCCTg-3': Reverse 5'-gCTgAAgAAgTCgCCATCg-3'), (753 bp, Forward 5'-CgATggCgACTTCTTCAgC-3': Reverse 5'-CTTggCgATggTCATgCCA-3'), (598 bp, Forward 5'-TggCATgACCATCgCCAAg-3': Reverse 5'-gAACCAgCATCCAgCACAg-3'). The primers were synthesized by the Nucleic Acid Facility at Thomas Jefferson University (TJU). All fragments were gel purified from 1% agarose gels using Quiagen's gel purification kit (Qiagen, CA) and the forward primers of each fragment was used in the sequencing reactions carried out by TJU-Nucleic Acid Facility.

Determination of mRNA expression by Competitive RT-PCR. Competitive RT-PCR for ALDH was developed by first designing PCR primers to amplify a 584 base pair fragment from the Sprague-Dawley rat cDNA, kindly donated by Dr. Henry Weiner

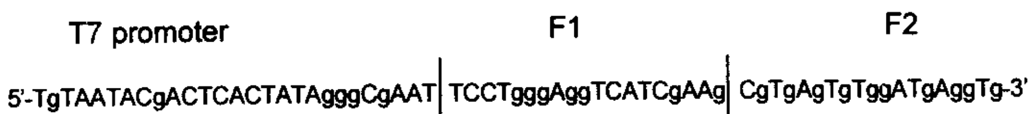
of Purdue University. The primers (specific to mitochodrial ALDH) used to generate the 584 base pair fragment (refered to as a parent fragment) were as follows: forward (**F**) 5'-ACgTggTggTgATgAAAgTg-3' and reverse (**R1**) 5'-gATgAAgTAACCACggTCTg-3'. The parent fragment was amplified from the cDNA template under the following conditions: denaturation at 95° C for 3 min followed by 30 cycles at 95°C –1 min, 63 °C –30 sec, 72 ° C 1.2 min and a final extension at 72 ° C for 10 min and the resulting band was purified from a 1% agarose gel. An internal standard, which served as a competitor for primers of the parent fragment during reverse transcription (as recombinant RNA, rcRNA) and PCR (as first strand cDNA) reactions, was constructed using a similar technique to that previously (23). The same forward primer mentioned above and a new reverse primer (NRP), comprised of the previous reverse primer sequence (R1) and an added reverse primer sequence (R2), was used to amplify a smaller internal standard fragment of 429 base pairs.



The pGEM®-T Easy Vector (Promega, WI) containing the 429 bp recombinant DNA fragment (rcDNA) was transformed into DH5α Competent E. coli cells according to the manufacture's protocol (Life Technologies, NY). The plasmid DNA was linearized using Not I restriction enzyme and then purified before use as template in the *in vitro* transcription reaction. The *in vitro* transcription reaction for generating RNA to be used as competitor in the RT-PCR reaction was carried out using the RiboProbe® In Vitro Transcription System (Promega, WI). The *in vitro* transcription

and RNA purification was performed as recommended in the Promega technical manual.

A streamlined procedure similar to that reported by Vanden Heuvel et al. (24) for design of a recombinant RNA (rcRNA) was used to make the GDH internal standard which eliminated any subcloning and relied only on PCR to obtain the rcDNA template for *in vitro* transcription. Primers were designed to amplify a 782 base-pair parent fragment (forward **(F1)** 5'-TCCTgggAggTCATCgAAg-3', reverse **(R)** 5'-AACTgCTTCTCgCTggCTg-3'). The cDNA template for GDH was obtained by reverse transcription using random hexamers (pd(N)6 20 ng/20 μl RT reaction) (Amersham Pharmacia Biotech, NJ) and Omniscript RT (Qiagen, CA) from RNA isolated from H4 cells. The GDH parent fragment primers were then used to amplify the 782 bp fragment by PCR using similar conditions to those described above for ALDH using 55° C for primer annealing. The PCR product was run on a 1% agarose gel and the 782 bp parent fragment was purified from the gel. The purified fragment was then amplified with a newly designed forward primer and the same reverse primer as mentioned above. The new forward primer was constructed as shown below. F1 = previous forward primer sequence and F2 = new forward primer sequence.



The smaller fragment was then used as the DNA template for *in vitro* transcription to make RNA which served as the GDH internal standard for competitive RT-PCR reactions with sample RNA.

Sample analysis for ALDH or GDH mRNA expression was performed using 2 μg of total RNA spiked with a range of internal standard RNA (ALDH 0-100 pg and GDH 0-32 ng) depending on the anticipated gene expression. Omniscript RT was used for all the reverse transcription reactions according to the Qiagen protocol using random heximers (Amersham Pharmacia Biotech, NJ) to prime first strand cDNA synthesis. The parent fragment and internal standard bands were analyzed on a Kodac Digital Science Image Station 440CF (Eastman Kodak Co, NY) by analyzing the net intensity of the UV/flourescence emitted by the ethidium bromide in the bands. The actual amount of internal standard that was needed to compete for amplification with the parent fragment was determined by plotting the amount of internal standard against its corresponding net intensity ratio (internal standard band / parent fragment band). Using a linear relationship, the exact amount of internal standard needed to equally compete with the amount of sample mRNA present was determined from the linear regression equation when y=1.

Animals and Treatment.

Intravenous administration of ASO-9. Male Lewis rats (Harlan, Indianapolis, IN) weighing 200-300 g, were used. Prior to femoral vein catheterization all rats were acclimated for at least 3 days to their cages. All animals were maintained on a 12 hr light/dark cycle and had free access to laboratory rodent diet 5001 (PMI Feeds, Inc., St. Louis, MO) and tap water. Once animals were acclimated, surgery was performed to insert a femoral venous catheter comprised of a flexible Tygon plastic tubing (0.015 I.D X 0.03 O.D). Patency of catheters was maintained by using a lock solution containing heparin (444 units/ml), dextrose (22%) and streptokinase (16,667 units/ml), and a small piece of monofilament

which was inserted at the exposed end of the cannula to retain the lock solution. Animals were treated for 4 days at 15 mg/kg/day via intravenous catheters.

Intraperitoneal Administration of ASO-9.

Male Lewis rats as described above were surgically implanted with 2-ml osmotic pumps (Alzet Minipump 2 M1-1®) containing a solution of 25 mg/ml ASO-9 in PBS or PBS (controls) in the interperitoneal cavity. A priming dose of 20 mg/kg was administered i.p. following implantation of the pumps. The pumps delivered ASO-9 at 24 mg/kg/day. Three days after pump implantation, animals were deprived of water overnight while rat chow remained accessible. After the overnight fluid deprivation, on day 4 animals were offered 6% v/v ethanol as the only fluid and consumption was measured at hourly intervals for 5 hours. Rehydration is a prime physiological drive and animals will initially consume the fluid offered. After the initial bout, rejection to continue consuming the fluid freely available indicates an aversion to the fluid offered. The general protocol follows that recently described by us (Garver et al, 2000). Animals were videotaped to capture their behavior after ethanol presentation (see: Garver et al in http://arc.tju.edu/egarver)

Acetaldehyde Determination by HPLC. Plasma acetaldehyde was determined as previously described (18).

Statistics.

Statistical analysis was performed for the data sets in Microsoft Excel 1997.

RESULTS

Low-Km mitochondrial aldehyde dehydrogenase (ALDH2) in rat hepatoma cells and the liver of the Lewis rat. Lindahl and associates (25) have reported the existence of a mitochondrial ALDH2-1 in rat hepatoma H4-II-E-C3 cells (referred to as H4 cell line), with similar characteristics to ALDH2 in rat and human liver mitochondria. The ALDH2-1 cDNA sequence was reported for the Sprague-Dawley rat by Farres et al (22). Since the H4 cell line was derived from a tumor from an AXC-Buffalo rat cross, it was necessary to confirm that the ALDH2-1 cDNA sequence in the H4 cell line and in the inbred Lewis rat were similar to that reported for the Sprague Dawley rat. We confirmed by RT-PCR and subsequent sequencing that the cDNA sequences of this isozyme in H4 cells and in the Lewis rat are >99% homologous to the cDNA sequence for which PCR primers were designed, which comprised 96.7% of ALDH cDNA. We further confirmed the studies by Huang and Lindahl (25) showing that ALDH2 activity is present in mitochondria of H4 cells and has a Km for acetaldehyde below 1 μM. Thus, the mitochondrial ALDH activity measured in the H4 cells has a similar high affinity for acetaldehyde as that in rat and human liver mitochondria (26).

Reduction in ALDH2-1 activity of H4 cells incubated in the presence of ASO-9

Half-Life. When using an antisense molecule to inhibit specific protein synthesis, the half-life of the pre-formed protein must be considered. In order to determine the half-life of the mature mitochondrial ALDH2 we used cycloheximide (CHX) to arrest the synthesis of all cellular proteins, which allowed us to measure the decay of the existing ALDH2 enzyme. We first determined the minimal concentration of CHX required for maximal inhibition of protein synthesis in the H4 cell, measured by the

the rate of incorporation of ^{35}S-metioninine into total proteins. CHX at a concentration of 5 µg/ml produced the maximal inhibition of ^{35}S incorporation. At this concentration of CHX methionine-derived ^{35}S incorporation was inhibited by 85% over 24 hours. The residual incorporation could be due to posttranslational incorporation of ^{35}S-sulfate, and it was not further reduced by increasing the concentrations of CHX. Cell viability under these conditions exceeded 90%. Under these conditions, ALDH2 activity in the H4 cells was reduced by 56% from control values in 24 hours. The calculated half-life of the mature ALDH2 in the mitochondria was 21.6 hours. Such a half-life is in line with a half-life of 22 hours reported for Hela cells transduced with human ALDH2-1 (27).

Effect of ASO-9. We present here the results of antisense phosphorothioate deoxyoligonucleotide #9 (ASO-9), the most effective of 9 antisense molecules containing the TCCC motif studied. Data in Table 4.1 show that following a 24-hour incubation of H4 cells with ASO-9, mitochondrial ALDH2 activity was reduced by 54.9 ± 10.7% (mean ± SEM). Lipofectamine alone or lipofectamine + control oligonucleotide (s) showed no reductions in ALDH2 activity. Given a half-life of 22 hours, it was determined that the *de-novo* synthesis of ALDH2-1 was inhibited by >95% following the 24 hours of exposure to ASO-9 (Table 4.1) . While ASO-9 proved to be very effective in inhibiting the synthesis of ALDH2, the exact mechanism by which this molecule elicited its effects was unknown. Thus, studies were conducted to determine whether ASO-9 reduced ALDH2-1 mRNA levels.

Reduction in ALDH2 mRNA levels induced by ASO-9 on H4 cells

The steady-state level of ALDH2 mRNA was determined by quantitative competitive RT-PCR as described in Methods. Table 4.2 shows that following a 24 h treatment with ASO-9, ALDH2 mRNA in the H4 cells was reduced by 84.8 $\pm$ 4.7 %. This result suggests that the mechanism of action for ASO-9 is likely mediated by RNA hydrolysis by RNase H.

Specificity

(i) *Sequence.* The effect of minor sequence changes on the ASO-9 molecule was tested by incubating cells with phosphorothioate oligonucleotides containing 2-, 3- or 4-base mismatches compared to ASO-9. One of the mismatches was always located in the "TCCC" motif. The studies showed that mismatches of 2-, 3- or 4-bases in ASO-9 blunted the oligonucleotide's ability to reduce the levels of mRNA. The greater the number of mismatches incorporated into the sequence of ASO-9, the less effective the modified ASO-9 became (Table 4.2; experiments 4 and 6).

(ii) *mRNA levels* The specificity of ASO-9 was also demonstrated by determining mRNA levels of ALDH2 and of glutamate dehydrogenase (GDH), another mitochondrial enzyme, in the same cells. Figure 4.1 shows the reduction in mRNA ALDH2 afforded by ASO-9 (see: pannel A1) and its ineffectiveness on GDH mRNA levels (pannel B1)

In vivo inhibition of ALDH2 activity and mRNA by antisense oligonucleotide ASO-9

Since ASO-9 proved to be most effective in reducing ALDH2 activity in cell culture and was both sequence and gene specific for ALDH2, we determined whether this molecule could also reduce the activity of hepatic ALDH2 *in vivo*. The activity of

GDH was determined as a control mitochondrial enzyme. ALDH2 and GDH gene expression (mRNA) were also determined *in vivo*. Antisense ASO-9 was administered for 4 days via an indwelling femoral vein catheter with either ASO-9 (15 mg/kg) or PBS. Twenty four hours after the last dose of ASO-9 or PBS animals received an oral dose of 1 g/kg ethanol (from a solution of 7% w/v in water) and were sacrificed 60 minutes later for the determination of mRNA levels, enzymatic activities and plasma acetaldehyde levels. Treatment with ASO-9 led to a 50% reduction in ALDH2 mRNA compared to PBS control animals (Table 4.3). Administration of ASO-9 reduced ALDH2 activity by 38-45% and led to a four-fold increase in plasma acetaldehyde levels following ethanol administration, when compared to acetaldehyde levels in control animals that received the same dose of ethanol (Table 4.4) . Glutamate dehydrogenase activity and GDH mRNA levels were not affected by ASO-9, thus also demonstrating *in vivo* the specificity of ASO-9 on ALDH2 gene expression (Tables 4.3 and 4.4).

Inhibition of ethanol Consumption following treatment with ASO-9

Studies were performed to determine if ASO-9 could establish an aversion to ethanol and reduce ethanol consumption. The animal model used was essentially that developed by Garver et al (2000) to test a drug-induced aversion to ethanol. Rats were surgically implanted with osmotic pumps which delivered ASO-9 at 24 mg/kg/day intraperitoneally. Three days after pump implantation, animals were deprived of water overnight. Thereafter, animals were offered 6% v/v ethanol as the only fluid, and consumption was measured at hourly intervals for 5 hours. A reduction in ethanol consumption after the first bout indicates an aversion to the fluid offered. Cumulative ethanol consumption (mean $\pm$ SEM) at each hourly interval is shown in Figure 4.2. Initial ethanol consumption was similar both the

ASO-9 and control (PBS) groups during the first hour of ethanol presentation, amounting to 1.12 ± 0.09 g ethanol/kg (ASO-9) and 1.70 ± 0.68 g ethanol/kg (Controls). After the first hour of ethanol presentation, consumption in 1-5 hour interval was reduced significantly ($p<0.015$) in the ASO-9 group (0.48 ± 0.23 g ethanol/kg) when compared to the control group (1.22 ± 0.16 g ethanol/kg) (Figure 4.2). This equates to a 61% reduction in ethanol consumption in ASO-9 treated animals *after* experiencing the drug (ASO-9) ethanol reaction. Cumulative ethanol consumption at 5 hours (which includes the amount consumed in the first hour of presentation) was 1.599 ± 0.23 g ethanol/kg (ASO-9) and 2.928 ± 0.59 g ethanol/kg (controls) corresponding to a 45% reduction ($p<0.035$) in the total ethanol consumption in the ASO-9 treated animals when compared to controls (Figure 4.3A). This reduction is identical to the reduction (46%) in ethanol consumption experienced in earlier studies in rats treated with disulfiram a non–specific drug used to induce an aversion to ethanol (Figure 4.3B, data from Garver et al 2000).

DISCUSSION

The studies presented show that an antisense phosphorothioate deoxyoligonucleotide (ASO-9) was specific and effective in reducing the *de novo* synthesis of mitochondrial aldehyde dehydrogenase ALDH2-1 in rat hepatoma cells. Given a 22 hour half-life for the mature enzyme, an inhibition of 95% in ALDH2-1 synthesis was calculated to occur following a 24 hour incubation with ASO-9. Actual remaining activity at 24 hours was 55%.

Present knowledge indicates that there are two major mechanisms of antisense action: (i) ocupancy of mRNA and (ii) occupancy-activated RNase-H hydrolysis of the RNA targets (13). Our earlier studies on the mechansims of action of antisense phosphorothioate oligonucleotides (ASOs) which incorporate the TCCC motif in the molecule led to the the finding that steady-state mRNA levels are significantly reduced by these ASOs. Since RNase H only exists in the nucleus and the most effective ASOs had targets mainly in *intron* and *3'-untranslated regions*, it was postulated that the TCCC mediated mechanism of antisense action was localized in the nucleus (15).

In the studies presented here, nine ASO's were designed which incorporated the TCCC motif (15) targeting exons (a message also present in the nucleus), since only the cDNA sequence of the Sprague-Dawley rat ALDH2 was known at the time of starting these studies (22). We found that two of the nine antisense molecules (2 ASO's in 3'-UTR , and 7 ASO's in open reading frame including ASO-9) had inhibitory effects on the ALDH2 enzyme activity with ASO-9 being the most effective. Since ASO-9 targeted *exon* 2, it was possible that the antisense

mechanism of ASO-9 might have been due to occupancy-mediated inhibition of translation rather than to RNase H mediated hydrolysis. In order to determine if the mechanism of action followed translation arrest or a reduction in mRNA transcript levels, we determined the steady-state levels of mRNA and whether any reduction in mRNA levels correlated with the loss of ALDH2 activity. Analysis of ALDH2 steady-state mRNA by competitive RT-PCR demonstrated that ASO-9 indeed reduced the levels of mRNA by 85% which paralleled the calculated 95% inhibition of enzyme synthesis. These data suggest that the mechanism of action of ASO-9 is more likely one involving mRNA hydrolysis by RNase H, although some additional transcriptional arrest by occupation cannot be ruled out.

A true antisense mechanism was demonstrated by the fact that as the number of mismatches from the original ASO-9 molecule were increased from 2-, to 3- and 4-bases, the oligonucleotide's ability to inhibit ALDH mRNA levels was reduced. An oligonucleotide with 4-base mismatches from ASO-9 was essentially inactive. Furthermore, ASO-9 was specific for its ALDH2 target since ALDH2 mRNA levels were reduced while the mRNA levels for GDH, another mitochondrial enzyme, remained constant.

Despite the variation in basal mRNA levels in the H4 cells, the inhibition afforded by ASO-9 over 6 experiments was quite constant and averaged 85%. During *in vitro* studies it was noted that basal ALDH2 mRNA levels varied between experiments. In each of these experiments, serum was added at 6 hours and the serum lots varied from one experiment to another. Previously, it has been shown that hormones such as insulin, progesterone and dihydrotestoterone, found at different levels in serum lots, can increase or reduce the expression of ALDH2 by as much

as 50% (28). In addition, H4 cells at various passages were used over the course of the experiments and we found that the ALDH2 activity could be reduced by as much as 50% following many (over 5) passages.

When "naked" (without liposomal conjugation) ASO-9 was administered intravenously for 4 days and animals were sacrificed 24 hours after the last dose of the oligonucleotide, hepatic ALDH2 mRNA was reduced by 50% and ALDH2 activity was reduced by 40%. Administration of ethanol to animals pre-treated with ASO-9 elicited a four-fold increase in plasma acetaldehyde levels versus that in control (PBS) animals. In the same animals, ASO-9 did not modify the activity of mitochondrial GDH, thus again indicating the specificity of action of this antisense oligonucleotide seen in cell cultures.

We further determined if treatment of ASO-9 would establish an aversion to ethanol consumption in rats. We have previously reported the development of a rat model to measure a drug-induced aversion to ethanol using disulfiram as the aversive test drug (Garver et al., 2000). Animals treated with ASO-9 showed a 61% reduction in alcohol consumption *after* experiencing the ASO-ethanol reaction when compared to control animals. Overall, ASO-9 showed an effectiveness in the same order as that reported previously for large doses of disulfiram.

Theoretically, an antisense phosphorothioate oligonucleotide of 17-21 bases with a sequence complementary to the primary RNA transcripts or the mRNA sequence can inhibit gene expression through Watson-Crick DNA-RNA hybridization in a specific manner because a sequence of this length is unlikely to repeat more than once in the human genome. Here, we demonstrated that ASO-9 did not affect

GDH activity or mRNA levels which strongly suggested that its effects were not due to non-antisense effects on another protein. In addition to specificity of antisense oligonucleotides for their target, antisense oligonucleotides have been shown to have minimal side effects over a wide range of doses (29). Both the specificity and lack of serious side effects of a well tested antisense oligonucleotide would be advantageous when compared with disulfiram. Disulfiram displays a large number of side effects, is non-specific for its target and requires daily administration, which has markedly reduced its clinical use (30, 31). The half-life of phosphorothioate oligonucleotides is 48 hours in rodents (32-34) while in humans, antisense molecules have been reported to have a half-life of 6 to 14 days (35). The protracted half-life in humans would make it possible to reduce the frequency of administration, maintaining the desired efficacy and improving compliance. Disulfiram is the only drug that inhibits aldehyde dehydrogenase which is approved in the U.S. for the treatment of alcoholism. Controlled studies show that daily administration of disulfiram is indeed effective in the treatment of alcoholism (36, 37), although disulfiram has been criticized for not lengthening the time elapsed until full abstinence is broken (38). However, since patients must experience the effects of the disulfiram-ethanol reaction _before_ the aversive effects take place, the time to break the abstinence is not expected to be affected by the drug. The main problem with disulfiram relates to the lack of compliance by patients with medication-taking, mainly due to sensory and motor neuropathies, optic neuritis, orthostatic hypotension and hypersensitivity reactions (30, 31, 39-41). In a number of studies in which disulfiram was administered under supervision its effectiveness in reducing alcohol consumption was clearly seen (42). However, due to its side effects, doses that are fully therapeutic are rarely achieved. At the doses normally employed (250-300 mg daily) less than 50% of patients display the disulfiram-

ethanol reaction (43, 44). Thus, there is the obvious need to develop alternatives to disulfiram to mimic the strong protective effects of a low ALDH2 activity seen in Asians carrying the ALDH2-2 allele.

Studies presented in this communication provide a strong proof of principle that inhibition of ALDH2 gene expression can mimic the ALDH2-2 Asian phenotype, resulting in (i) low liver mitochondrial ALDH2-1 activity, (ii) elevated plasma acetaldehyde levels following the administration of ethanol and (iii) an aversion to ethanol, shown as a reduction in alcohol consumption.

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FIGURE LEGEND

Figure 4.1. Quantitation of ALDH2-1 and GDH Steady-State mRNA by Competitive RT-PCR. Panels A1 and A2 show ALDH2 mRNA expression and Panels B1 and B2 show GDH mRNA expresion. H4-II-E-C3 hepatoma cells were incubated with lipofectamine plus in the presence of 1 μM ASO-9 (A1 and B1) or the 4-base mismatch of ASO-9 (A2 and B2, nucleotide sequence in Table 2). The concentration shown represents the pg of ALDH2 mRNA competitor added/μg total RNA (Panels A1 and A2) and ng GDH mRNA competitor added/μg total RNA(Panels B1 and B2). As can be observed, lesser competitor was necessary to compete with ALDH2 mRNA in ASO-9 treated cells than in cells treated with the 4-base mismatch of ASO-9 (control). In three replicates, the relative concentration of ALDH mRNA for 4-base mismatch control cells was 21.8 ± 8.3 pg/mg total RNA; that for the ASO-9 treated cells was 5.6 ± 0.5 pg/mg total RNA. ASO-9 did not affect the GDH mRNA levels (N.S.) when compared to the 4-base mismatch oligonucleotide. In three replicates, the relative concentration of GDH mRNA for 4-base mismatch control cells was 9.65 ± 0.19 ng/mg total RNA; that for the ASO-9 treated cells was 9.26 ± 0.18 ng/mg total RNA.

Figure 4.2. Effect of ASO-9 on ethanol consumption following an 18-hour water deprivation period. Data show cumulative ethanol consumption (g ethanol/kg) by animals during the first 5 hours of access to a 6% ethanol (v/v) solution following an 18-hour water deprivation. Prior to the study, rats received ASO-9 (24 mg/kg/hr) or PBS for 4 days from implanted osmotic pumps. Values represent mean ± SEM for 5 animals. Ethanol consumption was not significantly

different in the two groups in the first hour of fluid re-access. However, after the primal re-hydration bout (and after experiencing a combined ethanol-ASO-9 effect), rats that had received ASO-9 consumed 61% less ethanol than control animals in the following four hours (0.48 vs 1.23 g ethanol/kg; $p<0.015$).

Figure 4.3. Comparison of the inhibitory effect on ethanol consumption of ASO-9 (24 mg/kg/day) for 4 days (data from the present study) and of disulfiram administration (100 mg/kg/day) for 4 days (data from Garver et al 2000). The effect on ethanol consumption of ASO-9 and of disulfiram (a non-specific inhibitor of ALDHs), was determined following an 18-hour water deprivation period of inbred Lewis rats. Data show total ethanol consumption (g ethanol/kg) by animals during 5 hours (zero to 5 hours) of access to a 6% ethanol (v/v) solution following an 18 hour period of water deprivation of 18 hours. Control for disulfiram was carboxymethyl cellulose in saline administered orally 2 hours prior to fluid re-access. Control for ASO-9 was saline (phosphate buffered) delivered continuously from the osmotic pumps, which may account for the differences in fluid consumption (6% ethanol) in the two control groups. The inhibitory effect on ethanol consumption of the two drugs (ASO-9 and disulfiram) was identical (45 vs 46% NS).

TABLE 4.2

Reduction of ALDH2 mRNA Levels by ASO-9

	ALDH2 mRNA (pg mRNA/µg total RNA)	Inhibition
Experiment #1		
Control Oligo (1.0 µM)	12.5	NA
ASO-9 (1.0 µM)	2.5	80%
ASO-9 (0.5 µM)	2.0	84%
ASO-9 (0.25 µM)	1.5	88%
Experiment #2		
Control Oligo (1.0 µM)	100	NA
ASO-9 (1.0 µM)	3	97%
Experiment #3		
Control Oligo (0.5 µM)	25	NA
ASO-9 (0.5 µM)	5	80%
Experiment #4		
2 bp mismatch of ASO-9 (1.0 µM)	7.5	NA
ASO9 (1.0 µM)	2.5	67%
Experiment #5		
Lipofectamine Plus (no ASO)	23.4	NA
ASO9-(1.0 µM)	4.7	80%
Experiment #6		
3 bp mismatch of ASO-9 (1.0 µM)	12.95	NA
4 bp mismatch of ASO-9 (1.0 µM)	21.8 ± 8.3	NA
ASO-9 (1.0 µM)	5.6 ± 0.5	74%*

Control oligonucleotide in experiments 1-3: 5'-CgTCTTCACTTCCgTgTAggC-3'

2 base mismatch of ASO-9 in experiment #4: 5'-TCCTCg TTgTTCgCTTCg gCT-3'

3 base mismatch of ASO-9 in experiment #5: 5'-TCCTC*g* TTgTTC*g*CATCg gCT-3'

4 base mismatch of ASO-9 in experiment #6: 5'-TCC*A*C*g* TTgT*A*C*g*CATCg gCT-3'

* Inhibition versus the 4-base pair mismatch. Mean inhibition by ASO-9: 84.8 ± 4.7% (based on 6 experiments)

TABLE 4.3

Reduction of The Steady-State Levels of ALDH2-1 and GDH mRNA Following Treatment with PBS or ASO-9 Following Intravenous Administration for 4 days (15 mg/kg/day).

	ALDH2-1 (pg of mRNA/µg total RNA)	GDH (ng of mRNA/µg total RNA)	Inhibition*
PBS			
1	13.0	2.6	NA
2	15.3	2.6	NA
3	16.1	2.3	NA
4	19.6	—	NA
	16 ± 1.4	2.5 ± 0.1	
ASO-9			
1	7.4	2.3	53.8
2	9.6	2.7	40.0
3	6.4	2.6	60.0
4	8.4	—	47.5
	8.0 ± 0.7	2.5 ± 0.1	50.3 ± 4.2%

*Inhibition for each animal in ASO-9 group calculated from mean ALDH2-1 mRNA expression of PBS group

t-test of PBS verses ASO-9 treatment, $p < 0.007$

TABLE 4.4

In Vivo Effect of ASO-9 Intravenous Administration (15 mg/kg/day for 4 days) on the Activity of Liver ALDH2-1, GDH and Plasma Acetaldehyde Levels Following Administration of Ethanol (1 g/kg).

Treatment Group	**ALDH** Specific Activity (nmol/min/mg protein)	**GDH** Specific Activity (µmol/min/mg protein)	**ALDH/GDH** (Ratio)	**Plasma Acetaldehyde** (µM)
PBS				
1	58.9	6.4	9.2	1.5
2	48.2	6.4	7.5	1.9
3	42.9	7.1	6.0	1.7
4	58.9	8.4	7.0	3.6
	52.2 ± 4.0	7.1 ± 0.5	7.4 ± 0.7	2.2± 1.0
ASO-9				
1	37.5	7.7	4.9	7.5
2	37.5	7.7	4.9	6.1
3	32.2	8.4	3.8	7.5
4	42.9	8.4	5.1	8.1
5	37.5	7.7	4.9	9.4
6	32.2	7.7	4.2	11.4
	36.6 ± 1.6 [*]	7.9 ± 0.2 [‡]	4.6 ± 0.2 [§]	8.3 ± 1.9 [II]

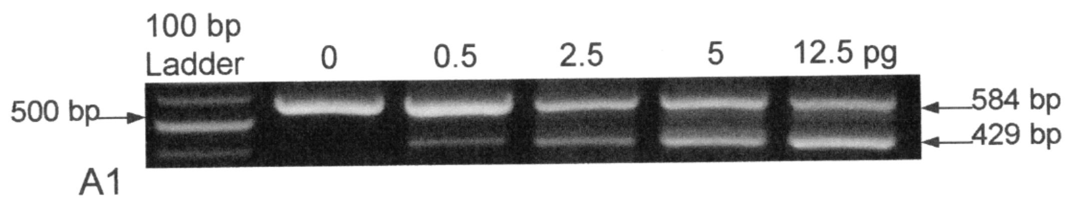
ASO-9 (15 mg/kg/day) was administered iv. to rats for 4 days. Twenty four hours after the last dose of the oligonucleotide animals received 1 g/kg ethanol orally. Group values are means ± sem

[*] Effect of ASO-9 on mitochondrial ALDH activity: **$p<0.01$**

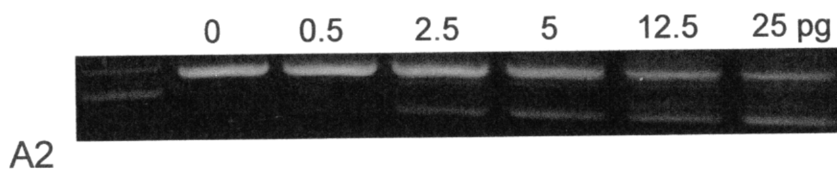
[‡] Effect of ASO-9 on mitochondrial GDH activity: NS

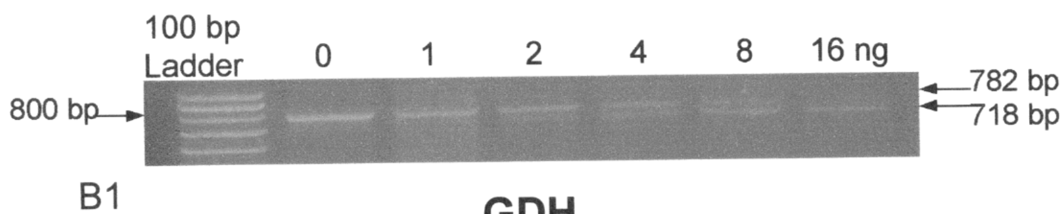
[§] Effect of ASO-9 on ALDH/GDH ratio: $p<0.015$

[II] Effect of ASO-9 on plasma acetaldehyde levels: $p<0.002$

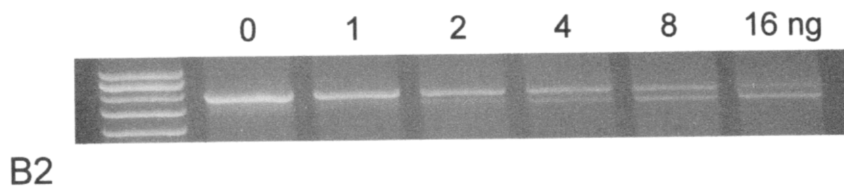


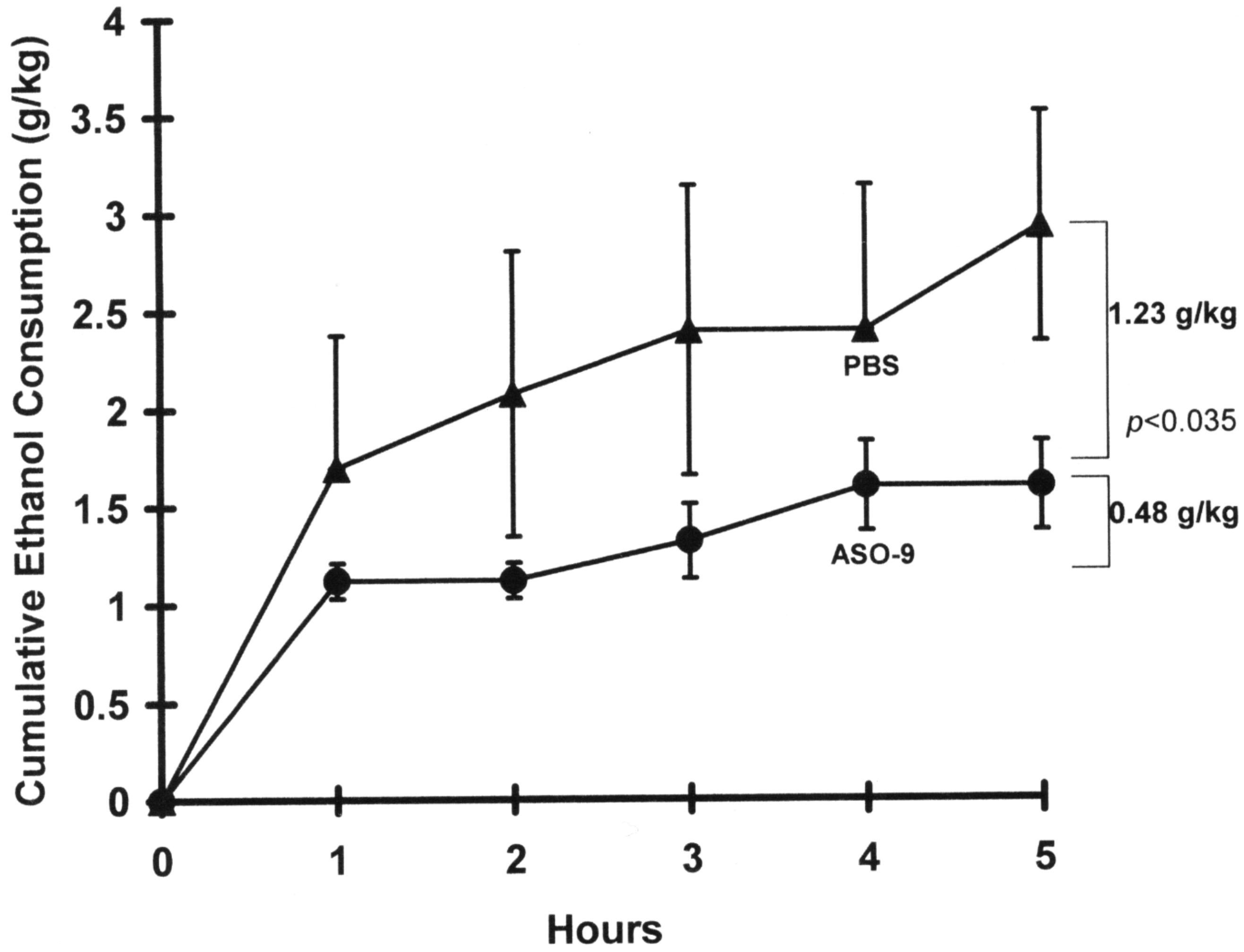
ALDH

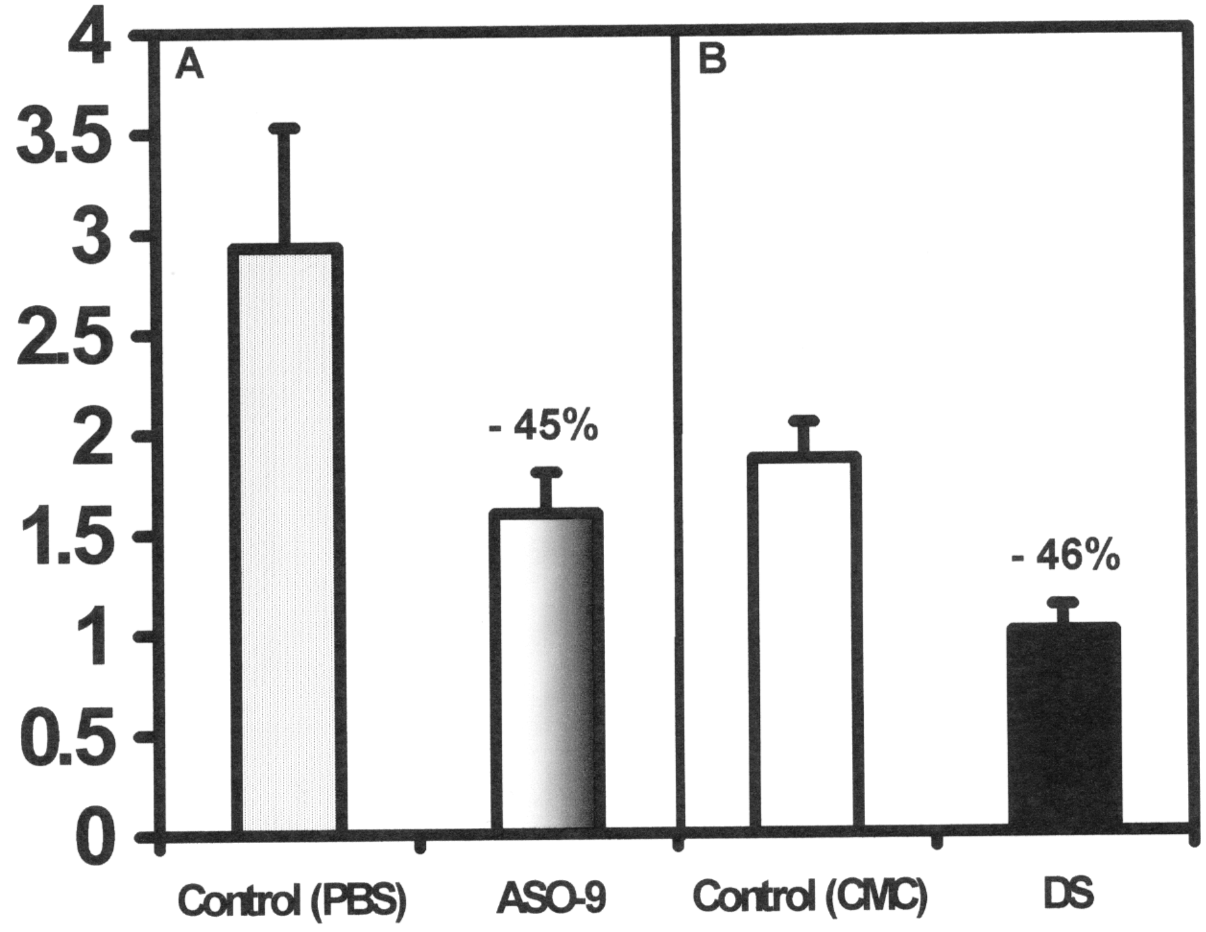




GDH




Cumulative Ethanol Consumption (g/kg)
4
3.5
3
2.5
2
1.5
1
0.5
0
0
1
2
3
4
5
Hours
PBS
ASO-9
1.23 g/kg
p<0.035
0.48 g/kg



Cumulative Ethanol Consumption (g/kg)
4
3.5
3
2.5
2
1.5
1
0.5
0
A
B
- 45%
- 46%
Control (PBS)
ASO-9
Control (CMC)
DS

Section V

TRANSDUCTION OF RAT HEPATOMA H4-II-E-C3 CELLS WITH HUMAN ALDH2*1 OR ALDH2*2 cDNA.

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Manuscript: not submitted

ABSTRACT

Gene therapy is an alternative approach to antisense-mediated gene regulation for the development of a new aversive medication that has the potential to suppress mitochondrial aldehyde dehydrogenase (ALDH2) for a protracted period of time (1 month to a lifetime) following a single treatment. The feasibility of the gene therapy approach for long-term suppression of ALDH2 was evaluated in *in-vitro* studies with the H4-II-E-C3 rat hepatoma cell line. Since the rat and the human ALDH2 are >80% identical in nucleic acid sequence, share >95% amino acid homology (Farres *et al.*, 1989), and have Km's of < 1 μM, it was envisioned that the subunits of the rat and the human enzymes could combine to form the ALDH2 enzyme. The H4-II-E-C3 cell line was transduced with human cDNAs for both ALDH2*1(wild-type) and ALDH2*2 (mutant) using a non-competent retrovirus. The inclusion of a mutant human ALDH2*2 monomer into H4 cells resulted in an enzyme with significantly less activity than that of the ALDH2 enzyme in H4-II-E-C3 cells transduced with retroviral vector backbone or human wild-type (ALDH2*1) with *p*-values of <0.00004 and <0.0002, respectively. These preliminary studies warrant further investigation of a suitable viral delivery system, such as the adenovirus, adeno-associated virus, or Lenti virus for delivery of the human mutant ALDH2*2 transgene for expression in the rat. The ultimate goal of the pre-clinical work in the rat is to be able to proove that humans could be provided with a mutant ALDH2*2 transgene for longer term suppression of ALDH2*1 as an alternative to disulfiram for the treatment of alcoholism.

INTRODUCTION

The most likely protective genetic factor that an individual can have to prevent the development of alcoholism is a single point-mutation in the mitochondrial aldehyde dehydrogenase gene (ALDH2*1 $\xrightarrow{\text{Glu 487 to Lys 487}}$ ALDH2*2) (Yoshida and Dave, 1985; Yoshida *et al.*, 1985). This point-mutation has been shown to reduce the k_{cat} and elevate the k_m for NAD+ in essence eliminating the enzyme's capacity to metabolize the acetaldehyde accumulation as a result of ethanol consumption (Steinmetz *et al.*, 1997). The accumulation of the toxic acetaldehyde results in a dysphoria characterized by dizziness, nausea, hypotension and palpitations (Mizoi *et al.*, 1983). The point- mutation in the ALDH2*1 gene is a dominant mutation in that incorporation of one or more of the monomeric subunit product/s into the forming tetrameric structure of the enzyme leads to a reduction in ALDH2's ability to catalyze the oxidation of the toxic acetaldehyde into its non-toxic product, acetate (Xiao *et al.*, 1997).

The gene therapy approach is a viable option to the development of a new aversive medication. Previous work by Farres et al in 1994 with recombinant ALDH2 protein purified from bacteria demonstrated that the incorporation of a mutation (E487K) into the rat ALDH2 monomer resulted in the formation of a ALDH2 tetramer that had a 60% reduction in k_{cat} while incorporation of the same mutation in the human monomer led to the formation of a ALDH2 tetramer with a much greater reduction of 91% in k_{cat} (Farres *et al.*, 1994). Therefore, we have studied the reduction in mitochondrial aldehyde dehydrogenase activity by incorporating a human mutant monomer (ALDH2*2) into the mature ALDH2 tetramer in rat hepatocytes with hopes of establishing larger reductions in ALDH2

activity. Provided that the incorporation of a human mutant ALDH2 monomer into the forming tetrameric structure decreased the enzyme activity *in-vitro*, then delivery of the mutant human monomer via a viral delivery system in rats would be warranted.

The rat and the human ALDH2 are >80% identical in nucleic acid sequence, share >95% amino acid homology (Farres *et al.*, 1989), and have Km's of < 1 μM. Thus, the monomeric subunits of the rat and the human might be expected to combine and form the tetrameric structure of the ALDH2 enzyme. However, it remained unknown as to whether a human monomer could combine with the rat monomer to form an active tetrameric form of the ALDH2 enzyme; and in addition, whether a mutant human monomer/s would have the capacity to render a combined species tetramer less active. If the later was demonstrated, then the use of gene therapy might be demonstrable in the rat and open the possibility of pre-clinical studies for a new approach to the treatment of alcoholism.

Human cDNA's encoding for the wild-type (ALDH2*1) and mutant (ALDH2*2) monomers in retroviral vectors were used in the studies. If *in-vitro* studies could demonstrate that the transduction of the mutant human monomer/s could lead to significant reductions in hepatocyte ALDH2 activity; delivery of the mutant human ALDH2*2 gene to rat hepatocytes *in-vivo* may lead to significant accumulations of acetaldehyde. These accumulations of acetaldehyde may be able to establish an aversion to ethanol in the rat which could potentially be maintained for prolonged periods through a single delivery of the ALDH2*2 human gene with one of the new gutless or helper-dependent adenoviral vectors (Morral *et al.*, 1999) or adeno-associated virus (Chao *et al.*, 2000).

The studies presented here were designed to address whether the delivery of the human ALDH2*1 and ALDH2*2 genes via a retrovirus for transduction of the rat hepatoma cell line H4-II-E-C3 significantly reduced the mitochondrial aldehyde dehydrogenase activity. While the studies demonstrated that the gene therapy approach was successful in significantly reducing ALDH2 activity by as much as 70-80%, this thesis concentrated primarily on the antisense approach to prove the principle that inhibition of ALDH2 in the rat could lead to aversion to alcohol consumption.

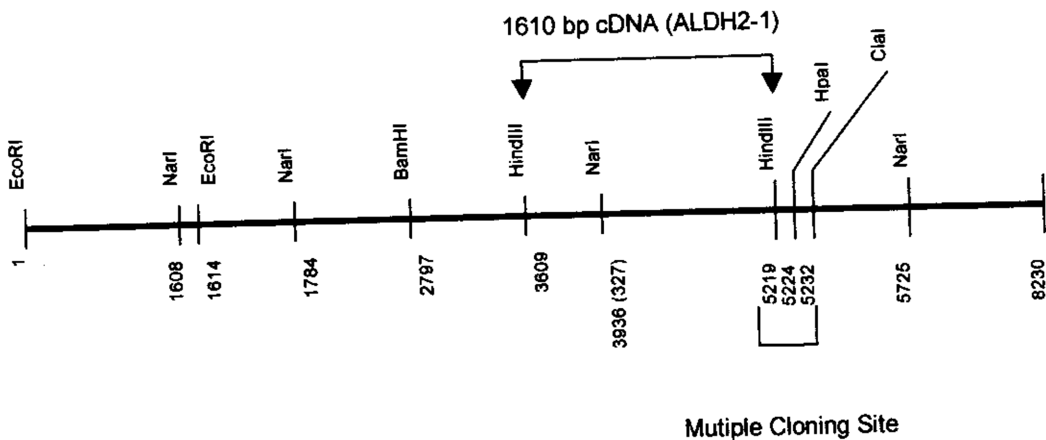
MATERIAL AND METHODS

Vector Preparation:

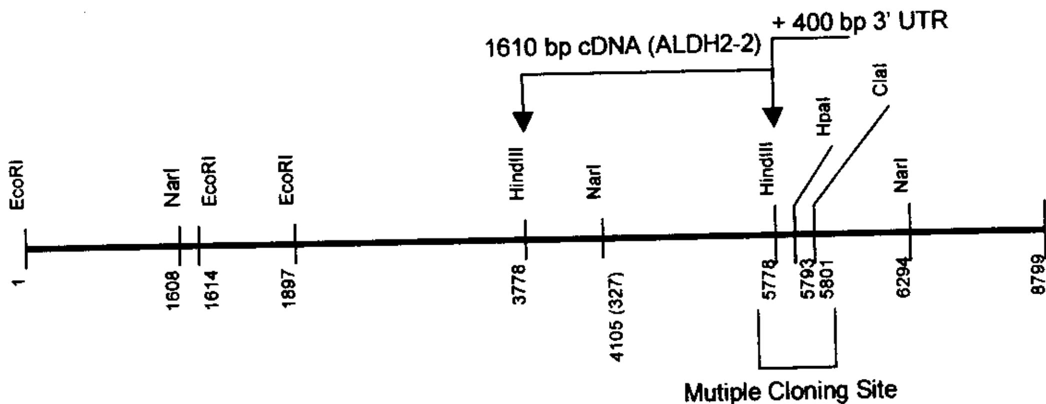
The retroviral vectors (pLNCE and pLHCK3'UT) were sent to our laboratory as a gift of Dr. David Crabb (University of Indiana). Approximately 7 mg of DNA was eluted from 3M filter paper with TE buffer (300 μl). In order to obtain enough vector DNA to transfect the PA317 amphotropic retrovirus packaging cell line (below), the vector DNA eluted from the filter paper was used to transform Subcloning Efficiency™DH5α™ Competent E. coli cells Cat. #18265-017 (GIBCO BRL Life-Technologies, Rockville, MD). The transformation proceeded essentially as describe by the manufacture's protocol.

Retroviral Vectors:

LNCX vector with ALDH2-1 (pLNCE)



LHCX vector with ALDH2-2 (pLHCK 3'UT)



The competent cells were thawed on wet ice and a 50 µl aliquot of the cells was placed into a chilled 1.5 ml microcentrifuge tube. Vector DNA (5 µl of filter paper eluant) was pipetted through the cells while being dispensed. The tube was gently mixed and the cells were placed back on ice for 30 minutes in order for the cells to take up the vector DNA. The cells were then heat-shocked for 20 seconds at 37° C and placed immediately on ice for 2 minutes. Room temperature LB medium (250 µl) was added to the microcentrifuge tube and a hole was placed into the cap with an 18 gauge sterile needle. The cap was then closed and the tube was placed in the shaking-incubator at 37° C and 250 rpm for 2 hours for expression. At the end of 2 hours, 100 µl of the cells were spread on LB agar plates containing 100 µg/ml ampicillin and 50 µg/ml Xgal and

incubated at 37° C overnight. White colonies containing vector DNA were selected after overnight incubation and placed into a conicle tube containing 5 ml of LB medium with 100 μg/ml ampicillin for additional overnight incubation at 37° C in the shaking-incubator at 250 rpm. Expanded E. coli was then lysed for vector DNA collection according to Qiagen's protocol for their plasmid mini-prep kit or by the cheaper method using Lithium Chloride as desribed in the Current Protocols in Molecular Biology (Chanda, 1994-1998). The isolated and purified vector DNA was subjected to restriction digests with Hind III to confirm the presence of either ALDH2*1 or ALDH2*2 human cDNA.

The following is an example of the Hind III digest set-up:

13.8 μl nuclease-free H_2O

2.0 μl Hind III 10X restriction Buffer E

3.0 μl DNA (0.6-1μg)

0.2 μl Bovine Serum Albumin (BSA, 10 μg/μl)

1 μl Hind III restriction enzyme (Promega, Cat. #R6041)

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20 μl Total Reaction Volume

Control Vector Preparation

The pLNCE vector containing the wild-type human cDNA (ALDH2*1) was digested with Hind III to liberate the 1610 bp cDNA. The digest was run on a 1% agarose gel containing ethidium-bromide and the 6620 bp band was electroeluted at 120 V for 30 minutes in 1 X TAE using a Spectra-por molecularporis membrane tubing (M.W. cut 3,500, cat. #132720, Las Angles, CA). The DNA was purified from the 1 X TAE by extracting with 1 volume of TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1[pH 4.5]), vortexing for 1

minute and centrifugation at 12000 X g for 2 minutes. The upper aqueous phase was transferred to a fresh tube and 1 volume of chloroform:isoamyl alcohol (24:1) was added and vortexed for 1 minute followed by centrifugation at 12000 X g for 2 minutes. Again, the upper aqueous phase was transferred to a fresh tube and 0.5% volume of 7 .5 M ammmonium acetate and 2.5 volumes of 100% ethanol were mixed and placed at –70 °C for a minimum of 30 minutes. The Epindorf® tube was then centrifuged at 12000 X g for 20 minutes and the supernate was removed. The DNA pellet was washed with 1 ml 70% ethanol (v/v). The DNA pellet was air dried in the speed-vac and reconstituted in DNase-free water. DNA concentrations were then determined on by UV spectrophotometry at 260/280 nm.

The backbone of the pLNCE vector with the Hind III sticky ends was ligated using T4 DNA ligase (cat. # M180A, Promega, Madison, WI).

Cell Culture:

Infectious virus was prepared using the PA317 amphotropic retrovirus packaging cell line (American Type Culture Collection #CRL 9078). These cells were grown in Dulbecco's modified Eagle's medium containing 4.5 g/L of L-glutamine (Mediatech,Cellgro®,VA), 10% fetal bovine serum (Life Technologies, NY) in 5% CO_2 at 37° C.

Transfection of PA317 Cells:

The PA317 cell line was transfected with the purified vector DNA by using Calcium Phosphate to complex with the DNA. Two solutions were prepared to form the $CaPO_3$-DNA complex for transfection of PA317 cells. Solution #1 of

Hepes buffered saline (HeBS, 2X) was prepared as follows: 2.4 g NaCL, 0.111 g KCl, 0.0564 g $Na_2HPO_4 \bullet 7H_2O$, 0.3 g Dextrose purchased from Fisher (Pittsburg, PA) and 1.5 g HEPES acid Cat. # H3375 from Sigma (St. Louis, MO) were dissolved in sterile water and brought to a pH 7.07 with 5-6 drops of 10 N NaOH and filter sterilized (150 ml filter flask, Cat # Corning, NY). Solution #2 of Calcium Chloride (2.5 M) was prepared by dissolving 36.74 g of $CaCL \bullet 2H_2O$ in 100 ml of HEPES(10 mM, 2.603 g/L).

In a polypropylene tube (Falcon 352063, 12X75 mm, Beckton Dickinson Labware, Franklin Lakes, NJ) containing 0.5 ml of solution #1, Vector DNA (10-20 μg) was added and mixed (Tube 1). In a second 5 ml polypropylene tube, 0.5 ml of solution #2 was added (Tube 2), and then the contents of Tube 1 were slowly added to Tube 2 while the solution in Tube 2 was constantly bubbled using a 2 ml disposable pipette and mechanical pipetter allowing the DNA to form a fine white precipitate. The mixture was incubated for 30 minutes at room temperature prior to addition to the PA317 cells. The final 1 ml $CaPO_3$-DNA complex was added to a 100 mm culture dish that had 1 X 10^6 cells plated in 8 ml of culture medium 24 hours prior to this addition of $CaPO_3$-DNA complex. Cells were incubated overnight (18 h) at standard culture conditions with the $CaPO_3$-DNA complex and then glycerol shocked to enhance DNA uptake the next morning.

The glycerol shock was prepared as follows:

Glycerol stock solution – (50% W/V) add 50 g glycerol to 100 ml water, mix and filter sterilize.

Glycerol Shock Solution (15% glycerol) – add 3 ml of sterile 50% glycerol stock to 5 ml sterile 2X HeBS and 2 ml sterile water and mix.

Glycerol Shock – medium was aspirated from $CaPO_3$-DNA complex treated cells at 18 h, and 4 ml of the 15% glycerol solution was placed into the 100 mm culture dish for 2 minutes at 37° C. The glycerol shock was removed and cells were washed with once with 1X HeBS followed by the addition of standard prepared culture medium (8 ml). The cells incubated for 72 hours under standard culture conditions for infectious viral particles to be released into the culture medium.

Virus Collection:

Infectious virus was collected at 72 hours. The medium (8 ml) containing the infectious virus was removed from the cells and placed into a 15 ml conicle tube (Corning, NY). Cellular debris was removed from the medium by centrifugation at 1000 X g. The viral supernatant was removed without disturbing the debris pellet and infectious virus supernatant was used immediately or stored at -70° C.

Infection and Selection:

Prepared virual supernate containing human ALDH2-1 or 2-2 cDNA was used to infect the rat hepatoma cell line H4-II-E-C3 (ATCC CRL-1600) that was purchased from American Type Culture Collection (Rockville, MD). The H4-II-E-C3 cells were infected by placing 1 ml of the viral supernate into 5 ml of prepared culture medium. In addition, hexadimethrene bromide (polybrene) Cat. #H9268 from Sigma Chemical Co (St Louis, MO)at a concentration of 4 µg/ml was added to the final 6 ml volume of culture medium to enhance viral infection. The cells were cultured at standard conditions overnight (18 h), and the next morning the

medium containing the viral supernate was removed and replaced with prepared culture medium. The cell were then cultured for 48-72 hours to allow for the expression of the transgene. At the end of 48-72 hours, Geneticin G418 (Neomycin) (added 1 μl/ml culture medium of a 580 mg/ml PBS stock) Cat. #G5013 from Sigma Chemical Co (St Louis, MO) or Hygromycin (added 4 μl/ml culture medium of a 50 mg/ml PBS stock) Cat. #843555 from Boehringer Mannheim (gmbH Germany, Corp. IN, USA) were added to the ALDH2*1 or ALDH2*2 transduced H4-II-E-C3 cells, respectively.

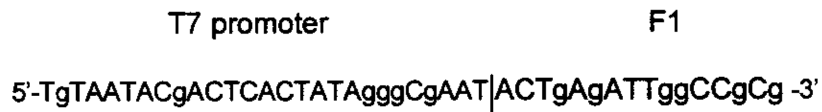
Assay Reagents:

All the reagents used in mitochondrial isolation and subsequent analysis of aldehyde dehydrogenase or glutamate dehydrogenase activity are as described in the materials and methods (supplement to manuscripts).

Quantitation of Transgene by Competitive RT-PCR:

The streamlined procedure similar to that desribed by Vanden Heuvel et al for design of a recombinant RNA (rcRNA) was used to make the human ALDH2 internal standard as described for rat ALDH2 competitive RT-PCR above (see Figure 5.1) (Vanden Heuvel *et al.*, 1993). Primers were designed to amplify a 357 base-pair parent fragment (forward (**F1**) 5'-ACTgAgATTggCCgCg-3', reverse (**R1**) 5'-CTgTTgCTTCCCCgTg -3'). Using the pLNCE vector DNA, the human ALDH2 parent fragment (357 bp) was amplified by PCR using the primers F1 and R1. The PCR settings were as follows: 94° C for 3 minutes, 94°C (1 min.) - 55° C (0.5 min.) - 72° C (1 min.) (30-50 cycles) and a final extension at 72° C for 10 min. The PCR product was run on a 1% agarose gel and the 357 bp parent fragment was purified

from the gel. The purified fragment was then amplified with newly designed forward and reverse primers. The new forward primer was constructed as shown below. F1 = previous forward primer sequence and T7 promoter = new forward primer sequence.



The new reverse primer contained a new sequence at its 3'-end (R2) which was added on to the previous reverse primer sequence (R1)(see below).

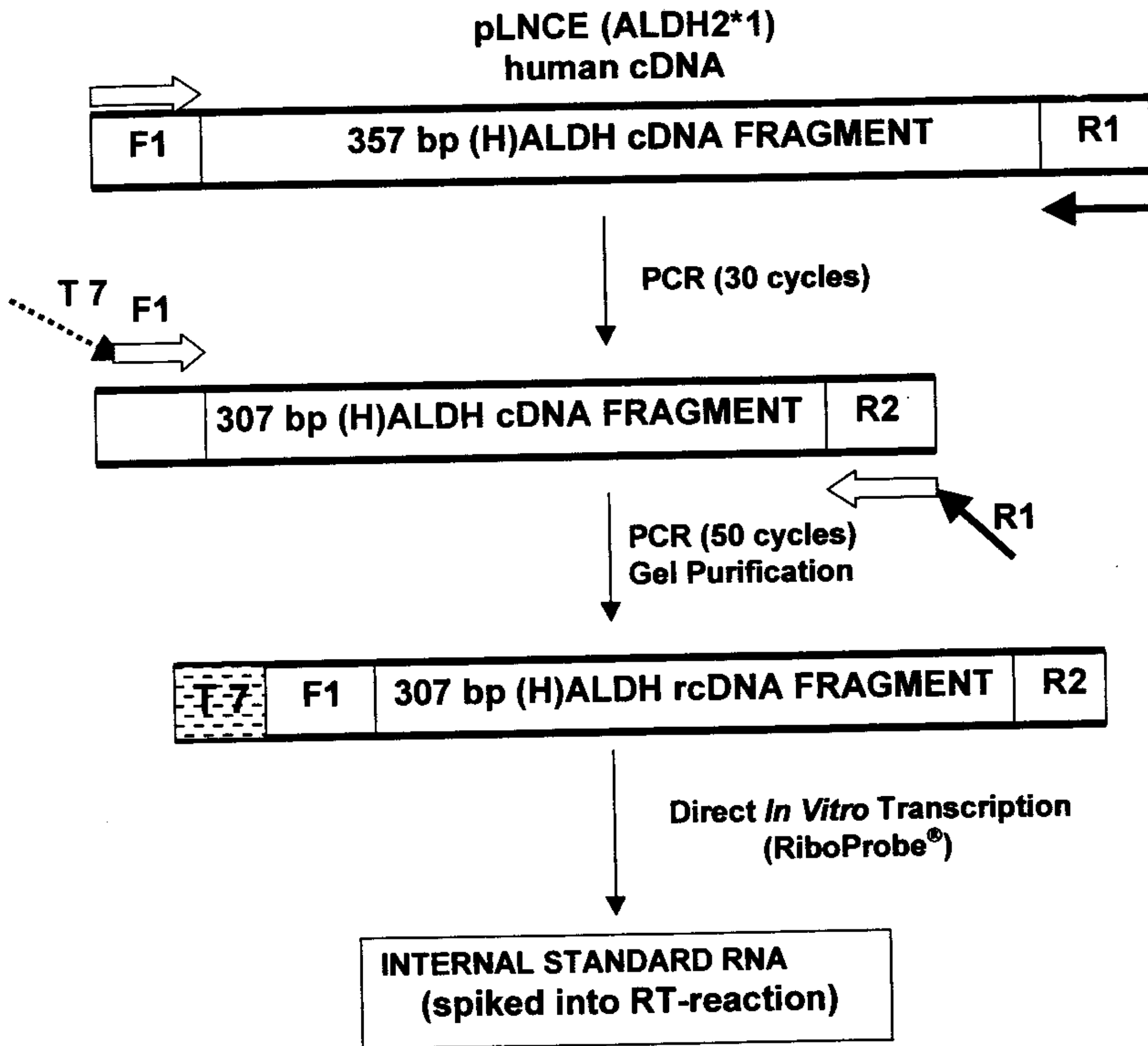


Figure 5.1.

The smaller 307 bp fragment obtained from the amplification using the above described primers was then used as the DNA template for *in vitro* transcription to make RNA. This served as the human ALDH2 internal standard for competitive RT-PCR reactions with sample RNA. The final internal standard band when amplified with the parent fragment primers during competitive RT-PCR was 280 bp in length as the 27 bp T7 sequence was not amplified.

RESULTS

Confirmation of Human ALDH2 cDNA in Vectors

In order to confirm the presence of the two different forms of cDNA and the integrity of the constructs a restriction digest was performed. Following a restriction digest with Hind III, vector DNA and liberated human cDNA insert sizes were confirmed on a 1% agarose gel containing ethidium- bromide.

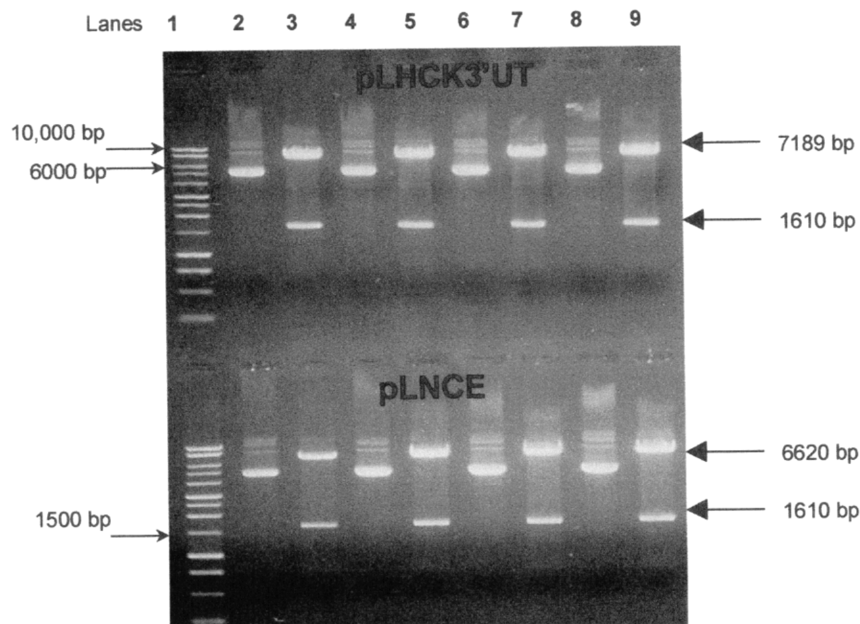


Figure 5.2. Hind III digested vector DNA resulted in two bands. One band at 1610 bp representing ALDH2*1 or 2*2 cDNA and another band at either 6620 bp or 7189 bp for wild-type (pLNCE) or mutant (pLHCK3'UT) vectors, respectively. Lane 1, 1 KB DNA ladder; Lanes 2,4,6 and 8, undigested vector DNA; Lanes 3,5,7 and 9, Hind III digested vector DNA.

Control Vector Preparation

Control vector without a cDNA insert was prepared to determine the baseline ALDH2 activity without the human wild-type or mutant cDNA in H4-II-E-C3 cells transduced with vector backbone alone. The pLNCE vector was modified such that the wild-type cDNA ALDH2*1 was liberated using Hind III to digest the vector DNA. The two bands (1610 bp cDNA, 6620 bp vector backbone) were separated on a 1% agarose gel and the 6620 bp vector backbone DNA was purified from the gel by electroelution for ligation.

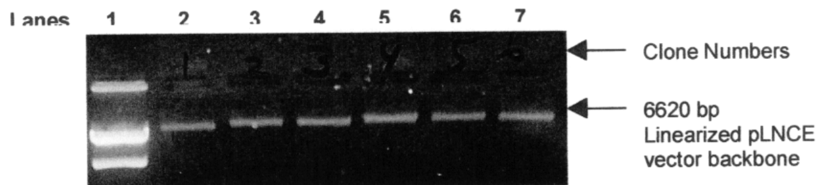


Figure 5.3. Empty pLNCE vector DNA isolated from DH5α E. coli that were transformed with the ligated vector. Lane 1, undigested pLNCE vector backbone; Lanes 2 to 7, Hind III digestion (linearized).

ALDH Activity in Transduced H4-II-E-C3 Cells

Empty pLNCE (Control) The ALDH2 activity in these cells was used as the standard for which H4 cells that were transduced with wild-type (pLNCE) or mutant (pLHCK3'UT) were compared to for the basal level of expression. Cells that were transduced with the pLNCE vector maintained the expression of the Neomycin resistance gene. The mean ± sem specific activity that was obtained for this group of clones (N=14) was 51.6 ± 1.0 nmol/min/mg soluble mitochondrial

protein. An example of the ALDH activity measured in one of these clones is depicted in Figure 5.4.

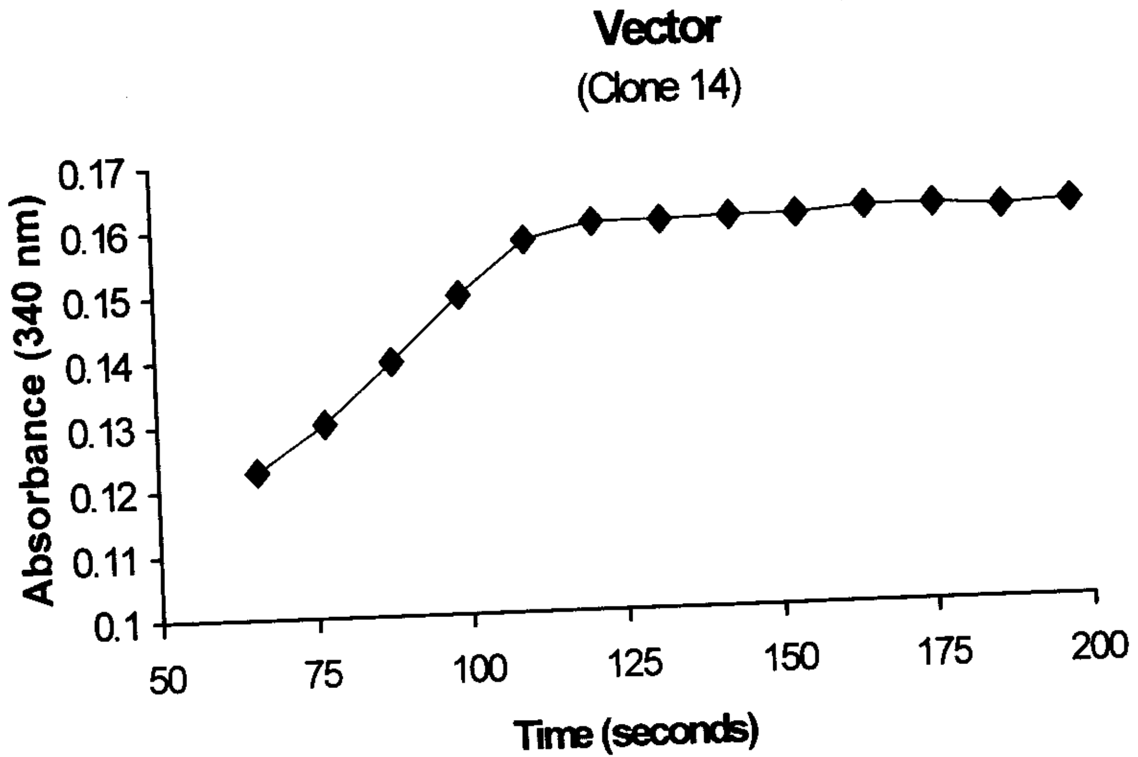


Figure 5.4. Endogenous rat ALDH2 activity measured in 150 μg of solubilized mitochondrial protein isolated from a H4-II-E-C3 cell (clone 14) transduced with the retroviral vector backbone, without human ALDH2 cDNA.

<u>pLNCE (Wild-Type ALDH2*1)</u> In order to determine if a functional ALDH2 tetramer could be produced by combining species monomers, H4 cells were transduced with the wild-type human cDNA (ALDH2*1). These transduced cells seemed to maintain the ALDH2 activity that was observed in the control goup which only had the pLNCE vector backbone incorporated into the genome. The

mean ± sem specific activity that was obtained for this group of clones (N = 15) was 54.7 ± 2.6 nmol/min/mg soluble mitochondrial protein. Although the mean activity for this group was similar to H4 cells transduced with pLNCE vector backbone, in some wild-type clones, ALDH activity was increased to as much as 52% more than in the control group. An example of the ALDH activity measured in one of these pLNCE wild-type clones is depicted in Figure 5.5.

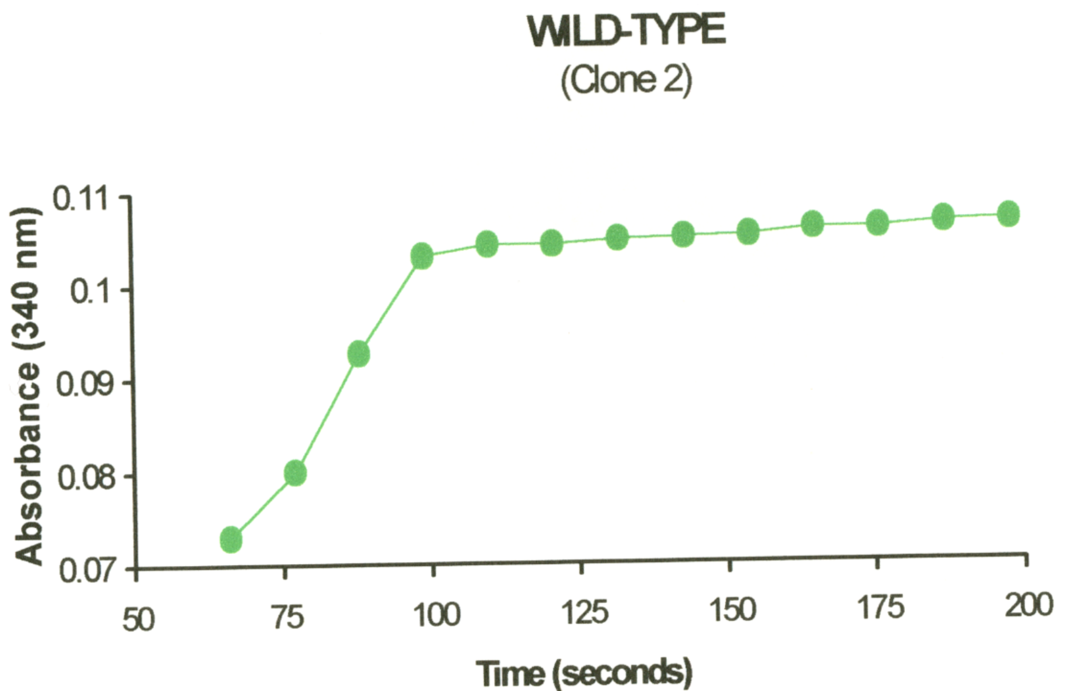


Figure 5.5. Combined human/rat ALDH2 activity measured in 150 μg of solubilized mitochondrial protein isolated from a H4-II-E-C3 cell (clone 2) transduced with human ALDH2*1 cDNA.

<u>pLHCK3'UT (Mutant ALDH2*2)</u> H4 cells were transduced with the mutant human cDNA ALDH2*2 to see if the incorporation of a mutant monomer into the tetrameric structure would decrease the enzyme activity. Cells transduced with

the mutant human cDNA decreased the ALDH2 activity that was observed in the control goup which only had the pLNCE vector backbone incorporated into the genome. The mean ± sem specific activity that was obtained for this group of clones (N = 22) was 40.4 ± 2.1 nmol/min/mg soluble mitochondrial protein. These clones had reduced in ALDH2 activity when compared to either vector backbone (control) or wild-type (pLNCE) transduced cells with p-values of <0.00004 (Vector vs Mutant) and <0.0002 (Wild-Type vs Mutant). While all the propionaldehyde (substrate) was converted to propionacetate within 2 minutes by ALDH2 from vector (control) or wild-type clones; the ALDH2 in some mutant clones still had not exhausted the added substrate until 4 minutes (230 sec) Figure 5.6.

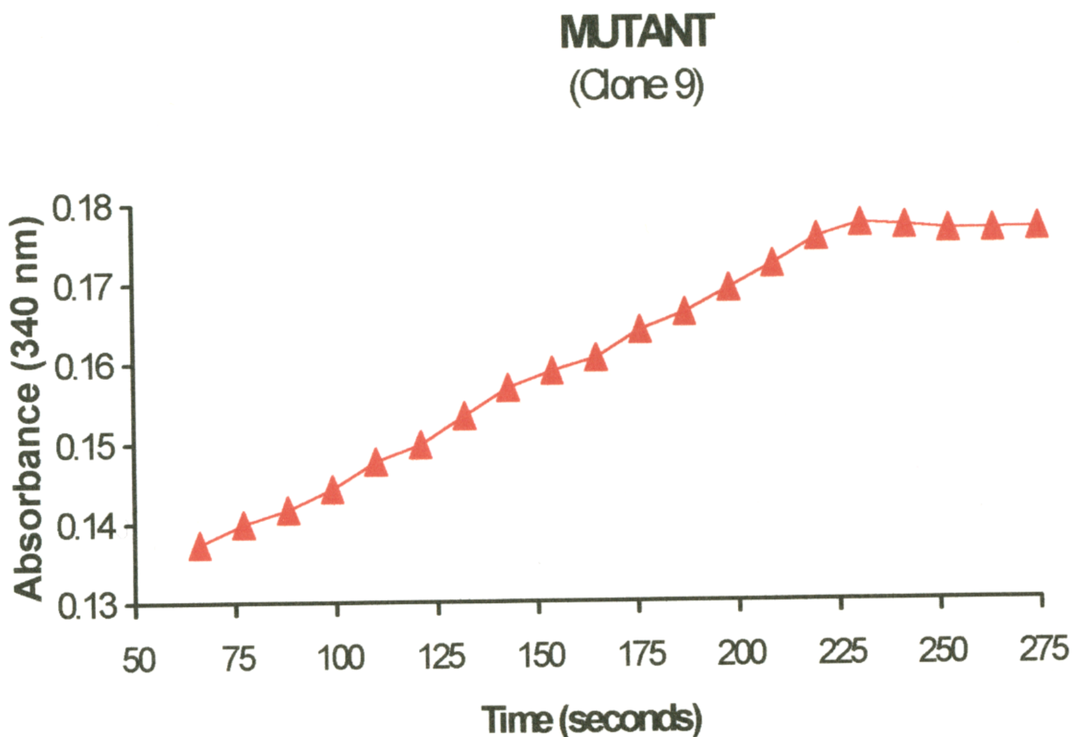


Figure 5.6. Combined human/rat ALDH2 activity measured in 150 μg of solubilized mitochondrial protein isolated from a H4-II-E-C3 cell (clone 9) transduced with human ALDH2*2 cDNA.

Trends in Transduced Clone Groups

A bar graph below (Figure 5.7) represents the percentages of clones from each group (vector backbone-control, wild-type or mutant) of transduced H4-II-E-C3 cells that had ALDH2 specific activities of 15 to 75 nmol/min/mg of soluble

mitochondrial protein. Table 5.1 summarizes the mean and range of ALDH2 specific activity for each tranduction group, as well as, the p-values with respect to vector backbone basal specific activity. The range of ALDH2 specific activity observed in the general population of vector and wild-type clones was 45 to 60 with several wild-type clones obtaining increased activities of 75 nmol/min/mg of soluble mitochondrial protein.

However, the mutant clones had a general population range of ALDH2 specific activity of 30 to 55 nmol/min/mg of soluble mitochondrial protein, and two of the clones had markedly lower specific activities of only 15 nmol/min/mg of soluble mitochondrial protein.

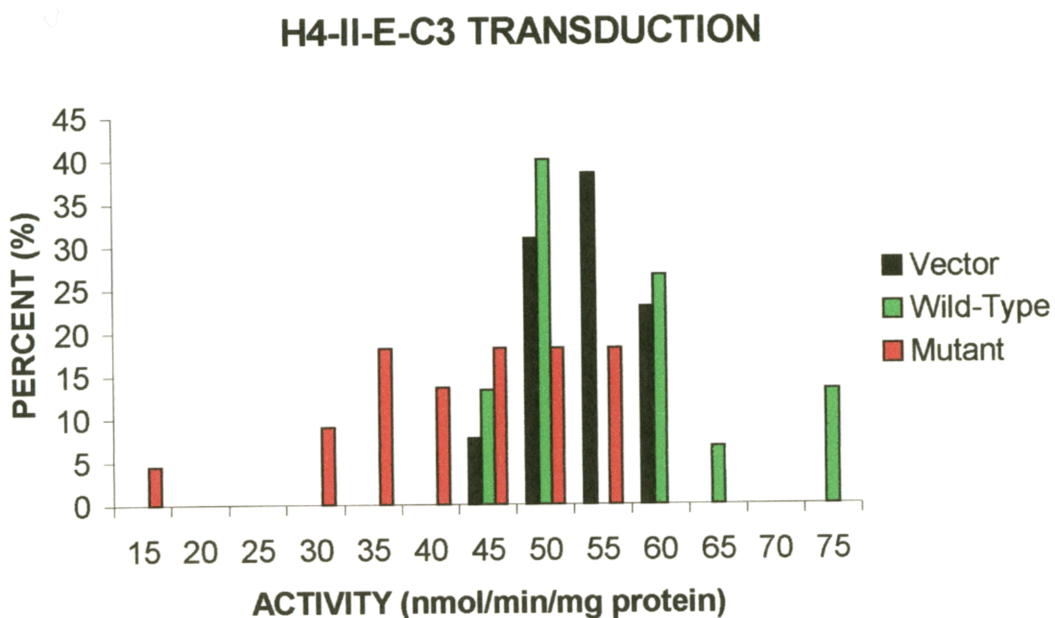


Figure 5.7. The percent of clones out of the total number of clones measured for each group (Vector,N=14, Wild-Type, N=15 or Mutant,N= 22) that had a particular ALDH2 specific activity.

Table 5.1. Summarization of Mitochondrial Adehyde Dehdrogenase Specific Activities for Transduced H4-II-E-C3 Cells.

TRANSDUCTION TYPE	NUMBER OF CLONES TESTED	SPECIFIC ACTIVITY (nmol/min/mg protein)*	P-VALUE***
Vector (no cDNA)	N = 14	51.6 ± 1.0 (46 – 57)**	NA
Wild-Type (ALDH2*1)	N = 15	54.7 ± 2.6 (42 – 78)	<0.14
Mutant (ALDH2*2)	N = 22	40.4 ± 2.1 (15 – 54)	<0.00004

* Value represent mean ± sem

**Numbers in parentheses represent the range of specific activity

****p-values* represent comparison of transduction type with vector baseline values

Glutamate Dehydrogenase Activity

The mitochondrial enzyme glutamate dehydrogenase (GDH) was measured in the mutant transduced H4 cells to determine if another unrelated mitochondrial enzyme's activity was being affected as a result of the incorporation of the ALDH2*2 transgene. In early studies prior to construction of the pLNCE vector backbone as a control, the GDH activity was measured in non-transduced H4-II-E-C3 cells and in mutant (pLHCK3'UT) transduced H4-II-E-C3 cell clones. The GDH activity remained constant in three mutant clones (1,7 and 10) while ALDH2 inhibition of 58.9%, 32.7% and 50.1% was observed respectively, when compared to the non-transduced H4-II-E-C3 cells. The p-value in a *t*-Test

comparing non-transduced H4-II-E-C3 cells to the three mutant clones was $p < 0.8$, and in Figure 5.8 it is shown that the GDH activities parallel each other.

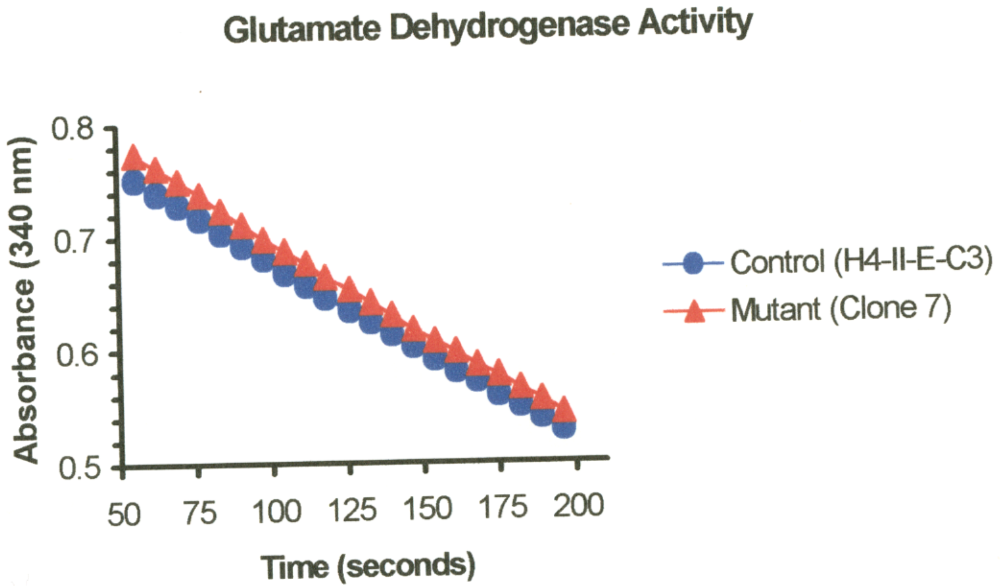


Figure 5.8. Glutamate Dehydrogenase activity measured in 25 μg of solubilized mitochondrial protein isolated from a H4-II-E-C3 cell (clone 7) transduced with human ALDH2*2 cDNA verses non-transduced H4-II-E-C3 cells.

DISCUSSION

Incorporation of the human ALDH2*1 gene with a mitochondrial signal peptide sequence into the H4-II-E-C3 cell line led to the formation of a functional combined species tetramer. Furthermore, some clones that contained the human wild-type ALDH2*1 gene actually had substantial increases in mitochondrial aldehyde dehydrogenase activity when compared to the basal level found in H4 cells that were only transduced with the retroviral vector backbone (see figure 5.7).

Likely, the inclusion of a mutant human ALDH2*2 monomer into H4 cells led to a combined enzyme species that was significantly less active than either retroviral vector backbone or human wild-type (ALDH2*1) transduced H4-II-E-C3 cells. Furthermore, in early studies with H4 cells transduced with the mutant human ALDH2*2 monomer, we found that the glutamate dehydrogenase activity remained constant in non-tranduced H4 cells when compared to mutant human ALDH2*2 transduced H4 cells (Figure 5.8). Incorporation of the mutant human ALDH2*2 did not effect GDH, also a mitochondrial enzyme, and suggests that the effect of the expressed ALDH2*2 monomer was specific to ALDH2.

The final desired goal of a gene therapy approach for the treatment of alcoholism would be to incorporate a mutant human monomer into the functional human tetramer such that accumulation of acetaldehyde would lead to dysphoria and ultimately ethanol aversion. Previously, we were able to demonstrate that the same ethanol aversion established with Disulfiram in humans could be established in the rat (Garver *et al.*, 2000). If expression of a mutant human

monomer could be sustained at high levels in the rat's liver, the resulting acetaldehyde accumulation may lead to substantial ethanol aversion as is seen in Asians that are either hetero or homozygotes for ALDH2*2.

The advantage that a gene therapy approach might have as compared to gene regulation (antisense), is that the potential for longer lasting effects from a single administration could led to better patient compliance. With the advent of viral vectors such as the gutless adenoviral vectors or the helper-dependent adenovirus vectors which can deliver a transgene quite efficiently to the liver (Kahn, 2000), it has become possible through a single administration to be able to maintain transgene expression for a month or more (Aurisicchio *et al.*, 2000). Recently an example of the sustained expression was shown by Thule and Liu that have shown in rats that they were able to create a liver targeted insulin transgene by engineering glucose responsive elements into a hepatic promoter containing an inhibitory insulin response sequence that was used for the treatment of diabetes mellitus using one of these adenoviral vectors that lasted for 12 weeks (Thule *et al.*, 2000). In addition, in 1999 Morral et al demonstrated that administration of a helper-dependent adenoviral vector carrying a human α1-antitrypsin transgene maintained transgene expression for over one year after a single administration (Morral *et al.*, 1999).

The disadvantage of a gene regulatory approach is that delivery of an antisense molecule may have to be as frequent as twice a week; thus, patient complience would be less obtainable under this approach when compared to the gene therapy approach which can sustain expression of a transgene for months or years (Morral *et al.*, 1999).

The studies presented here represent the first demonstration that introduction of a human transgene for ALDH2*1 and ALDH2*2 can increase or reduce the activity, respectively, of the rat enzyme. However, further studies should be performed to determine if the amount of human ALDH2*1 or 2*2 transgene expression can be correlated with the increase or decrease of ALDH2 activity measured in the isolated mitochondria from individual clones. Since a competitive RT-PCR assay was developed to quantitate and distinquish the human transgene mRNA expression from that of the endogenous rat ALDH2*1, it might be possible to make a correlation between the mRNA expression of the transgenes and the increase or decrease in the ALDH2 activities in individual clones transduced with human ALDH2*1 or 2*2, respectively. Although there is no gaurantee that the mRNA expression will parallel the ALDH2 protein synthesis, direct protein quantitation from the transgenes is not readily obtained without the modification of the net charges of wild-type or mutant homotetramers. Preliminary attempts to separate the rat and human heterotetramers by IEF or SDS-PAGE using a rat ALDH2 polyclonal antibodies for detection were unsuccessful in resolving the ALDH2 heterotetramers (data not shown). However, recently Zhou and Weiner were able to modifiy the human ALDH2*1 and ALDH2*2 cDNA's through mutagenesis techniques such that the net charge of the homotetramers produced from the cDNA's of ALDH2*1 or ALDH2*2 were different in that the ALDH2*1(E487) subunit was more negative than the ALDH2*2 (K487) subunit while the activity of these new homotetramers remained identical to the parent homotetramer activity despite the incorporated mutations (Zhou. and Weiner, 2000). The change in the wild-type or mutant subunits charges allowed for separation of coexpressed ALDH2*1/2*2 (E/K) heterotetramers by DEAE-Sephacel column by FPLC and subsequent Western

blotting analysis with anti-ALDH antibody following SDS-PAGE (Zhou and Weiner, 2000). Hence, if the expression of ALDH2 mRNA from the human transgenes does not parallel protein synthesis, a correlation to increases or decreases in ALDH2 activity as a result of a combined species heterotetramer could be made by modifying the net charges of the homotetramers produced from mutagenesis of the cDNA's such that the protein could be directly quantitated.

According to the results of Zhou and Weiner the ALDH2 tetramer functions as a dimer of dimers, and as such, the incorporation of one mutant monomer or subunit into one dimer renders that dimer inactive while the other dimer maintains its enzymatic activity in its tetrameric structure (Zhou and Weiner, 2000). This resulting in half of the site reactivity equating to the tetramer having a 50% reduction in enzymatic activity (Zhou and Weiner, 2000).

In order to see if the combine species heterotetramer follows the same model as that proposed by Zhou, and to make a correlation between monomer expression and activity, H4-II-E-C3 clones transduced with either the human ALDH2*1 or ALDH2*2 with a modified net charge would be advisable if competitive RT-PCR was not successful in providing a correlation between transgene expression and enzymatic activity increases (human ALDH2*1) or decreases (human ALDH2*2). Nonetheless, these initial studies are promissing for the gene therapy approach to the establishment of ethanol aversion in the rat. The present studies warrant further investigation into a suitable viral system, such as the adenovirus, adeno-associated virus, or Lenti virus for delivery of the human mutant ALDH2*2 transgene for long-term expression in the rat.

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Section VI DISCUSSION

Pre-Clinical Animal Model.

The first task at hand in developing a new aversive medication was to develop a pre-clinical animal model that would allow us to determine if the test drug (antisense) was effective in establishing an aversion to ethanol consumption. This was accomplished using disulfiram, the only U.S approved aversive medication, as the model drug in the rat. The rat was an ideal pre-clinical model because of the similarity of the mitochondrial aldehyde dehydrogenase between humans and rats in terms of amino acid and nucleic acid sequences, as well as, the Km (< 1.0 μM). This isozyme characterized by a low Km plays the major role in the metabolism of acetaldehyde formed after alcohol consumption in both species.

One disadvantage of the rat model was its inherent dislike for the consumption of ethanol. Past studies demonstrated that the rat had to be either selectively bred or placed into a ethanol-sweetner "fade-out" process in order to get these animals to consume large amounts of ethanol (Samson, 1986, Lumeng *et al.*, 1995). Furthermore, these animals had to be placed into a limited access paradigm in order to get the animals to binge up to 0.9 g ethanol/kg in 30 minutes (Samson, 1986).

Using a modification of the technique of schedule-induced polydipsia first described in 1961 by Falk, ethanol naive rats were induced to consume 0.7-1.5 g ethanol/kg within an hour of fluid presentation. This pattern of consumption was

advantageous in several ways. A commercially available stock animal (Lewis rats) could be induced to consume ethanol without breeding or training.
Most importantly by comparison with previously described models, is that in this model the animals were able to rapidly and clearly associate their dysphoria with the consumed ethanol in the presence of the aversive medication (disulfiram and antisense). Although we did not test for ethanol aversion in rats selectively bred or trained for alcohol consumption in the presense an aversive medication like disulfiram, it is very unlikely that either of these animal models would have readily associated their ethanol consumption with the dysphoria after being trained or selected for alcohol consumption.

The above models had been used in the testing of anti-craving medications. Naltrexone, the non-selective opiod receptor antagonist has been effectively shown to reduce consumption of ethanol in the selectively bred UchB and AA rats strains (high drinkers) in a voluntary selection situation (Quintanilla and Tampier, 2000, Parkes and Sinclair, 2000). In addition, Remoxipride, a dopamine D2 antagonist was tested in Long-Evans rats after they were trained to self-administer ethanol in daily 30 minute operant sessions using the sucrose-substitution procedure for its ability to reduce the desire for a second drink (Files *et al.*, 1998). Studies with Remoxipride produced an approximate 50% reduction in the number of ethanol presentations per session at the highest dose suggesting that it may have a role as an adjunct in the treatment of excessive alcohol consumption (Files *et al.*, 1998). These studies demonstrate the usefulness of rats that have been selective bred or trained by the sucrose-substitution procedure to consume alcohol for testing of anti-craving medications.

Following treatment with ASO-9, rat's consumption of an ethanol-containing solution was markedly reduced despite the intense drive of animals to rehydrate. The animal model that we designed was very effective at demonstrating an established aversion to ethanol in the presence of ASO-9 measured as a 45% reduction in ethanol consumption. This reduction in ethanol consumption of ASO-9 treated rats was essentially identical to the 46% reductions observed in disulfiram treated animals. One likely explanation for the identical reductions in ethanol consumption observed after treatment with ASO-9 or disulfiram is that small increases in plasma acetaldehyde can result in a physiological cascade that produces the dysphoria that leads to the aversion to ethanol consumption. One observation in support of this explanation is that the plasma acetaldehyde levels 1 hour after ethanol administration in rats pre-treated with disulfiram (100 mg/kg, i.p bolus) or ASO-9 (24 mg/kg/day i.p. constant infusion) had plasma concentrations of 59.6 ± 11.1 μM and 8.3 ± 1.9 μM, respectively. In the absence of ALDH inhibitors, the levels of acetaldehyde after alcohol administration are of the order of 2 μM. Since disulfiram non-specifically inhibits all aldehyde dehydrogenases and not just ALDH2, as ASO-9, it is expected that animals treated with disulfiram would have a higher plasma acetaldehyde level after ethanol administration. However, the studies presented here were done at only one dose level and no attempt was made to determine a dose-response curve for either disulfiram or ASO-9 with regard to plasma acetaldehyde levels and reductions in ethanol consumption. Hence, further dose-response studies could provide some insight into the plasma acetaldehyde levels that are needed in order to obtain an aversion to ethanol consumption. From the data obtained it

would be reasonable to hypothesize that a 4-fold increase in acetaldehyde levels to 8 μM is enough to induce a strong aversion to ethanol.

We have not determined the long-term effects on ethanol consumption in animals treated with ASO-9. In order to determine the long-term effects of an antisense molecule targeted to ALDH2, one could consider using one of the selectively bred strains of rats that have an innate preference for ethanol. For instance, if one were to take an ethanol naïve selectively bred rat (i.e AA, UchB, or P) pre-treated with ASO-9 that was subjected to an 18 hour period of schedule-induced polydipsia with food freely available prior to first ethanol presentation, then it may be possible to demonstrate the long-term effects of ASO-9 because control animals would have an innate preference for alcohol consumption in the absence of an aversive medication.

Gene Regulation.

Our antisense targeted to mitochondrial aldehyde dehdrogenase proved the principle that specific inhibition of this gene's expression in the rat results in the protective Asian phenotype. The resulting Asian phenotype, characterized by increases in circulating acetaldehyde upon ethanol consumption, associated dysphoria, and ultimately the development of an aversion to alcohol consumption was observed in the rat model.

In our studies we demonstrated some specificity of ASO-9 for its target through mismatches of bases in the oligonucleotide (*in-vitro*), lack of effects on another mitochondrial enzyme glutamate dehydrogenase (*in-vitro and in-vivo*), and

degradation of the targeted ALDH2 mRNA indicated a true antisense mechanism. Although RNase H activity in the nucleus on the ASO-9/ALDH2 pre-mRNA hybrid could explain the reduction in the steady-state ALDH2 mRNA levels observed, reductions in ALDH2 mRNA by an occupancy mechanism cannot be ruled out without further studies from those presented. Since ASO-9 targets a region of ALDH2 pre-mRNA in close proximity to an exon/intron boarder, it may be that proper splicing is impaired as others have shown in cell free systems (Dominski and Kole, 1993), or that the ALDH2 pre-mRNA or mRNA secondary or tertiary structure has been altered such that stability and/or the degradation rate of the ALDH2 RNA has been affected (Crooke, 1998).

The phosphorothioate backbone of an antisense oligonucleotide does have some disadvantages. The polyanionic characteristics of PS-oligos have been associated with complement activation and prologation of activated partial thromboplastin time (aPTT) (Galbraith *et al.*, 1994, Henry *et al.*, 1997 and Shaw *et al.*, 1997). This effect occurs at doses beyond the ones used therapeutically (Crooke, 1998). An initial concern about a phosphorothioate oligonucloeotides (PS-oligo) was their ability to stimulate the immune system (Agrawal and Zhao, 1998). However, such an effect of PS-oligo is dependent on the presence of CpG motifs (Agrawal, and Zhao, 1998). The severity of the immune stimulation depends on the position of the CpG motif and its flanking sequence in the PS-oligo (Levin, 1999). A CpG motif in which CG residues are flanked by two purines (A or G) at the 5' end and two pyrimidines (C or T) at the 3'end (e.g. AACGTT) has been shown to be particularly active at inducing B-cell proliferation and the release of cytokines (Krieg *et al.*, 1995). These cytokines include IL-6,

IL12, TNF-α, gamma-IFN, as well as, chemokines (Klinman *et al.*, 1996, Pisetsky, 1996 and Zhao *et al.*, 1997).

Although ASO-9 had a CpG motif at its 3' end, the flanking residues TT and GC did not fit the rule of two purines (A or G) at the 5' end and two pyrimidines (C or T) at the 3'end for maximum immune stimulation (ASO-9 contained 5'-TT**CpG**GC-3'). However, It was noted after 8 days of interperitoneal administration of ASO-9 (24 mg/kg/hr) that some animals had fluid present in the abdominal cavity which suggested some toxicity was present by this method of administration. Additional experiments were not performed to determine the cause of this observed toxicity, however, it does suggest that there may be non-sequence specific side effects occurring at the selected dose during repetitive administration over 8 days.

It would seem based on clinical experience with other ASO's that a mixed backbone oligonucleotide (MBO) should be considered for further development of an antisense to target ALDH2 in humans in order to minimize the possibility of toxicity. While PS-oligos have been promising as the first generation of oligonucleotides, efforts have been made to further improve their biological potency and safety profile. Second generation MBO's (see Figure 6.1) contain segments of phosphorothioate oligodeoxynucleotides and appropriately placed segments of modified oligodeoxy- or oligoribonucleotides (Agrawal, 1996). The phosphorothioate linkages maintain the MBO's capacity to be a substrate for RNase H assisting in the degradation of target RNA. The other segments of the MBO's contain non-ionic methylphosphonate which is very resistant to nucleases or 2'-O-alkyloligoribonucleotides which improves target affinity as well as stability

in the presence of nucleases (Agrawal, 1996). These MBO's significantly increase *in-vivo* stability, which may reduce the frequency of administration; and they have less severe side-effects due to their reduced polyanionic nature and immunogenic response (Zhang *et al.*, 1995, Zhao *et al.*, 1996).

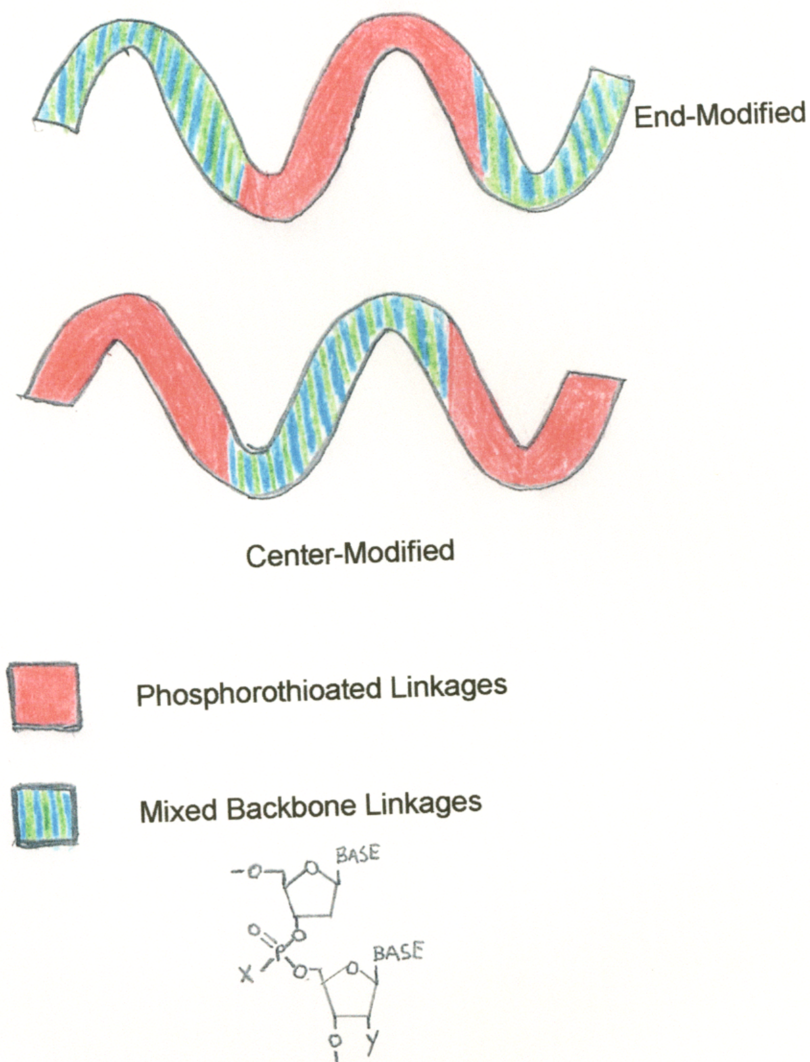

Figure 6.1

Mixed backbone oligonucleotides (MBO's) have the advantage of retaining their ability to act as a substrate for RNase H because of the presence of some phosphorothioate portions in the oligonucleotide while other modified backbone linkages in the oligo minimize the the side-effects inherent in PS-oligos. The positioning of the modified-oligo or oligoribonucleotide in a PS-oligo is

critical for its desired properties. Placement of a methylphosphonate linkages (X = CH_3, Y = H) at the ends reduces the overall polyanionic-related side effects, and increases the *in vivo* stability by protecting both ends of the PS-oligo from nuclease degradation. Placement of 2'-O-methylribonucleotide (X= S, Y = OCH_3) can provide increased affinity and *in vivo* stability. This increased stability of the oligo equates to longer duration of action and less dosing required in order to maintain efficacy. If an oligo is more stabile *in-vivo*, then it is likely that there will be fewer degradation metabolites decreasing the potential for unwanted side effects from such metabolites. Placing the modified segment of oligodeoxynucleotide or oligoribonucleotide in the center of the PS-oligo provides also protects from nuclease degradation and metabolite formation.

The ideal situation for administration of an ASO targeted to human ALDH2 would be to have an ASO that can be administered orally, however, PS-oligos have been shown to have a poor oral bioavailability (<2%) when compared to subcutaneous (30.9%) or intraperitoneal (28.1%) administration (Nicklin *et al.*, 1998). On the other hand, MBO's have been very promising in pre-clinical trials in mice where they were administered orally (Wang *et al.*, 1999). Recent studies have shown that partially substituted 2'-O-(methoxyethyl) derivatives of phosphorothioate oligonucleotides (ribose 2-O $CH_2CH_2OCH_3$) increase oral bioavailability of intact drug from 0.3% to 5% by preventing intestinal degradation and increasing absorption. Bioavailability determined from ^{35}S label was 38% (Geary *et al.*, 2001).

The MBO tested was targeted to the regulatory subunit isozyme I (RIα) of cAMP-dependent protein kinase (PKA), and increased expression of this subunit of PKA is correlated with cell proliferation and neoplastic growth (Cho-Chung and Clair, 1993). The MBO tested (GEM231®) was modified at the 3' and 5' ends of the

oligonucleotide where four of the nucleosides were 2'-O-methylribonucleosides with the middle linkages of the oligonucleotide being phosphorothioated (Wang, *et al.*, 1999). The results from these studies are impressive with an oral bioavailability of >48% with a long elimination half-life of 60.8 hours measured in the tumor tissues of the mice xenografts which indicated a significant accumulation and retention of the MBO (Wang *et al.*, 1999).

GEM231® was recently tested in a phase I clinical trial in cancer patients in which the safety and pharmacokinetic profiles were tested in 2-h IV infusions twice a week in patients with refractory solid tumors. These studies found no treatment-related complement activation or thrombocytopenia at any dose level and high plasma concentrations of GEM231® were well tolerated without significant acute toxicities (Chen *et al.*, 2000).

Given the results discussed above with an MBO, it is likely that an orally available MBO may make into clinical trials in the near future and hopes of an orally administered antisense targeting human ALDH2 looks promising as an alternative to disulfiram for the treatment of alcoholism.

Future Direction for ALDH2 Antisense. In order to justify the use of an MBO for oral administration in humans, studies would have to be initiated in the rat to demonstrate the efficacy and antisense mechanisms of an orally available MBO of ASO-9. In addition, a new ASO should be designed to target human ALDH2 and *in-vitro* studies should be performed in a suitable human hepatocyte cell line such as the HepG2 cell line. Further toxicity testing of an ASO-9 MBO would be required by the FDA for safety assessment and justification for first administration

in man of the orally available human ASO targeted to ALDH2. Since esophageal cancer could be a risk with high levels of acetaldehyde exposure (see Considerations and risk assessment below), it may be required to ascertain the tissue exposure to acetaldehyde following antisense administration in the presence and absence of alcohol consumption.

Gene Therapy.

The proof that transduction of a human dominate negative mutant ALDH2*2 cDNA into the rat hepatocyte's genome reduced the rat's hepatocyte ALDH2*1 activity holds promise of long-term suppression of ALDH2 activity for alcoholics trying to maintain abstience. Although competitive RT-PCR was developed to differentiate the human from the rat ALDH2 transcripts, further experiments were not performed to determine the ALDH2*2 expression in various mutant clones compared to clones transduced with vector backbone alone.

Further Research Suggestions. If the human mutant ALDH2 transcript was quantitated by competitive RT-PCR in clones that had differences in reduction of the ALDH2 activity, it might be possible to correlate the amount of ALDH2 activity reduction with that of the transgene expression. For example, one could postulate that the activity in a clone that had a high expression of the human mutant ALDH2*2 transgene would have an overall ALDH2 activity that was significantly reduced from the clones transduced with vector backbone alone. Conversely, if a clone was tranduced with the human ALDH2*1 cDNA and had high expression, then the overall ALDH2 activity in that clone may supercede the activity of clones transduced with vector backbone alone. Nonetheless, despite

the lack of a well defined mechanism by which the human mutant transgene reduces the overall ALDH2 activity in the transduced rat hepatocytes, the data presented in section III of this thesis provides evidence that the human ALDH2*2 cDNA could be introduced into the rat as a pre-clinical gene therapy model because transduction with human ALDH2*1 or vector backbone did not reduce the overall ALDH2 activity.

One could consider delivering the human ALDH2*2 cDNA via a viral delivery system such as the adeno-associated virus (AAV) to a rat that has been selectively bred for its desire to consume ethanol (i.e. AA, P, UchB, HAD ect.). The expression of the human mutant ALDH2 monomer in the liver of one of the selectively bred rats could lead to the long-term suppression of mitochondrial aldehyde dehydrogenase activity.

*Considerations and risk assessment of aversive therapy that results in acetaldehyde accumulations due to ALDH2*1 activity reduction.* An aversive medication such as disulfiram, cyanamide or ASO-9 can lead to increased blood or plasma acetaldehyde levels which has been associated with some types of cancer. Acetaldehyde has been shown to produce respiratory tract tumors in hamsters exposed to acetaldehyde vapors at 1650-2500 ppm/hr/day, 5 days/week for a period of 52 weeks demonstrating that high exposure levels can increase the risk of producing respiratory tract cancer (Feron *et al.*, 1982). This data suggest that acetaldehyde exposure to any tissue would have to be sustained for a long period of time or at very high levels in localized tissues in order to cause cancer. Nonetheless, one must consider the risks of cancer, pancreatitis and/or alcoholic cirrhosis versus the benefit of aversive therapy for

alcohol dependence given the high morbidity and mortality associated with alcoholism (Chick, 1999).

One way in which the risk of having increases in circulating acetaldehyde as a result of reduced ALDH2 activity has been assessed is through genotyping in some of the Asian population with or without alcohol dependence. Individuals that are heterozygous or homozygous for the point mutation (E487K) are afforded a protection against the development of alcohol abuse (Harada *et al.*, 1982, Goedde, *et al.*, 1983, Higuchi, 1994, Thomasson *et al.*, 1991, Tu and Israel, 1995). Since these individuals are less likely to consume alcohol just as in an individual treated with an aversive medication, increases in circulating acetaldehyde are also reduced in these individuals. Hence, the risk of these individuals developing cancer, pancreatitis and/or alcoholic cirrhosis of the liver are reduced. As shown in Table 6.1, ALDH2 genotyping in Japanese or Chinese alcoholics demonstrates that the prevalence of heterozygotes (ALDH2*1/2) and homozygotes (ALDH2*2/2) is significantly reduced in comparison to the normal ALDH2*1/1 genotype for both alcoholic cirrhosis and pancreatitis patients. Furthermore, a comparison of alcoholic cirrhosis and pancreatitis patients verses healthy controls revealed that there are more patients with cirrhosis or pancreatitis that have the 1/1 genotype and less of the 1/2 genotype (p-values <0.001). This suggests that the ALDH2*1/2 genotype represents a negative risk factor or protection against the development alcoholic cirrhosis and/or pancreatitis.

A comparison in Table 6.2 of the ALDH2 genotypes (1/1, 1/2, 2/2) shows that no single genotype was more prevalent in Japanese alcoholics with either

hepatocellular or oral cavity cancer when compared to healthy controls. However, there was an increased risk of Japanese alcoholics with the 1/2 genotype of having oropharyngolaryngeal or esophageal cancer. One possible explanation for the increased risk of development of oropharyngolaryngeal or esophageal cancer in Japanese alcoholics with the 1/2 or 2/2 genotype is that local ALDH activities may influence regional concentrations of acetaldehyde. One study demonstrated high ADH and low ALDH activities in the esophageal mucosa (Yin *et al.*, 1993) which suggests that intracellular acetaldehyde may accumulate locally during alcohol ingestion in this tissue and as such, the ALDH 1/2 or 2/2 genotype could result in substantial accumulations of acetaldehyde.

Given the data presented in Tables 6.1 and 6.2, it appears as though the benefit of being able to reduce the alcohol intake in an alcoholic as a result of administration of an aversive medication such as ASO-9 outweighs the risk of developing cancer from circulating acetaldehyde. This is in line with the recent findings of Chick in which there is no evidence to suggest that disulfiram causes cancer (Chick, 1999). In addition, if alcoholics are treated with an aversive medication like ASO-9, these individuals should be monitored for evidence of a continuation of alcohol consumption through biochemical markers in order to assess the efficacy of the aversive medication. By using biochemical markers such as the improved diagnosis of carbohydrate-deficient transferrin (CDT) in conjunction with gamma-glutamytansferase (GGT) reported by Sillanaukee, physicians should be able to minimize a patient's exposure to acetaldehyde at high levels or for a protracted time (Sillanaukee and Olsson, 2001).

Overall, the studies presented in this thesis represent the building blocks for a new aversive medication as an alternative to disulfiram without all the side effects. It is conceivable that someday an alcoholic may be able to acquire the same protection afforded to some of the Asian population with the naturally occurring ALDH2*2 allele/s.

TABLE 6.1 Risk of Alcoholic Cirrhosis or Pancreatitis According to Genotype of Asian Alcoholics

	Patient No.	ALDH2 GENOTYPE 1/1	1/2	2/2	ALDH2/2 Allele Effect	Reference
Alcoholic Liver Disease	47 Disease	85.1%*	14.9%*	0%	-72%	Enomoto *et al.*, 1991
	18 Controls	45.0%	45.0%	10%		
(cirrhosis)	31 Disease	80.6%*	19.4%*	0%	-68%	Tanaka *et al.*, 1996
	66 Controls	39.4%	53.0%	7.6%		
(cirrhosis)	75 Disease	86.7%*	13.3%*	0%	-73%	Chao *et al.*, 1997
	100 Controls	50.0%	41.0%	9.0%		
(cirrhosis)	116 Disease	84.5%*	15.5%*	0%	-68%	Chao *et al.*, 2000
	105 Controls	51.0%	40.0%	9.0%		
Alcoholic Pancreatitis	48 Disease	83.3%*	16.7%*	0%	-66%	Chao *et al.*, 1997
	100 Controls	50.0%	41.0%	9.0%		
	87 Disease	84.5%*	15.5%*	0%	-68%	Chao *et al.*, 2000
	105 Controls	51.0%	40.0%	9.0%		

*$p < 0.001$ *vs* non-alcoholic controls

TABLE 6.2 Risk of Cancer According to Genotype of Asians

		ALDH2 GENOTYPE				
	No.	1/1	1/2		*p*-Value	Reference
Non-Alcoholic						
Hepatocellular Carcinoma	85 Disease	57.6%	40.0%	2.4%	NS	Takeshita, T., *et al.*, 2000
	101 Controls	53.5%	37.6%	8.9%		
Oral Cavity Cancer	92 Disease	52.1%	45.7%	2.2%	NS	Katoh, T., *et al.*, 1999
	147 Controls	53.1%	41.5%	5.4%		
Alcoholics						
Oropharyngolaryngeal Cancer	19 Disease	47.4%	52.6%*	NA	*<0.001	Yokoyama, A., *et al.*, 1998
	487 Controls	91.0%	9.0%*	NA		
Esophageal Cancer	71 Disease	45.1%	54.9%*	NA	*<0.001	Yokoyama, A., *et al.*, 1998
	487 Controls	91.0%	9.0%*	NA		

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