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Cytosolic Acetaldehyde Dehydrogenase (*Ahd-2*) in Mice: cDNA Sequence, Molecular Characterization and a Possible Role in Ethanol Sensitivity.

by

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Submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario

January, 1992

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ABSTRACT

The influence of genetic factors on alcoholism and relative sensitivity to alcohol has been recognized but remains elusive. In mammals, ethanol is primarily oxidized by alcohol dehydrogenase (ADH) to form acetaldehyde. Acetaldehyde is the toxic metabolite responsible for most alcohol-related adverse effects. Acetaldehyde dehydrogenase (ALDH) irreversibly oxidizes acetaldehyde to acetate. Individuals with a mutant allele for mitochondrial ALDH experience a severe reaction to ethanol, caused by elevated acetaldehyde levels. Also, C57BL/6J, alcohol preferring mice are reported to have increased ALDH enzyme activity after ethanol feeding while BALB/c and 129/ReJ (alcohol avoiding) mice do not. The significance of ALDH in regulating the relative level of acteldehyde and the adverse effects of ethanol consumption is now well recognized.

The genes of alcohol metabolism in strains of mice with variable ethanol responses were examined. C57BL/6J mice showed increased *Adh-1* and *Ahd-2* mRNA, BALB/c mice a small increase in *Adh-1* mRNA and no change in *Ahd-2* mRNA, while 129/ReJ mice had decreased levels of both mRNA species after ethanol challenge. The increased levels of *Ahd-2* mRNA in C57BL/6J mice after ethanol consumption correlated to the reported increases in AHD-2 enzyme activity. Furthermore, a correlation between ethanol preference of the strains and banding patterns on Southern blots produced by hybridization with a partial cDNA probe for *Ahd-2* was noted.

The complete cDNA for *Ahd-2* was sequenced and includes 1503 nucleotides coding for 501 amino acids. Two regions of the *Ahd-2* gene were sequenced and compared among the three strains studied. Only minor differences were observed in both the promoter and Antabuse binding regions. Variable responses to ethanol in these strains cannot be explained by the observed DNA sequence differences observed. Promoter region analysis revealed a number of possible enhancer binding sequences. Binding of proteins to these sequences and possible effects of ethanol on transcriptional regulation in the strains are not known. Partial sequence of an additional gene, '*Ahd-2* related', 90.8% identical to *Ahd-2* was obtained. Its function in ethanol metabolism and sensitivity, however, remains to be determined.

For Ian, my best friend.

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GLOSSARY

ADH	alcohol dehydrogenase enzyme
Adh-1	gene coding for α ADH subunit in humans
Adh-1	gene coding for ADH-A2 in mice
Adh-2	gene coding for β ADH subunit in humans
Adh-3	gene coding for γ ADH subunit in humans
ADH-A2	alcohol dehydrogenase enzyme in mice
AHD-2	cytosolic acetaldehyde dehydrogenase enzyme in mice
Ahd-2	acetaldehyde dehydrogenase gene in mice
Ahd-2 related	a sequence transcribed in the mouse liver which is similar, but not identical to *Ahd-2*.
AHD-5	mitochondrial acetaldehyde dehydrogenase enzyme in mice
Ahd-5	gene coding for AHD-5 in mice
ALDH	acetaldehyde dehydrogenase
Aldh-1	gene coding for ALDH1, cytosolic acetaldehyde dehydrogenase in humans.
Aldh-2	gene coding for ALDH2, mitochondrial acetaldehyde dehydrogenase in humans.
bp	base pair
C/EBP	CCAAT enhancer binding protein, a rat liver transcription factor
cDNA	complementary DNA
DNA	deoxyribonucleic acid
kb	kilobase
MOPS	3-(n-morpholino) propanesulfonic acid
mRNA	messenger ribonucleic acid
pADHm16	cDNA probe used to detect mouse *Adh-1* mRNA
PCR	polymerase chain reaction
pG121	cDNA probe used to detect mouse *Ahd-2* mRNA
RFLP	restriction fragment length polymorphism

RNA	ribonucleic acid
RT-PCR	reverse transcription and polymerase chain reaction
S.E.M.	standard error of the mean
SB-2	cDNA clone for BALB/c *Ahd-2* cDNA
SBg-5	gDNA clone for BALB/c *Ahd-2*
SSC	standard saline citrate
tsp	transcription start point

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Chapter 1

INTRODUCTION

1.1 The Genetic Basis of Alcoholism

Alcoholism has been defined as a pathological state of physical or psychological dependence on alcohol but its multifactorial nature makes patient diagnosis difficult (Li, 1977). The disease generally progresses along a continuum, beginning with alcohol use, progressing to abuse and addiction. Addiction to alcohol is also difficult to pinpoint as it occurs through a number of phases which include psychological and behavioural changes (Barnes et al., 1987). The typical alcoholic patient exhibits a persistent and progressive pattern of heavy drinking despite the repeated serious personal problems such as arrest, job-loss, and poor health it causes (Schuckit, 1986).

It has been estimated that 90% of the population from industrialized Western cultures will consume alcohol at some point in their lifetime. One in three males in the United States will be temporarily afflicted with alcohol related problems and approximately 10% of all men and 3-5% of all women will become alcoholic (Schuckit, 1986). Although a large percentage of the population drinks, over one-half of all the alcohol in the U.S. is consumed by one-tenth of the population (Schuckit et al., 1985). The estimated economic cost of alcohol abuse in the U.S. is over $100 billion per annum and is the direct cause of over 10,000 fatalities. Alcohol

use has been linked to one quarter of all traffic injuries and has also been associated with suicide, crime, absenteeism, and drowning (Chambers, 1990). Alcoholism causes a number of serious and potentially fatal health problems. Ethanol consumption can lead to malnutrition in the alcoholic via altered protein metabolism or damage to the gastrointenstinal tract. Myopathy, liver disease (i.e. fatty liver or cirrhosis) and alcohol-induced neurological disorders are also common (Leo, 1982; Lieber, 1982). Approximately one-quarter of all hospital beds are believed to be filled by patients with alcohol related afflictions (Fawcett et al., 1987).

Due to its enormous social and personal impact, alcoholism has been studied for centuries. Although it has been accepted that alcoholism 'runs in families', 'familial' does not necessarily imply 'genetically inherited' (Goedde and Agarwal, 1987). Modern studies have attempted to dissect the genetic factors influencing alcoholism from the environmental factors by different approaches including familial and twin, adoption, and animal models (Schuckit, 1987).

Early family studies demonstrated a three to four-fold increased risk for alcoholism in the children of alcoholics, without an increased risk of other psychiatric disorders (Schuckit, 1985). Fifty-four percent of monozygotic twins studied showed similar drinking habits in contrast to twenty-eight percent of dizygotic twins, indicating a genetic predisposition to alcohol consumption (Goedde and Agarwal, 1987). Comparison of the lifetime occurrence of alcoholism in relatives of alcoholics showed that the risk for developing the disease is increased two-fold for second degree relatives, four-fold for first degree relatives and ten-fold for monozygotic twins (Schuckit et al., 1985). In addition, studies on alcohol elimination rate from the body have shown that the range of variation in the rate is

almost three-fold in the general population, including dizygotic twins. However, elimination rates in monozygotic twins were found to be much more similar, indicating a genetic influence on alcohol metabolism (Schuckit et al., 1987).

Careful analysis of the life histories and drinking patterns of patients from early studies revealed a number of different subtypes of alcoholism. Researchers began to focus on primary alcoholism, that is 'alcoholism which occurs in the absence of any pre-existing psychiatric disorders' (Schuckit, 1986). Alcoholics were also categorized based on age of onset, behavioural and social problems, and frequency of required treatment. Type 1, or milieu-limited, alcoholism is characterized by mild alcohol abuse, late age of onset (often in the patient's 30's or 40's), and the absence of criminal behaviour. Chronic drinking is often initiated by an environmental stress such as job loss or divorce (Hittle and Crabb, 1988) and occurs in equal frequency in men and women (Schuckit et al., 1987; Cloninger, 1987). In type 2, or male-limited alcoholism, heavy drinking begins early in their teens and is often associated with criminal behaviour. Type 2 alcoholics generally have repeated treatments for their severe alcohol abuse. A third, less common category of alcoholics is known as 'antisocial'. These patients are characterized by violent repetitive criminal behaviour in males and hypochondriac tendencies in females. Alcohol abuse in these patients is often very severe and is generally untreated (Schuckit et al., 1985).

A number of studies have examined the sons of alcoholic fathers. They, termed family history positive (FHP) subjects, differ from family history negative (FHN) subjects, or the sons of non-alcoholic fathers. Both FHP and FHN subjects anticipate similar effects from alcohol consumption

and both show similar blood alcohol concentrations after drinking (Schuckit, 1986, 1990). However, the FHP subjects perceived themselves to be less intoxicated after drinking. They also showed less decrement in cognitive and psychomotor skills, such as body sway, than FHN subjects with the same blood alcohol concentration. It was hypothesized that these individuals may not possess the necessary physiological cues to recognize intoxication. This may lead to increased alcohol tolerance and addiction (Schuckit, 1986; 1990).

Alcohol abuse was found to be significantly greater in the sons of Type 2 alcoholic fathers, even when the sons were adopted by non-alcoholic parents (Schuckit. et al., 1985). Daughters adopted from Type 1 alcoholic parents had significantly greater alcohol abuse than daughters of non-alcoholic biological parents or daughters from Type 2 alcoholics. Sons from Type 1 families were not at an increased risk for alcoholism unless the adoptive father had low socioeconomic status (Schuckit et al., 1985). Thus genetic influences of alcoholism may vary, depending on the subtype or category.

The above findings and others support the hypothesis that alcoholism is a multifactorial problem. Genetically influenced factors such as ethanol metabolism, personality, tolerance and psychological disorders likely interact with environmental factors, such as socioeconomic status and culture, to produce a level of predisposition to alcoholism. An in depth examination of the many different facets of the disease is essential for a better understanding of the phenomenon of alcoholism.

Animal studies have indicated that genetic factors influence alcohol consumption and preference. Different inbred mouse and rat strains show significantly different and highly heritable levels of voluntary alcohol

consumption (McClearn and Rodgers, 1979; Festing, 1979). It is also possible to selectively breed animals to increase or decrease voluntary consumption (Eriksson, 1973). The importance of animal studies to alcohol research will be discussed in greater detail below.

1.2 Ethanol Metabolism

The pathway of ethanol metabolism in mammals is relatively simple and well defined. Most ingested ethanol is oxidized to acetaldehyde in the liver by the NAD^+-dependent cytosolic enzyme alcohol dehydrogenase (ADH; E.C. 1.1.1.1) (Lieber, 1977; Bosron and Li, 1988). ADH is also located in the stomach, intestines, lung, kidney, heart and skeletal muscles. The contribution to ethanol oxidation from these tissues, however, is nominal (Li, 1983). A small proportion of ethanol is metabolized in the liver by the peroxisome enzyme catalase (E.C. 1.11.16) and by the membrane-associated electron transport system (MEOS) (Bosron and Li, 1988). There has been some debate as to the relative contribution of these systems to alcohol elimination. Studies using the ADH-deficient deermouse *Peromyscus maniculatus* have provided some evidence that, at least in the absence of hepatic ADH, other pathways can be exploited for alcohol metabolism (Burnett and Felder, 1980). Deermice with the ADH null genotype are able to eliminate ingested alcohol, although at a reduced rate, despite their lack of ADH. Other reports have indicated that MEOS-related alcohol metabolism in humans is approximately 1/100th the rate of the ADH pathway, indicating that the normal contribution of the alternate pathways is minimal (Lieber and DeCarli, 1972). MEOS activity, however, can be induced by chronic alcohol use and thus it may play a

more prominent role in the alcoholic liver (see Ehrig et al., 1990 for review). The ethanol-inducible isozyme of the P-450 system is named P450IIE1. The P450IIEi gene, which has been characterized in the rat, also has a human counterpart (Umeno et al., 1988). Studies have indicated the possible involvement of ethanol in post-translational modification of the P450IIE1 protein (Lieber, 1991).

Oxidation of ethanol leads to the production of acetaldehyde, a highly toxic metabolite (Bosron and Li, 1988). It has been implicated in most, if not all, alcohol-related aversive reactions (i.e. nausea, facial flushing) and health problems including cirrhosis of the liver (Lieber, 1988). The irreversible oxidation of acetaldehyde to acetate is catalyzed by the NAD^+-dependent oxidoreductase acetaldehyde dehydrogenase (ALDH E.C. 1.2.1.3). In humans, this occurs primarily in the hepatic mitochondria (Goedde and Agarwal, 1990). There has been considerable controversy over the relative importance of different forms of ALDH with different subcellular locations in acetaldehyde metabolism.

1.2.1 Alcohol Dehydrogenase

In humans, the ADH enzymes have been classified into three distinct groups (Vallee and Bazzone, 1983). Class I enzymes consist of homo- or hetero-dimeric metalloenzymes randomly formed from 374 amino acid residue α, β, or γ subunits. These subunits are coded for by the structural genes *Adh-1*, *Adh-2*, and *Adh-3*, respectively (Smith et al., 1971). Sequencing of the genes for α, β, and γ has revealed a high degree of similarity in their amino acid sequence, nucleotide sequence, genomic size and number of exons (Yoshida et al., 1988). All three genes have been

mapped to human chromosome 4q21-4q24 (Smith et al., 1985). In the early fetal liver, α is the only class I subunit expressed. In the second and third trimesters both α and β subunits are expressed. All three subunits are detectable in the liver shortly after birth (Bilanchone et al., 1986). Nucleotide sequencing and Northern blot analysis have indicated that all three genes produce transcripts with variable lengths due to multiple polyadenylation sites (Smith et al., 1985). Class I enzymes are the enzymes primarily responsible for ethanol metabolism in the human liver. The other two classes, class II (π ADH) and III (χ ADH) generally oxidize medium or longer chain alcohols and are not considered to be important in ethanol metabolism (Holmes et al., 1986a).

A number of polymorphisms have been noted for the β and γ class I ADH enzymes. Three allelic polymorphisms for *Adh-2* (β_1, β_2, and β_3) and two for *Adh-3* (γ_1 and γ_2) are known (Smith et al., 1971). In Caucasians, β_1 has an allele frequency greater than 0.95 and β_2 of less than 0.05 (Hittle and Crabb, 1988). In Orientals the ratios are reversed, with a frequency of approximately 0.32 and 0.68 for β_1 and β_2, respectively (Ehrig et al., 1990). A third allele β_3 or $\beta_{Indianapolis}$, has a frequency of 0.16 in Afro-Americans (Bosron et al., 1983). The frequency ratios of γ_1 and γ_2 are 0.6/0.4 for Caucasians and 0.9/0.1 for both Orientals and Afro-Americans (Ehrig et al., 1990). A recent study examining ADH genotypes in Chinese men has implicated a possible association between the absence of the β_2 and the γ_1 alleles and alcoholism (Thomasson et al, 1991). The results suggest that individuals having the $\beta_2\beta_2$ homodimer, which metabolizes ethanol 20 times faster than $\beta_1\beta_1$, and $\gamma_1\gamma_1$, which metabolizes twice as fast as $\gamma_2\gamma_2$, produce higher transient levels of acetaldehyde. This

may discourage excessive drinking due to the aversive and unpleasant physical effects of higher levels of acetaldehyde.

1.2.2 Acetaldehyde dehydrogenase

Humans have four distinct classes of ALDH. $ALDH_2$ (E_2) is localized in the mitochondrial subcellular fraction and is found in the liver, kidney, intestine, brain, scalp, fibroblasts, and white blood cells (Goedde and Agarwal, 1990; Helander et al., 1988). $ALDH_2$ metabolizes more than 50% of the acetaldehyde formed from the breakdown of ethanol in the liver (Goedde and Agarwal, 1990). $ALDH_2$ has also been detected in small amounts in the fetal liver (Smith et al. 1985). The gene for $ALDH_2$ (*Aldh-2*) is 44 kb long and has 13 exons (Hsu et al., 1988). *Aldh-2* has been localized to chromosome 12q24 and produces a 2.35 kb mRNA species (Hsu et al., 1986). $ALDH_1$, (E_1) is a cytosolic enzyme found in the liver, kidney, intestine, brain, scalp and red blood cells (Goedde and Agarwal, 1990; Helander et al., 1988). The gene for $ALDH_1$ (*Aldh-1*) has been assigned to human chromosome 9q21. It is approximately 53 kb long and produces a 2.1 kb mRNA species (Hsu et al., 1989). The K_m values of $ALDH_2$ and $ALDH_1$ for acetaldehyde are 3 μM and 32 μM, respectively, both within the range of physiological levels after alcohol consumption (Chambers, 1990; Ryzlak and Pietruszko, 1989). $ALDH_2$ and $ALDH_1$ are tetrameric enzymes with unequal subunits of approximately 54,000 daltons (Harada et al., 1980). The subunits of $ALDH_2$ and $ALDH_1$, however, do not form hybrid molecules. Nucleotide and amino acid sequencing have shown that the mitochondrial and cytosolic forms of human ALDH share a 68% positional identity, with only 27% of the changes being conservative

(Goedde and Agarwal, 1990; Hsu et al., 1988; 1989). Other studies comparing the cytosolic and mitochondrial nucleotide and amino acid sequences in various species have shown that intraclass identity (i.e. identity between human cytosolic and horse cytosolic ALDH) is higher than intraspecies identity (i.e. identity between human mitochondrial and human cytosolic ALDH) (Goedde and Agarwal, 1990; Lindahl and Hempel, 1991). These results suggest that the divergence of the mitochondrial and cytosolic forms of the enzyme may pre-date the ancestral separation of horse and man (Jornvall et al., 1987).

A link between an ALDH2 polymorphism and alcoholism has also been suggested. This association appears much stronger than the correlation between alcoholism and the ADH polymorphism mentioned earlier. One half of all Orientals exhibit a severe reaction to ethanol. Symptoms include facial flushing, nausea, and tachycardia (Agarwal and Goedde, 1987). This response has been associated with a single nucleotide and corresponding amino acid substitution at position 487 (GAA [glu] to AAA [lys])of ALDH2. Individuals with variant ALDH2 (mutant) express a protein which cross reacts with ALDH2 antibodies but is functionally inactive (Yoshida et al., 1984). In heterozygotes the ALDH2 mutant allele is dominant over the normal allele, so that tetramers having at least one mutant subunit are not enzymatically active (Crabb et al., 1989; Thomasson et al., 1991). The severe reaction experienced by these individuals upon ethanol consumption is believed to provide a protective effect against alcoholism. That is, individuals with mutant ALDH2 usually refrain from excessive drinking due to initial discomfort and are therefore less likely to become addicted to alcohol (Harada, 1989). A recent report, however, has indicated that when heterozygotes (ALDH2 normal/ALDH2 mutant) do

drink they have a higher incidence of alcoholic hepatitis and/or cirrhosis (Enomoto et al., 1991). This is presumably caused by the damaging effects of relatively higher acetaldehyde levels in the liver. No $ALDH_2$ mutant homozygotes were reported in the survey. A small percentage (5 to 10%) of Caucasians are also 'alcohol flushers' (Yoshida et al., 1989). These individuals have normal $ALDH_2$ activity but have a decrease in $ALDH_1$ (cytosolic) activity. Again these individuals show normal protein levels, as detected by cross reaction to $ALDH_1$ antibodies. The nature of the $ALDH_1$ mutation and its possible protective effects has not been determined.

The severe and unpleasant reaction caused by high acetaldehyde levels has been exploited as a treatment for alcoholics. Antabuse (disulfiram) is administered as a supportive drug to patients undergoing psychotherapy. Antabuse irreversibly inhibits approximately 90% of all $ALDH_1$ activity and may partially inhibit $ALDH_2$ activity (Goedde et al., 1983; Helander et al., 1988). Abstinence is reinforced by the unpleasant side-effects of high acetaldehyde levels experienced by patients who attempt to consume alcohol.

There are two other classes of ALDH in humans, $ALDH_3$ (E_3) and $ALDH_4$ (E_4). $ALDH_3$ has been isolated from both the stomach and the liver and has been described by two different groups. Kurys et al., (1989) isolated the enzyme from human liver and found that it was precipitated by anti-$ALDH_1$ and anti-$ALDH_2$ antibodies, was not inhibited by Antabuse, and had a K_m for acetaldehyde of approximately 40 µM. They concluded that the enzyme was glutamic γ-semialdehyde dehydrogenase, an ALDH tetramer. Yin et al. (1988) detected a polymorphic enzyme in the human stomach which they also describe as $ALDH_3$. The enzyme has up to five different forms, a K_m value for acetaldehyde of 83 mM, and is a dimer.

The relationship between the enzymes examined by the two groups and the role of ALDH3 in ethanol metabolism has not been demonstrated (Ehrig et al., 1990). ALDH4 has a very high K_m value for acetaldehyde and is probably not functional in acetaldehyde catabolism (Ehrig et al., 1990).

1.3 Animal Models

Animal models are essential for studying the metabolic effects of ethanol, particularly the role of ADH and ALDH. Ethical considerations limit even non-invasive experimentation in humans. Patients undergoing treatment for alcoholism or recovering alcoholics cannot be given ethanol. The drinking patterns of subjects of post-mortem liver ADH and ALDH enzyme activity and mRNA studies are often unknown or are unreported by sympathetic health care workers wishing to 'protect' the patient. Analysis of the response of family history positive subjects to ethanol requires extensive long term (10 to 20 years) follow-up to determine a possible correlation to the development of alcoholism. In addition, under estimation of self-reported drinking by patients is a chronic problem. The complex social environment which interacts with genetically influenced factors to produce alcoholism cannot be duplicated in the laboratory. However, animal models can be effectively used to analyze the biological effects of alcohol and the genetic factors controlling alcohol preference, metabolism, and consumption.

Some animal studies on the effects of alcohol have used sheep (Sanny and Weiner, 1987), horses (Jornvall et al., 1987) or baboons (Ainley et al., 1988), but a majority of researchers work on rodents, particularly rats and mice. A variety of genetically well-defined inbred strains of rats and mice

are commercially available to researchers. Some of these strains have been characterized in ethanol consumption, preference and tolerance (McClearn and Rodgers, 1979, Eriksson, 1973). Liquid diets, which deliver clinically relevant doses of alcohol while maintaining adequate nutrition in the test animals, were developed in the early 1960's in response to the direction of alcohol research (Lieber and DeCarli, 1989). Aversion to ethanol is overcome by this feeding method as no other food or water sources are available to the animal. Thus, the Lieber-DeCarli liquid diet can be used on both alcohol-preferring and alcohol-avoiding animals. Previously, ethanol was orally administered as part of the animal's drinking water. This method, however, was fraught with problems including low levels of consumption and low blood alcohol concentrations (Lieber and DeCarli, 1989).

In mice, ADH-A_2 (a homodimer) is coded for by the gene *Adh-1* and is considered to be structurally analogous to human Class I β_1 ADH. *Adh-1* is expressed in a temporal and tissue-specific manner and is the primary liver enzyme using ethanol as its substrate (Algar et al., 1983, Balak et al., 1982). Sequencing of *Adh-1* has revealed that the gene is 13 kb long and the coding region is 84% identical to the human β_1 coding region. The number and location of introns is identical to its human counterpart, with the intron size and intron/exon junction sequences being well conserved (Ceci et al., 1987; Zhang et al., 1987). The *Adh-1* gene has been localized to chromosome 3 in the mouse and produces an mRNA 1.4 kb in length (Holmes et al., 1986a; Patterson et al., 1987). Glucocorticoid response elements (GREs) were located in the promoter region of both mouse *Adh-1* and human *Adh-2* (β) (Duester et al., 1986; Zhang et al., 1987). GREs may alter the transcription rate of contiguous genes in

response to corticosteroids (Payvar et al., 1983). Both ADH-A_2 enzyme activity and *Adh-1* mRNA levels were increased in the kidney of female A/J mice after treatment with testosterone (Ceci et al., 1986). Expression in the liver, which is constitutively higher than in the kidney, did not show this concomitant induction (Ceci et al., 1986; Carr et al., 1989). A similar study using cultured rat hepatocytes showed that dexamethasone, a corticosteroid, induced the rat class I ADH enzyme activity and corresponding mRNA levels in the cell lines (Hittle and Crabb, 1988).

Mice from different inbred strains express different levels of liver ADH-A_2 enzyme and the corresponding *Adh-1* mRNA (Zhang et al., 1987; Patterson et al., 1987). A restriction fragment length polymorphism (RFLP) associated with the *Adh-1* locus, which correlated to the level of ADH-A_2 expression in the different mouse strains, was detected (Zhang et al., 1987). Sequencing of the first intron in the coding region of the gene revealed that the mouse strains with lower ADH-A_2 expression (BALB/c and DBA/2J) had a 101 base pair (bp) alternating purine-pyrimidine deletion. This deletion is responsible for the *Adh-1* associated RFLP pattern. The authors hypothesized that this region, which closely resembles sequences in the SV40 enhancer region, may act as a transcriptional enhancer element in the high activity mouse strains (C57BL/6J and YBR/Ki) (Zhang et al., 1987).

The murine enzyme ADH-C_2 is comparable to human class II (π) ADH and murine ADH-B_2 to human class III (χ) ADH. ADH-C_2 is expressed in the stomach, male and female reproductive tissues, the kidney, and the lung. It is a dimeric enzyme with broad substrate specificity (Algar et al., 1983). ADH-B_2 has a wide tissue distribution and generally oxidizes medium chain alcohols (Algar et al., 1983). The cDNA of ADH-

B2 was recently sequenced and shown to be 92.8% identical to human χ-ADH at the amino acid level and 87% identical within the coding region at the nucleotide level (Edenberg et al., 1991).

The two major enzymes of acetaldehyde oxidation in the mouse liver, AHD-2 and AHD-5, are homologous to human cytosolic ALDH and to human mitochondrial ALDH, respectively (Manthey and Sladek, 1989). The *Ahd-2* gene has been localized to the centromeric end of chromosome 19 (Timms and Holmes, 1981). It codes for a dimeric enzyme with a subunit weight of approximately 53,000 daltons, similar to the human cytosolic ALDH subunit weight (Harada et al., 1980). Mouse AHD-2 also has similar K_m values for acetaldehyde and NAD and is inhibited by disulfiram (Algar and Holmes, 1986). AHD-2 is not detected in fetal and neonatal tissues. It first appears in the liver at 3 to 6 weeks, at near adult levels (Timms and Holmes, 1981). In the adult, it is expressed predominantly in the liver but is also found in the pancreas, testis, stomach, and lung in low levels (Timms and Holmes, 1981).

Unlike the rat cytosolic ALDH, which shows very low activity in the liver unless induced by phenobarbitol, mouse AHD-2 is constitutively expressed. AHD-2 activity in the mouse liver can be stimulated by phenobarbitol, but to a lesser degree than in the rat (Holmes, 1985; Dunn et al., 1989). The rat phenobarbitol-induced (cytosolic) ALDH cDNA encodes a protein 501 amino acids in length and shows a strong conservation with human cytosolic ALDH (Dunn et al., 1989). Rat cytosolic ALDH is believed to be a tetrameric enzyme with a subunit weight of approximately 54,000 daltons. The enzyme, however, is in very low abundance in the rat liver and is not believed to play an important role in ethanol metabolism for this reason (Smolen et al., 1981). In contrast,

cytosolic forms account for the bulk (70%) of the total ALDH activity in the mouse liver. In addition to AHD-2, betaine aldehyde dehydrogenase (AHD-9), and AHD-7, which has a low affinity for acetaldehyde are also found in the cytosolic fraction (Sladek et al., 1991).

AHD-5, a dimeric enzyme with a high affinity for acetaldehyde is found in the mouse hepatic mitochondria (Algar and Holmes, 1986; Sladek et al., 1991). AHD-5 is expressed in the liver, kidney, lung, and testis (Timms and Holmes, 1981). Unlike AHD-2, AHD-5 is expressed throughout development but with a lower activity in the fetal and neonate stages (Timms and Holmes, 1981). AHD-1, a mitochondrial enzyme with a low affinity for acetaldehyde, is also found in both the liver and the kidney (Algar and Holmes, 1986). Succinic semi-aldehyde dehydrogenase (AHD-12) and AHD-3, a microsomal enzyme have also been detected in the mouse liver (Sladek et al., 1991). The relative contributions of cytosolic and mitochondrial aldehyde dehydrogenases to acetaldehyde oxidation in the ethanol metabolism pathway in the mouse liver has been the subject of considerable debate. AHD-5 is known to have a higher affinity for acetaldehyde than AHD-2, but it is found in much lower levels in the liver (Smolen et al., 1981). However, the K_m for the co-enzyme binding factor NAD of AHD-2 is lower than that of AHD-5 (Algar and Holmes, 1989). It is believed that both enzymes probably function in acetaldehyde metabolism, especially during chronic alcohol consumption (Algar and Holmes, 1986; 1989). In the rat, mitochondrial ALDH is unequivocally primarily responsible for the oxidation of acetaldehyde produced by ethanol consumption (Smolen et al., 1981; Farres et al., 1989). The cDNA for rat mitochondrial ALDH has been sequenced and was shown to have an open reading frame of 1557 bp, or 519 amino acid residues. The mature

subunit shares a 96% identity to the amino acid sequence of human mitochondrial ALDH (Farres et al., 1989).

1.4 Acetaldehyde Dehydrogenase and Sensitivity to Ethanol

Researchers have examined differences in ethanol metabolism between individuals and its possible effects on alcohol sensitivity and predisposition to alcoholism. A number of studies have indicated that ethanol alters the activity of the enzymes which metabolize it. In 1984, Jenkins et al. found that cytosolic ALDH ($ALDH_1$) activity was significantly reduced in the livers of alcoholic patients. Mitochondrial ALDH ($ALDH_2$) activity did not show this same reduction. When alcohol intake was eliminated (6 month period of abstinence), the activity returned to normal values. Thus alcohol consumption by the patients was depressing $ALDH_1$ activity. Other researchers also found a significant decrease in $ALDH_1$ activity in the livers of alcoholics (Thomas et al., 1982). When the patients were examined after 4-34 weeks of abstinence, however, the activity levels remained low. ADH activity, which was also decreased in the primary analysis, returned to normal levels, indicating that the reductions were not due to irreversible liver damage. The researchers concluded that reduced cytosolic ALDH activity and subsequent altered acetaldehyde metabolism may be a primary defect of alcoholism (Thomas et al., 1982).

Several studies have also examined the effect of ethanol on ADH and ALDH activity in mice. Wang and Singh (1985) reported genotype-specific induction and repression of ADH activity in inbred strains of mice given 15% (v/v) ethanol in their drinking water over a 6 week period.

Only BALB/c mice, an alcohol avoiding strain, had consistently elevated levels of ADH activity over the time course of the experiment. The effect of ethanol on the alcohol metabolizing enzymes appears to be dependent on the feeding method as different results were obtained by Wei and Singh (1988). In this study, the alcohol avoiding (BALB/c and 129/ReJ) and the alcohol preferring (C57BL/6J) strains fed the Lieber DeCarli liquid diet had a decrease or no change in ADH enzyme activity in the liver, respectively, as compared to control-fed animals (Wei and Singh, 1988). Liver cytosolic ALDH enzyme activity, on the other hand, was found to be increased in C57BL/6J mice but was decreased or had no apparent change in BALB/c and 129/ReJ mice (Wei and Singh, 1988).

1.5 Objectives

Acetaldehyde is the toxic metabolite in the ethanol metabolism pathway. It is responsible for most, if not all, of the adverse physiological effects associated with alcohol consumption (Lieber, 1988). Differences in acetaldehyde production and clearance may contribute to an individual's relative sensitivity to ethanol. Increased cytosolic ALDH enzyme activity has been reported in C57BL/6J mice, an alcohol resistant and preferring strain, after ethanol feeding and has been postulated to be responsible for its relative resistance to ethanol (Singh, 1986; Wei and Singh, 1988). It was the aim of this project to characterize the genes involved in alcohol metabolism in the mouse, particularly *Ahd-2*, at the molecular level. Changes in the steady-state levels of *Adh-1* and *Ahd-2* mRNA in a number of alcohol preferring (C57BL/6J; Rodgers, 1966) and alcohol avoiding (BALB/c and 129/ReJ; Rodgers, 1966) mouse strains were examined to

assess possible changes in the availability of mature transcripts after ethanol feeding. Differences in *Ahd-2* at the genomic DNA level among mouse strains with different ethanol preferences were evaluated using restriction fragment length polymorphisms (RFLPs). The cDNA of *Ahd-2* from a BALB/c cDNA library was sequenced and compared with the cDNAs of enzymes with reportedly similar functions from different species (Hsu et al., 1989; Dunn et al., 1989). Several regions of *Ahd-2* were examined in detail in three different strains of mice to detect possible genotype differences which could affect transcription of the gene or its activity. The promoter region of *Ahd-2* was sequenced from BALB/c, C57BL/6J and 129/ReJ genomic DNA to detect possible strain-specific sequence differences in transcriptional control. The sequence of the region surrounding *Ahd-2*'s putative Antabuse binding site was also examined for strain specific changes. The structure of the *Ahd-2* gene in BALB/c was partially characterized. In addition, a novel PCR product coding for a partial cDNA of a transcript related to *Ahd-2* was dete‹ .ed. The results of these experiments are discussed in detail with respect to possible relationships to ethanol preference and sensitivity.

Chapter 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Animals

Six different inbred strains of mice (*Mus musculus*) were used in this study: BALB/c, C57BL/6J, C3H/HeSnJ, C3H/HeAnl/*Cas-1*^{b} (Cs^{b}), 129/ReJ and Swiss Webster (SW). BALB/c and SW breeding stocks were obtained from Canadian Breeding Farms, St. Constant, Charles River, Quebec. Cs^{b} mice were provided by Dr. T.W. Clarkson, the University of Rochester, New York. The other strains were obtained from the Jackson Laboratory, Bar Harbor, Maine.

The mice were brother and sister mated and housed in a 14 hour light: 10 hour dark photoperiod, thermostatically controlled (23°C) room at the animal quarters of the Department of Zoology, the University of Western Ontario. Commercial mouse chow (Purina Canada Inc.) and water were available *ad libitum*.

2.1.2 Chemicals

Unless otherwise noted, molecular biology chemicals were obtained from BDH Chemicals Canada Ltd. (Toronto, Ontario), BRL Gaithersburg,

Maryland), Difco (Detroit, Michigan), Fisher Scientific Co. (Philipsburg, New Jersey), Gelman Sciences (Ann Arbor, Michigan) or Sigma Chemical Co. (St. Louis, Missouri).

Deionized water was made RNase-free by treating with 0.1% diethyl pyrocarbonate (DEPC) overnight and autoclaving. Phenol for RNA extractions was melted at 65 °C and saturated with DEPC-treated deionized H_2O. Salt-saturated phenol for DNA extractions was prepared by melting 200 g of phenol and adding 40 ml 2 M Tris, pH 7.4 and 52 ml double distilled H_2O. The aqueous phase was removed and 40 ml 2 M Tris pH 7.4, 10 ml m-cresol, 0.4 ml β-mercaptoethanol and 0.1% 8-hydroxyquiniline were added. Both were stored in dark bottles at 4 °C.

2.1.3 DNA

Plasmid DNA was isolated by alkaline lysis as described in Davis et al., (1986) with the following exceptions. Mini-preps of plasmid DNA were isolated from 3 mls of overnight cultures and no lysozyme was used to lyse the bacteria. Plasmid DNA isolated for sequencing reactions was extracted once with phenol saturated with 50 mM sodium acetate (NaOAc) pH 4.0, then once with chloroform:isoamyl alcohol (24:1) (Weickert and Chambliss, 1991). Bluescript vector DNA and the XL-1 Blue host bacteria were obtained from Stratagene (La Jolla, California).

DNA samples used for RFLP analysis from C57BL/6J*, C57BL/10J, C57BL/KsJ, C3H/HeJ and DBA/2J inbred strains of mice were obtained from the Jackson Laboratory, Mouse DNA Resource, Bar Harbor Maine.

Primers synthesized for library screenings, the Polymerase Chain Reaction and DNA sequencing are given in Table 1. The primers were

Table 1. A summary of the oligonucleotide primers and their sequences, listed 5' to 3', used in cDNA and gDNA library screenings, the Polymerase Chain Reaction experiments, and DNA sequencing reactions. All primers were synthesized by Dr. G. Hammond, London Regional Cancer Centre, London, Ontario.

Primer	Sequence (given 5' to 3')
P_1	GCAATCTGAAGAGAGTC
P_2	GCTGCACAAAGAAGCCT
P_3	AAGGAGTGTTGAGCGAG
P_4	CTTCAAGTCGGCCAGTG
P_8	GTTAGGCAGATTTGTGGTTCTCTG

manufactured by Dr. G. Hammond, London Regional Cancer Centre, London, Ontario. The M13 universal sequencing primer and the T3 Bluescript sequencing primer were obtained from Pharmacia (Upsala, Sweden) and Stratagene (La Jolla, California), respectively.

2.2 METHODS

2.2.1 Ethanol Administration

Twenty-one day old male mice from the alcohol preferring/resistant strain (C57BL/6J) and the alcohol avoiding/sensitive strains (BALB/c and 129/ReJ) were weaned and maintained on mouse chow diet until they were approximately 6 to 10 weeks old. Weight-matched litter-mates were then placed in individual cages and given an ethanol or isocaloric (maltose-dextran) control liquid diet (Lieber and DeCarli, 1982; BioServe Inc. Frenchtown, New Jersey, U.S.A.). The ethanol dose for the experimental animals was increased gradually. On days 1 to 3, the animals were given 1% ethanol. This was increased to 3% on day 4 and to 5% for days 8-14. The mice were weighed daily and their approximate diet consumption from the preceeding 24 hours recorded. Mean daily weight gain/loss ± S.E.M. and average daily consumption ± S.E.M. were calculated using the Macintosh software program StatWorks (version 1.1) for each strain on days 1, 3, 5, 7, 9, 11 and 13 for all mice on the diet at that point. Statistical differences (Student's t-tests) between the treatment groups and between the strains were cal ulated using StatWorks. On day 15, surviving mice were sacrificed by cervical dislocation and the liver removed for RNA isolation. Blood alcohol concentrations were determined on day 15.

Blood was collected from a cardiac puncture, after cervical dislocation, in a heparinized capillary tube. Analysis was performed using the Sigma Diagnostics Blood Alcohol Concentration Kit, procedure 332-UV (Sigma, St. Louis, Missouri) and a Beckman DU-8 spectrophotometer.

2.2.2 RNA Analysis

2.2.2.1 RNA Isolation and Gel Electrophoresis

Total cellular RNA was isolated from the liver by acid guanidium thiocyanate-phenol-chloroform extraction (Chomcyznski and Sacchi, 1987). The tissue was washed in DEPC-treated H_2O, weighed and homogenized in a 1:10 (w/v) solution of denaturing solution (4 M guanidium thiocyanate; 25 mM sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$, pH 7); 0.5% sarcosyl; 0.1 M 2-mercaptoethanol). The RNA was extracted by adding 1/10th volume of 2 M NaOAc (pH 4.0), 1 volume of water-saturated phenol, 1/5th volume of chloroform-isoamyl alcohol (49:1) and centrifuging at 10,000 x g for 20 min. at 4 °C in a DuPont HB-4 rotor. The aqueous layer was then transferred to another tube and the RNA precipitated with isopropanol overnight. The RNA was then pelleted at 10,000 x g for 20 min. at 4 °C (DuPont HB-4 rotor) and dissolved in 1 ml of the above denaturing solution, precipitated with an equal volume of isopropanol, microfuged at 4 °C for 10 min. and the pellet was vacuum dried. The resulting RNA was dissolved in 500-1000 µl of water at 65°C for 10 min. and stored at -70°C. The RNA concentration was obtained by UV spectrophotometry using a Beckman DU-8 spectrophotometer.

A 1.4% agarose gel was made up with 20 mM MOPS, 5 mM NaOAc, 1 mM EDTA (Ethylenediamintetra-acetic acid disodium salt, pH 7.0) and 1.85% formaldehyde (0.66 M). Thirty μg of total cellular liver RNA was lyophilized and resuspended in 5 μl of DEPC-treated H_2O. Electrophoresis sample buffer (25 μl of 52.8% deionized formamide, 0.02 M MOPS [3-(n-morpholino) propanesulfonic acid], 5 mM NaOAc, 1 mM EDTA, (pH 7.0), 16.9% formaldehyde, 7% deionized RNase-free H_2O, 7% glycerol, 0.56% (w/v) bromophenol blue) was added and the samples denatured at 65 °C for 15 min. RNase-free ethidium bromide (EtBr, 1 μg) was then added, and the samples loaded on the gel (15 cm X 20 cm) and electrophoresed in 0.02 M MOPS, 5 mM NaOAc, 10 mM EDTA (pH 7.0) at 25 V for 18 hours. After electrophoresis, the gel was photographed using a 302 nm Spectroline transilluminator (model TR-302) with a Kodak Wratten #9 filter and Polaroid type 665 PN film (Fourney et al., 1988).

2.2.2.2 Northern Transfer

The gels were prepared for transfer by soaking in 0.05 M NaOH, 1 X Standard saline citrate (SSC; 0.15 M NaCl: 0.015 M $Na_3C_6H_5O_7 \cdot 2H_2O$, pH 7.2) for 15 min. followed by two ten minute soaks in 10 X SSC at room temperature. The gels were placed upside-down on a sheet of Whatman 3MM paper covering a polyurethane sponge. BioTrace (Gelman Sciences, Ann Arbor, Michigan) nylon membrane, cut the same size as the gel was wetted with distilled water, soaked in 10 X SSC for 5 min., and placed on top of the gel. Parafilm was placed around the edges of the BioTrace to prevent 'short-circuiting' of the transfer. Three pieces of 3MM paper, 10 cm of paper towels and a 200 g weight were placed on top of this. The

transfer, using 10 X SSC as the transfer buffer, was allowed to proceed for 6 hours, with frequent changes of the paper towelling during this time. The apparatus was then disassembled, the membrane removed and baked at 80 °C for 2 hours in a vacuum oven.

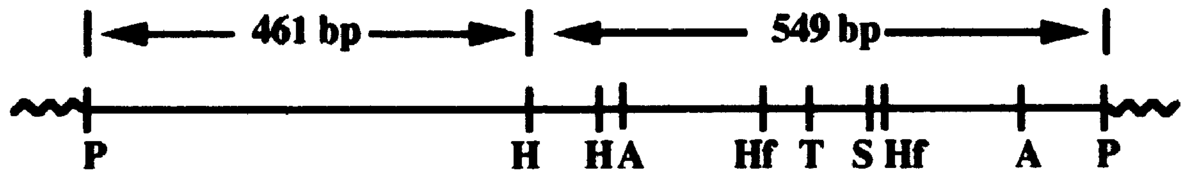
2.2.2.3 RNA Dot Blotting

Liver RNA (12 µg) was lyophilized and resuspended in 60 µl of 0.25 mM EDTA/0.1% SDS. An equal volume of 3:2 20 X SSC (3 M NaCl, 0.3 M $Na_3C_6H_5O_7 \cdot 2H_20$, pH 7.2):37% formaldehyde was added and the samples denatured at 65 °C for 10 min. After cooling on ice, the samples were diluted in 10 X SSC to 20 µg/ml, 2 µg/ml, 0.2 µg/ml and 0.02 µg/ml. Five hundred µl of each of these dilutions was applied to the BioTrace membrane previously equilibrated with 10 X SSC in a dot blot apparatus (BioRad, Richmond, California). The samples were vacuumed through the wells, washed with 200 µl of 10 X SSC and the membrane allowed to air dry before baking at 80 °C for 2 hours.

2.2.2.4 Probe labelling and Hybridization

The cDNA for the mouse *Adh-1* gene ($pADH_{m16}$; 1.0 kilobase long, including approximately 150 bp of the 3' untranslated region in pBR322; Ceci et al., 1987) was obtained from Dr. Michael Felder, University of South Carolina (Figure 1). The $pADH_{m16}$ insert in the vector pBR322 (200 ng) was nick translated according to the manufacturer's directions (Gibco BRL) using $\alpha^{32}P$ dCTP (3000 Ci/mmol; ICN Biochemicals). A partial cDNA clone for the mouse *Ahd-2* gene was obtained from Dr.

Figure 1. Restriction map of pADH$_{m}$16, a 1.0 kb long partial 3' cDNA for the mouse *Adh-1* gene including approximately 150 bp of the 3' untranslated region in pBR322 is shown. Obtained from Dr. Michael Felder, University of South Carolina. Wavy lines represent the pBR 322 vector; P, *Pst* I; H, *Hin*d III; A, *Alu* I; Hf, *Hinf* I; T, *Taq* I; S, *Sau* 3A.


461 bp
549 bp
P
H
H
A
Hf
T
S
Hf
A
P

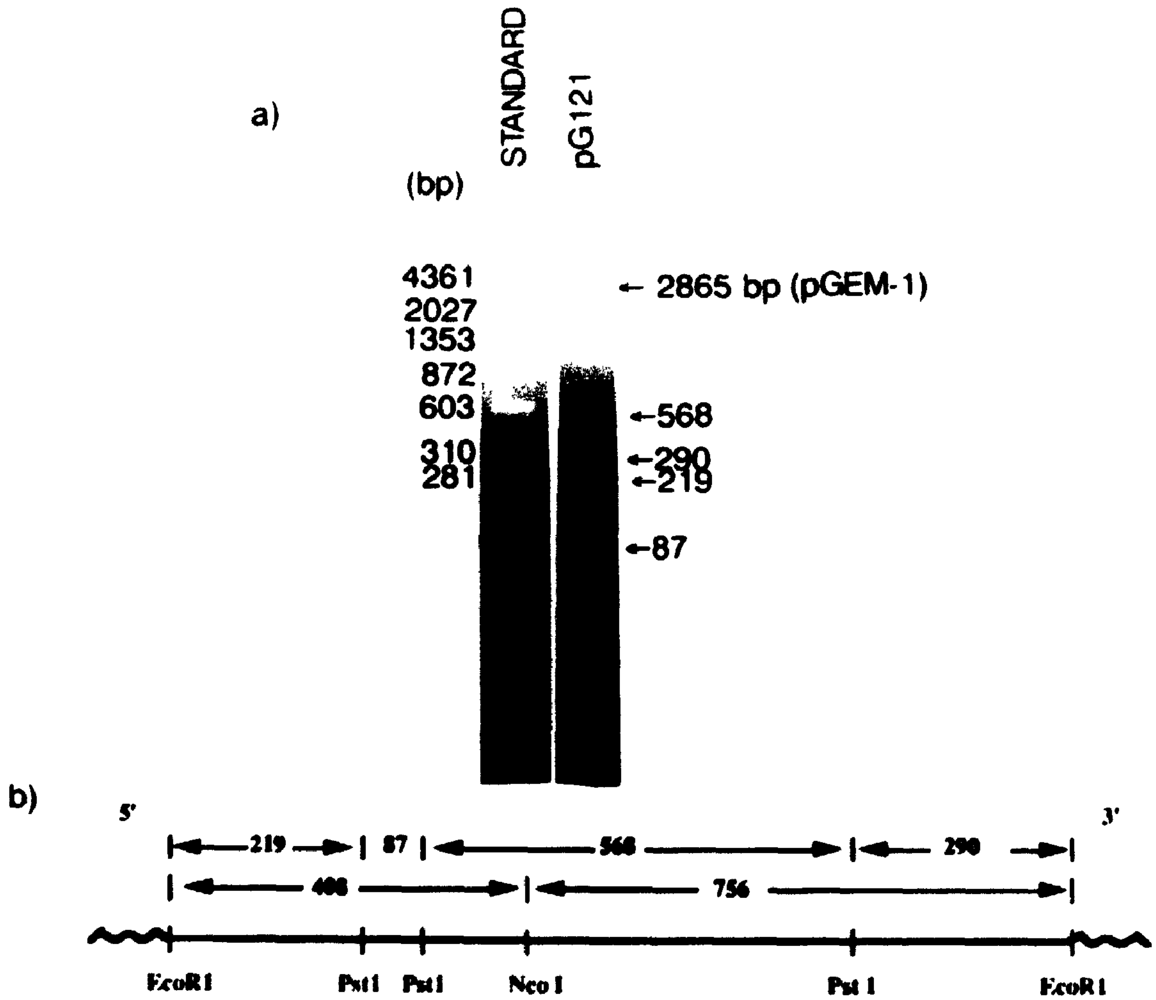
Steven Weaver of the University of Illinois at Chicago (pG121; 1164 bp containing 129 bp of the 3' untranslated region and 1035 bp coding for 345 amino acids; Rongnoparut and Weaver, 1988). The internal 568 bp *Pst* I fragment (Figure 2) of pG121 was isolated by digesting the recombinant plasmid with *Eco* RI and *Pst* I, running the sample on a 0.8% low melting point gel in 1 X TAE (40 mM Tris, 20 mM NaOAc, 1 mM EDTA pH 7.2) and excising the desired band. The agarose containing the gel fragment was then melted at 70°C for 10 min. and diluted with three volumes of sterile water. This solution was used directly in random prime labelling according to the manufacturer's directions using Gibco BRL's Random Primers Labelling System and $\alpha^{32}P$ dCTP (3000 Ci/mmol; ICN Biochemicals).

Membranes for both the Northern transfers and RNA dot blots were prehybridized according to manufacturer's directions in approximately 30 mls of 4.7 X SSPE (0.7 M NaCl, 50 mM $NaH_2PO_4 \cdot H_2O$, 5 mM EDTA), 4.7 X Denhardt's (0.5% polyvinylpyrrolidone, 0.5% bovine serum albumin, 0.5% Ficoll; Denhardt, 1966), 0.1% sodium dodecyl sulphate (SDS), 10% dextran sulphate, 0.34% Blotto (skim milk powder), 47% formamide and 188 µg/ml denatured salmon sperm DNA for 1-2 hours at 42 °C in a Hybrid-Ease chamber (Mandel Scientific). The radiolabelled cDNA probe was then denatured by boiling for 10 min. and added to this solution. The hybridization continued for 24-36 hours at 42 °C.

The hybridization solution was removed and the membranes washed once in 1 X SSC, 0.1% SDS (room temperature) for 15 min. The membranes were then removed from the Hybrid-Ease chamber and washed twice with 1 X SSC, 0.1% SDS at 52 °C for 15 min. in a pyrex dish, with gentle agitation. The membranes were then blotted dry, covered with

Figure 2. (a) A restriction digest of pG121 in pGEM-1 (Dr. Steven Weaver, The University of Illinois at Chicago), cut with *Pst* I and *Eco* RI and run on a 0.8% agarose gel is shown. Sizes were calculated using λ DNA-*Hin*d III/ØX-174 RF DNA- *Hae* III (Pharmacia, Upsala, Sweden) molecular weight markers as a standard.

(b) A corresponding restriction map of the cDNA probe pG121, (based on Figure 2a). The *Nco* I site in the insert which yields 408 and 756 bp fragments is also shown. The pG121 insert was carried in a pGEM-1 vector (Promega, Ottawa, Canada), represented by the wavy lines .


a)
(bp)
STANDARD
pG121
4361
2027
1353
872
603
310
281
← 2865 bp (pGEM-1)
←568
←290
←219
←87
b)
5'
3'
219
87
568
290
408
756
EcoR I
Pst I
Pst I
Nco I
Pst I
EcoR I

Saran wrap and used to expose a Kodak X-OMAT AR film with a Quanta Detail-T intensifier screen at -70 °C. The films were developed in a Picker Corporation automatic processor (model Data IV) and analysed with a Beckman LKB laser densitometer (UltraScan XL). Data for mRNA levels in ethanol-fed mice are presented in relation to values for genotype-matched control mice. Membranes were stripped prior to re-hybridization by soaking in boiling 0.1 X SSC, 0.1% SDS and allowing the solution to cool to room temperature. The efficiency of the stripping procedure was checked by autoradiography (manufacturer's instructions).

2.2.3 Southern Blot Analysis

2.2.3.1 Isolation of Genomic DNA

High molecular weight genomic DNA was isolated from the livers or brains of adult male mice from six different inbred strains; Swiss Webster, BALB/c, C57BL/6J, 129/ReJ, C3H/HeSnJ and Cs^b by the method of Canaani and Aaronson (1979). The tissue was minced on ice and gently homogenized in 10 volumes of extraction buffer (150 mM NaCl, 70 mM EDTA, pH 8.0). Pronase E (Boehringer Mannheim, Laval, Quebec) was added to the samples to a final concentration of 1 mg/ml, gently mixed, and SDS (20%) added to a final concentration of 1%. The samples were then placed in a 37°C waterbath to incubate overnight with gentle agitation. The next day the samples were extracted with 1 volume of salt-saturated phenol and 1 volume of chloroform:isoamyl alcohol (24:1) and centrifuged in an HB-4 rotor of an RC5C Sorval centrifuge at 659 x g for 20 min. The aqueous phase was transferred to a new tube and the extraction was

repeated with phenol and chloroform:isoamyl alcohol and then once with an equal volume of chloroform:isoamyl alcohol. The aqueous phase was then transferred to dialysis tubing prepared for DNA as described by Maniatis et al. (1982). The samples were dialyzed at 4 °C with gentle spinning against 1 M NaCl , repeated twice at 24 hour intervals; then in dialysis buffer (10 mM Tris pH 7.5, 1 mM EDTA) replaced twice at 48 hour intervals; followed by 10 mM Tris pH 7.5, 0.1 mM EDTA, replaced twice at 24 hour intervals. The DNA concentration was obtained by UV spectrophotometry using a Beckman DU-8 spectrophotometer.

2.2.3.2 Restriction Endonuclease Digestion and Gel Electrophoresis

Preliminary evaluation and screening for RFLPs was performed on DNA isolated from a limited number of strains (C57BL/6J, BALB/c and 129/ReJ) using 8 restriction enzymes (*Eco* RI, *Hae* III, *Hin*d III, *Msp* I, *Pst* I, *Rsa* I, *Taq* I, and *Xho* I; Pharmacia, Uppsala, Sweden) and 0.8% 15 cm X 20 cm gels electrophoresed for 16 hours. The analysis was repeated using DNA samples from additional strains. Genomic DNA (5µg) was digested overnight at 37°C with the appropriate One-Phor-All Plus (100 mM Tris-acetate, pH 7.5, 100 mM magnesium acetate, 500 mM potassium acetate) buffer concentration and one of the four previously determined polymorphic restriction enzymes (*Eco* RI, *Hin*d III, *Pst* I or *Rsa* I). The restriction enzyme digestions were stopped by adding one fifth volume of gel loading buffer (50 mM EDTA, pH 8.0, 0.125% bromophenol blue, 12.5% Ficoll) and the samples loaded onto a 0.8% agarose gel (8.5 cm X 8.5 cm) made up with 1 X TAE buffer and electrophoresed at 35 V

(approximately 35 mAmps) for 4 hours. The gels were stained by immersion in 2 μg/ml ethidium bromide in water for 20 min. and photographed as described in section 2.2.2.1.

2.2.3.3 Southern Transfer

The DNA was denatured by immersing the gel in 2.4 M NaCl, 2 M NaOH for 1 hour then neutralized in 0.6 M NaCl, 1 M Tris, pH 7.4 twice 30 min. each at room temperature with gentle agitation. The gel was set up for capillary transfer overnight using 20 X SSC and Zetaprobe nylon membrane (BioRad, Richmond California). The next morning the membrane was air-dried for 20 to 30 min. at room temperature, rinsed with 2 X SSC and the DNA fixed to the membrane by UV irradiation (302 nm) for 15 min. or cross-linking using a Stratagene Stratalinker (method modified from Sambrook et al., 1989).

2.2.3.4 Hybridization

The Zetaprobe (BioRad) membranes were pre-hybridized in heat-sealable bags following BioRad's instructions in 1 mM EDTA, 0.5 M $Na_2HPO_4 \cdot 7H_2O$, pH 7.2, 7% SDS for 30 min. at 65 °C. The solution was then removed and replaced with fresh pre-hybridization solution. Carrier DNA (5 mg herring sperm DNA; Boehringer Mannheim) was boiled with the radiolabelled 568 bp fragment of pG121 (section 2.2.2.4) for 10 min., allowed to cool on ice (10 min.) and added to the bag containing the membrane and the pre-hybridization solution. The blots were hybridized for 18 hours at 65 °C with gentle agitation.

The next morning the blots were washed in 1 mM EDTA, 40 mM $Na_2HPO_4 \cdot 7H_2O$, pH 7.2, 5% SDS at 65 °C for 30 min. A second wash in 1 mM EDTA, 40 mM $Na_2HPO_4 \cdot 7H_2O$, pH 7.2, 1% SDS at 65 °C for 30 min. was carried out, according to the manufacturer's recommendations. The blots were wrapped in Saran wrap and exposed to X-OMAT RP film (Eastman Kodak Company, Rochester, New York) at -70 °C, using a Quanta Detail-T intensifier screen. Films were developed using a Picker Corporation processor (model Data IV).

2.2.4 Bacteriophage Libraries

2.2.4.1 Plating and Transferring of Bacteriophage Libraries

Two different libraries were used in the following experiments. An adult male BALB/c mouse liver cDNA library in the bacteriophage λ gt 11 with an *E. coli* Y1090 host and an adult BALB/c mouse liver genomic DNA (gDNA) library in the bacteriophage EMBL-3 SP6/T7 with an *E. coli* LE 392 host were both obtained from Clonetech Laboratories, Palo Alto, California. All techniques for growing, transferring and hybridizing bacteriophage libraries are from *Current Protocols in Molecular Biology* (Ausubel et al., 1987). Large (150 mm) sterile polystyrene plates were used for primary screenings for both libraries. Host bacteria were grown overnight in LB media (1% tryptone, 0.5% yeast extract, 0.5% NaCl pH 7.0) with 0.2% maltose and 10 mM $MgSO_4$. The next morning 500 µl of the host bacteria were mixed with titred phage from the cDNA or gDNA library, incubated at room temperature for 20 min then 37°C for 10 min and plated on 150 mm plates (LB, containing 15 % agar) with 3 mls of

0.7% top agarose (1% tryptone, 0.8% NaCl, 0.7% agarose) to give approximately 25,000 plaques/plate. The cDNA plates were incubated at 42 °C for 4 to 8 hours until sufficient lysis had occurred. Y1090 are ampicillin resistant and all media used with this host contained 50 μg/ml ampicillin. The plates inoculated with the gDNA library in the LE 392 host were incubated for 3 to 8 hours at 37 °C until sufficient lysis had occurred.

After lysis the plates were refrigerated at 4 °C overnight. Nitrocellulose filters labelled with ballpoint ink were placed on the agarose surface for 2 min. to allow transfer of the phage particles. Orientation of the filters on the plates was marked with a 20-G needle and India ink before the filters were removed. After air drying for 15 min. the phage DNA was denatured by saturating the filters with 0.2 M NaOH/1.5 M NaCl for 1.5 min. The filters were neutralized by soaking in 0.4 M Tris-Cl (pH 7.6)/2 X SSC followed by 2 X SSC for 1.5 min. each. The filters were then baked in a vacuum oven for 90 min. at 80 °C .

2.2.4.2 Hybridization

The nitrocellulose filters were prehybridized in heat-sealable bags in 48% formamide, 4.8 X SSC, 20 mM Tris-Cl pH 7.6, 1 X Denhardt's solution, 10% dextran sulphate and 0.1% SDS at 42°C for 1 hour. The cDNA probes, representing different regions of the mouse *Ahd-2* gene, were random prime labelled as previously described, boiled for 10 min. with 1 ml of 2 mg/ml sonicated herring sperm DNA, and quenched on ice for 10 min. before being added to the filters. The hybridization was allowed to continue at 42 °C overnight.

The next morning the filters were removed from the bags, and washed three times in 2 X SSC/0.1% SDS, 15 min. each at room temperature. Then the filters were washed in a high stringency wash buffer 0.2 X SSC/0.1% SDS at 65°C (cDNA library) or 55°C (gDNA library) for 20 min. The filters were then wrapped in Saran wrap and exposed to X-OMAT RP film (Eastman Kodak Company, Rochester, New York) overnight at -70 °C, using a Quanta Detail-T intensifier screen. The films were developed using Kodak's GBX developer and fixer.

2.2.4.3 *Purification of Bacteriophage Clones*

Positively hybridizing plaques on the primary plates of the cDNA and gDNA libraries were identified by aligning orientation marks on the filters, film and primary plates. The plaques were isolated by inserting sterile toothpicks first into the area on the primary plate, and then onto a grid-marked secondary plate. Secondary plates were made by plating 200 µl of host bacteria (Y1090 for cDNA clones and LE 392 for gDNA clones) in 0.7% top agarose onto an 82 mm petri dish with 25 mls of LB agar. Approximately 30 stabs were picked for each positive primary clone. The plates were grown overnight at the appropriate temperature (42°C for cDNA plates and 37°C for gDNA plates). The phage were then transferred to nitrocellulose filters and hybridized as previously described. Positive plaques from the secondary screen were further purified by inserting a sterile toothpick into the positive plaque and placing it in 500 µl of SM (0.58% NaCl, 0.2% $MgSO_4.7H_2O$, 50 mM Tris-Cl pH 7.5, 0.1% gelatin) for 5 min. Serial dilutions of this stock were then plated. Plates with 10 to 50 well isolated plaques were used for transfer and hybridization, as above.

Single plaques, which gave a strong signal on this hybridization, were then used to make pure phage stocks. A sterile toothpick was inserted into the plaque and placed in SM for 5 min. Approximately 300 µl of host bacteria (Y1090 for cDNA clones and LE 392 for gDNA clones) grown overnight was incubated with the 500 µl pure phage stock at room temperature for 20 min., 37°C for 10 min. and then plated onto 82 mm LB agar plates with 0.7% top agarose. The plates were grown at the appropriate temperature (42 °C for cDNA plates, 37°C for gDNA plates) until confluent lysis was achieved. The agar was then covered with 3 mls of SM buffer containing 100 µl chloroform and incubated overnight at 4°C. The next morning the SM buffer was harvested from the plates and stored in a sterile glass tube at 4°C over chloroform. This stock was then titred (Ausubel et al., 1987) and used to make high titre lysates.

2.2.4.4 DNA Isolation from cDNA and gDNA clones

After obtaining pure phage stocks, high titre lysates were made to extract the phage DNA. Eighty-two mm petri dishes with LB agarose (UltraPure BRL) substituted for LB agar were used. Host bacteria (Y1090 for cDNA plates and LE 392 for gDNA plates) were inoculated with 50,000 plaques from the pure phage stocks, incubated for 20 min. at room temperature, 10 min. at 37°C and plated with 0.7% top agarose. The plates were allowed to grow to confluency at 42 °C (cDNA clones) or 37 °C (gDNA clones). Once confluency was reached, the plates were covered with 3 mls of SM buffer containing 100 µl of chloroform and incubated at 4 °C overnight. The next morning the lysate was harvested and an additional 2 mls of SM buffer was added to the plates. After storing in a

tilted position for 15 min. at room temperature this lysate was added to the previous harvest and spun at 16,488 x g for 10 min. The resulting high titre lysate was decanted off and stored at 4 °C over chloroform in a glass tube.

DNA was isolated from 10 mls of high titre lysate from the pure cDNA clones as follows. RNase A and DNase I (Boehringer Mannhein, Laval, Quebec) were added to the lysate at a final concentration of 1 µg/ml and the mixture allowed to incubate at 37 °C for 30 min. An equal volume of 20% polyethylene glycol (PEG, Sigma, St. Louis, Mo.), 2 M NaCl in SM was added and the tubes placed in ice water for 1 hour. The phage was then pelleted at 10,000 x g for 20 min. at 4°C, the supernatant completely removed, and the pellet resuspended in 500 µl of SM. The resuspended phage were then transferred to a microfuge tube and spun at 8,000 x g for 2 min at 4°C to remove any bacterial debris. The supernatant was then transferred to a new tube and treated with 5 µl of 10% SDS and 0.5 M EDTA pH 8.0 for 15 min. at 68°C. The phage DNA was extracted once with an equal volume of salt-saturated phenol and spun for 5 min. in an Eppendorf microfuge, then once with phenol/chloroform:isoamyl alcohol (24:1) and then once with chloroform:isoamyl alcohol (24:1), spinning for 2 min.. An equal volume of isopropanol was added to the supernatant and the phage DNA precipitated at -70°C for 20 min.. The DNA was pelleted at 12,000 x g for 15 min. at 4°C, washed with 100 µl of 70% ethanol, allowed to air dry and then resuspended in 50 µl of TE (10 mM Tris, 0.1 mM EDTA, pH 8.0) (Sambrook et al., 1989).

To extract DNA from the purified gDNA clones, 30 mls of high titre lysate was equilibrated to room temperature and 30 mls of TM buffer (50 mM Tris pH 7.4, 10 mM MgSO4) with 0.96 mg of DNase (Boehringer

Mannheim, Laval, Quebec) were added. This mixture was incubated at room temperature for 15 min. Then 1/10 volume of 5M NaCl and 3.3 g of PEG was added and gently mixed to dissolve completely. After incubating on ice for 15 min. the phage were precipitated by centrifuging at 12,000 x g for 10 min. at 4 °C. The supernatant was removed, the phage pellet dried and re-suspended in 0.9 mls of TM. The phage were extracted with an equal volume of chloroform twice, spinning for 5 min. in an Eppendorf microfuge. The aqueous phase was then transferred to a new tube, extracted once with an equal volume of Tris-saturated phenol with 10 mM EDTA (pH 8.0), and 200 mM NaCl and then once again with chloroform. The DNA from the phage was then precipitated with 2.5 volumes of ethanol in ice water for 10 min., spun down at 12,000 x g for 10 min. and washed with 100 µl of 70 % ethanol (Davis et al., 1986). The phage DNA was air dried and resuspended in TE (pH 8.0).

2.2.4.5 Restriction Analysis of the cDNA and gDNA Clones

Insert size from the cDNA clones was determined by digesting the phage DNA with *Eco RI* (Pharmacia, Upsala, Sweden) to remove the insert intact. The DNA fragments were then separated on a 0.8% agarose mini-gel. The clone with the longest insert, (SB-2), was digested with the restricition enzymes *Eco RI* and *Pst I* and compared to pG121 to determine 5' to 3' orientation. The most 5' fragment of this cDNA clone was then used to re-screen the gDNA library.

Recombinant clones from the gDNA library were partially restriction mapped using single and double digests of a limited number of restriction enzymes. Approximately 2 µg of phage DNA from the purified

gDNA clones were digested for 2 hours at 37°C with the appropriate buffer concentration and *Xho* I, *Eco* RI, *Bam* HI or *Suc* I (Pharmacia, Upsala, Sweden). The restriction enzyme digest was stopped by adding one-fifth volume of gel loading buffer (50 mM EDTA, pH 8.0, 0.125% bromophenol blue, 12.5% Ficoll) and the samples loaded onto a 0.5% agarose gel (15 cm X 20 cm) made up with 1 X TAE and electrophoresed at 30 V for 18 hours. λ DNA-*Hin*d III/ØX 174 RF DNA-*Hae* III molecular weight markers were also electrophoresed to determine band size. After running, the gels were stained by immersion in 2 µg/ml EtBr in H_2O for 20 min. and photographed as previously described. Insert size of the gDNA clones was determined by digesting the DNA with *Xho I*, which releases the insert from the EMBL3 SP6/T7 bacteriophage vector. Southern transfers and hybridization of the DNA were performed as in sections 2.2.3.3 and 2.2.3.4. The recombinant clones from the genomic library were hybridized with a number of cDNA probes including the entire pG121 insert (a 1164 bp *Eco* RI fragment), a 408 bp *Eco* RI/*Nco* I fragment and a 756 bp *Nco* I/*Eco* RI fragment, all from pG121 (see Figure 2) to aid in mapping.

2.2.4.6 Screening for the Promoter Region of Ahd-2

The gDNA library was then re-screened, as described above, with the most 5' fragment of the SB-2 cDNA clone used as a probe. From this screening one positive clone 'SBg-5b' was isolated. This clone and the clones from the initial gDNA library screenings (section 2.2.4.2) were then screened with P4, an oligonucleotide 17 bases in length corresponding to the 5' end of the *Ahd-2* gene (see Table 1). The 17-mer was labelled with

γ-^{32}P dATP and T4 Polynucleotide Kinase according to the manufacturer's directions (Stratagene, La Jolla California) and used to screen the selected gDNA clones for sequences corresponding to the 5' end of the gene. Southern blots were made as described previously (section 2.2.3.3) except that nitrocellulose (Scheicher and Schuell, Keene New Hampshire) was used as the membrane. DNA from each clone (1 µg in 10 µl TE) was heated to 95°C for 5 min. and 40 µl of 20 X SSC was added to the samples. Dot blots were generated by vacuuming the samples through the wells (BioRad, Richmond, California) onto a nitrocellulose membrane. Each well was rinsed with 100 µl of 20 X SSC, the filter removed from the apparatus and baked for 2 hours at 80°C (Davis et al., 1986). The blots were prehybridized in 6 X SSC (0.9 M NaCl, 90 mM $Na_3C_6H_5O_7 \cdot 2H_2O$ pH 7.2), 5 X Denhardt's, 0.05% sodium pyrophosphate, 100 µg/ml boiled salmon sperm DNA and 0.5% SDS at 42 °C for 1 hour, then hybridized in 6 X SSC, 1 X Denhardt's, 100 µg/ml yeast tRNA, 0.05% sodium pyrophosphate and 20 ng of the labelled P_4 oligonucleotide overnight at 42 °C. The next morning the blots were washed five times in 6 X SSC, 0.05% sodium pyrophosphate at room temperature, 10 min. each, and then the same solution at 48°C for 30 min. The blots were dried, wrapped in Saran wrap and exposed to film to generate autoradiograms.

2.2.5 DNA Sequencing

Plasmid DNA to be sequenced was isolated using the acid-phenol mini-prep procedure previously described (section 2.1.3). Approximately 1.5 to 2.0 µg of plasmid DNA containing the insert of interest was denatured in 0.4 M NaOH for 10 min. at room temperature, and an equal

volume of 1.0 M NaOAc pH 5.2 and 3 volumes of ethanol were added. The DNA was precipitated at -70 °C for 30 min. and then centrifuged at 12,000 x g for 10 min. at 4°C. The DNA was washed in 80 µl of 80% ethanol, re-centrifuged and allowed to air dry thoroughly.

The DNA pellet was then re-suspended in 10 µl of sterile water. To this solution 2 µl (0.80 µM) of the appropriate sequencing primer (M13 universal sequencing primer, T3 Bluescript sequencing primer, or a sequencing primer complementary to an internal region in *Ahd*-2; Table 1) and 2 µl of annealing buffer (Pharmacia T7 sequencing kit, Upsala, Sweden) were added. Annealing was allowed to proceed for 20 min. at 37 °C then the reaction was cooled to room temperature for at least 10 min.. Dideoxynucleotide sequencing of the DNA was performed using Pharmacia's T7 Sequencing Kit and α-^{35}S dATP from DuPont (Markham, Ontario).

Sequencing reactions were separated on 8% polyacrylamide 8M urea gels using a BioRad (Richmond, California, U.S.A.) SequiGene sequencing system (0.4 cm X 21 cm X 50 cm) and an LKB Bromma 2302 Multidrive XL 3.5 kV power pack (Pharmacia). The gels were electrophoresed at 50 °C for 2, 4, 6 or 8.5 hrs. Some extended sequencing reactions were performed using the 'read-long' reactions from the T7 Sequencing Kit and a 6% polyacrylamide 8 M urea gel. After electrophoresis, the gels were washed in 10% acetic acid/10% methanol for 20 min. and then in distilled water for 20 min. before drying onto Sequencing Gel filter paper (BioRad) for 1 hr. in a BioRad Model 583 gel drier. X-ray film (Kodak X-OMAT AR) was placed next to the dried gel in a film cassette and exposed 18 - 72 hrs. at room temperature. The film was then developed according to manufacturer's instructions using GBX developer and fixer.

When necessary, recombinant clones were subcloned to allow sequencing of internal regions. All ligations for subcloning experiments were performed according to Sambrook et al. (1989). Insert and vector DNA were isolated by running on low melting point agarose gels in 1 X TAE and excising the appropriate bands. The DNA was then combined in a 2:1 (insert:vector) molar ratio. One Weiss unit of bacteriophage T4 DNA ligase in 100 mM Tris-Cl pH 7.6, 10 mM $MgCl_2$, 20 mM dithiothreitol, 1 mM ATP was added and the ligation allowed to proceed overnight at 16 °C. The next morning the mixture was heated to 70 °C for 15 min. and the appropriate competent host bacterial cells transformed according to Davis et al. (1986). Vector DNA carrying identical cohesive ends was dephosphorylated prior to ligation with one molecular biology unit (MBU) of HK™ phosphatase (Bio/Can Scientific) in 33 mM Tris-acetate pH 7.8, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM β-mercaptoethanol and 5% bovine serum albumin at 30 °C for one hr. The reaction was then heated to 65 °C for 30 min. to inactivate the phosphatase.

Host cells were made competent by growing bacteria in LB until the cells reached an $O.D._{600}$ of 0.3 to 0.5 (measured on Beckman DU-8 spectrophotometer). The bacteria were spun at 2,500 x g for 5 min., resuspended in one-half volume of 50 mM $CaCl_2$ and placed on ice for 30 min. At this time the bacteria were re-pelleted (2,500 x g, 5 min) and resuspended in one-tenth volume of 50 mM $CaCl_2$ and stored at 4°C for 24 hours. Competent bacteria were transformed by inoculating 200 µl of competent cells with 10 µl of ligation reaction, as prepared above. The mixture was placed on ice for 30 min., heat shocked at 42°C for 2 min., 1

ml of LB was added and the cells grown at 37°C for one hr. before plating on LB plates with the appropriate antibiotics.

2.2.6 Polymerase Chain Reaction

2.2.6.1 Amplification of the Ahd-2 Promoter Region

Specific regions of the *Ahd-2* gene from the various mouse strains were amplified using the polymerase chain reaction (PCR). Genomic DNA from three strains of mice (C57BL/6J, BALb/c and 129/ReJ, previously isolated for RFLP analysis from mice housed in the Animal Quarters, Department of Zoology, U.W.O), was used as the template for amplification of the *Ahd-2* promoter region. Approximately 100 ng of genomic DNA was used in the initial reaction as the template, in addition to 100 picomoles of each of the two primers (P8 and P4 , see Table 1 for details), 0.25 mM dATP, 0.25 mM dCTP, 0.25 mM dGTP, 0.25 mM dTTP, 1 X reaction buffer (500 mM KCl, 100 mM Tris-Hcl, pH 8.8 at 25 °C, 15 mM $MgCl_2$, 1% Triton X-100) and 2.5 units of Taq DNA polymerase (Promega, Fisher Scientific). The reaction was brought up to 50 µl with sterile distilled water and sealed with 50 µl of mineral oil (Sigma, St. Louis, Missouri) Thermocycling was performed in a Perkin-Elmer Cetus Thermocycler under the following conditions. After an inital melt at 94 °C for 7 min., 30 cycles of 94 °C for 1 minute, 55 °C for 2 min., and 72 °C for 2 min. were performed. A 30 min. extension period (72 °C) followed the 30 cycles. The samples were stored at 4 °C until further analysis (personal communication, D. Zhu, Departments of Anatomy and Zoology, University of Western Ontario).

Products from this inital reaction were electrophoresed on a 1.8% low melting point agarose gel with 0.5 mg/ml EtBr in 1 X TAE at 60 V for approximately 2 hours. The products were visualized using a transilluminator and specific products, determined by appropriate size, were excised from the gel using a clean razor blade and placed in a sterile eppendorf tube. The agarose slice containing the DNA was then melted at 65 °C for 10 min. in the presence of 9 volumes of sterile water. A portion of this solution (10 µl) was then used as the template for a secondary amplification reaction in order to increase both the yield of the product and specificity. The same PCR reaction conditions and primers were used for the secondary amplification reaction.

2.2.6.2 Amplification of the Putative Antabuse Binding Region in Ahd-2

Total liver RNA was isolated (section 2.2.2.1) from mice of three strains (C57BL/6J, BALB/c, 129/ReJ), and two (C57BL/6J X BALB/c) recombinant inbred lines # 2 and #13. The RNA was used in a reverse transcription reaction to generate a cDNA template for the amplification of the *Ahd-2* putative Antabuse binding region. Approximately 4 µg of total RNA was brought up to 9.0 µl in DEPC-treated H2O and to this solution 4 µl of 5 X RT buffer (supplied by BRL, Gaithersburg, Maryland), 4 µl of 100 ng/µl Oligo dT's (Boehringer Mannheim, Laval, Quebec), 2 µl of a 10 mM solution of dATP, dCTP, dGTP and dTTP (Boehringer Mannheim), 0.5 µl of 40 U/µl RNAsin (Promega, Ottawa, Canada) and 0.5 µl of MMLV reverse transcriptase (200 U/µl, BRL) were added and incubated at

42 °C for 2 hours. The solution was then heated at 95°C for 10 min., placed on ice for 5 min. and then stored at -20°C until needed.

One quarter (5 µl) of the resulting cDNA mixture was used as the template in a preliminary PCR amplification of the *Ahd-2* putative Antabuse binding region. The template was incubated with 100 picomoles of each of the two primers (P_1 and P_2, Table 1), 0.25 mM dATP, 0.25 mM dCTP, 0.25 mM dGTP, 0.25 mM dTTP, 2 mM MgCl2, 1 X reaction buffer (500 mM KCl, 100 mM Tris-Hcl, pH 8.8 at 25 °C, 15 mM $MgCl_2$, 1% Triton X-100) and 2.5 U of Taq DNA Polymerase (Promega). The reaction was brought up to 50 µl with sterile distilled water and overlayed with 50 µL of mineral oil (Sigma, St. Louis, Mo.). Thermocycling was performed in a Perkin-Elmer Cetus Thermocycler, using the following conditions. After an inital melt at 94 °C for 7 min., 25 cycles of 94 °C for 1 minute, 60 °C for 2 min., and 72 °C for 2 min. were performed. A 30 min. extension period (72 °C) followed the 25 cycles. The samples were stored at 4 °C until further analysis. The yield and specificity of the product were increased by a second amplification cycle using the conditions specified above.

2.2.7 Subcloning and Sequencing of PCR Products

2.2.7.1 Subcloning

All PCR products were subcloned into Bluescript vector DNA (Stratagene, La Jolla California) for sequencing. The second amplification product (15 µl) was blunt ended with 5.0 µl of T4 DNA polymerase buffer (33 mM Tris-acetate pH 7.9, 66 mM potassium acetate, 10 mM magnesium

acetate, 0.5 mM DTT, 0.1 mg/ml bovine serum albumin) 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.5 mM dTTP and 10 U of T4 DNA Polymerase (Promega) and brought up to 50 µL with sterile distilled water. After incubating at 37°C for 30 min., the solution was brought up to 100 µl with sterile distilled water and extracted once with an equal volume of salt-saturated phenol/chloroform:isoamyl alcohol (24:1) and then once with chloroform:isoamyl alcohol (24:1). After this final extraction, one-tenth volume of 3 M NaOAc pH 5.2 and 3 volumes of 100% ethanol were added to the aqueous phase. The resulting solution was placed on ice for 30 min. The DNA was precipitated by centrifugation in a Sorval RC5C high speed centrifuge (DuPont) for 60 min. at 4°C, 12,000 x g. The DNA was then washed with 80% ethanol and allowed to dry before being dissolved in 5 µl of H_2O for 10 min. at 37°C.

The DNA was phosphorylated by adding 0.5 µl of 10 X ligation buffer (50 mM Tris-HCl pH 7.5, 7 mM $MgCl_2$, 1 mM DTT), 1 mM ATP and 5 U of T4 Polynucleotide Kinase and sterile distilled water to 10 µl. This was incubated at 37°C for 30 min., then at 70°C for 30 min. and then the DNA was isolated by running the solution on a 1.8% low melting point agarose gel in 1 X TAE and excising the band. This PCR fragment was then ligated into *Eco* RV cut and dephosphatased Bluescript vector (Stratagene, La Jolla California) as previously described (section 2.2.5).

2.2.7.2 Sequencing

After ligating the *Ahd-2* specific PCR fragments from the three strains of mice into Bluescript vector, the resulting recombinant DNAs were used to transform competent XL-1 Blue host bacteria (Stratagene, La

Jolla California), as previously described. The transformed bacteria were then plated on 50 µg/ml ampicillin, 12.5 µg/ml tetracycline LB agar plates (1% NaCl, 1% Bacto-Tryptone, 0.5% Yeast Extract) with X-Gal (5-Bromo-4-chloro-3-indoyl-β-D-galactoside, 20 µg/ml) and IPTG (Isopropylthio-β-D-galactoside, 0.1 mM). After incubation for 18-24 hours, white colonies were picked, grown in separate 5 ml tubes of LB with 50 µg/ml ampicillin and 12.5 µg/ml tetracycline overnight at 37°C, with constant rotation. The next morning the DNA was isolated by an acid-phenol 'mini-prep' (see section 2.1.3), digested with the appropriate restriction enzymes and electrophoresed on an agarose mini-gel to verify the presence of the desired insert in the plasmid. DNA from recombinant plasmids with an insert of the appropriate size was then sequenced as described in section 2.2.5 using M13 universal and Bluescript T3 sequencing primer. The PCR primers (P_4 and P_8, Table 1) were used to sequence internal regions of the promoter fragment, as previously described.

For the 369 bp PCR fragment surrounding the putative Antabuse binding region two sequencing strategies were employed. Initially, reverse transcription of total liver RNA, PCR amplification from the resulting cDNA template, blunt-ending of the PCR product, ligating and transformation of the recombinant DNA were carried out as previously described. LB (3 mls) with 50 µg/ml ampicillin and 12.5 µg/ml tetracycline was added to the transformation mix and the solution incubated with constant rotation overnight at 37°C. The solution was not plated to isolate individual colonies. The recombinant plasmid DNA was then extracted by an acid-phenol 'mini-prep' isolation and used for sequencing as described in section 2.2.5. using the two internal primers, P_1 and P_2

(Table 1). Since the DNA is a mixture of all the PCR amplified fragments, all possible sequences of this amplified region are expected. The use of sequencing primers complementary to the insert ensures that non-recombinant plasmid DNA is not sequenced (BioTechniques, Volume 11, 1991). The sequence of a gene product, '*Ahd-2* related', detected by the above procedure, was confirmed using sequence information derived from individually isolated clones, as previously described.

2.2.8 Primer Extension Analysis

Primer extension analysis is used to determine the possible transcription start sites of a gene. The putative transcription start sites for *Ahd-2* in different strains of mice were evaluated as follows. P4 (see Table 1) was labelled with γ ^{32}P dATP and T4 Polynucleotide Kinase as previously described (section 2.2.4.6). The primer was then dissolved in 50 mM Tris, 10 mM $MgCl_2$, 5 mM Tricine pH 8, 20 mM NaOAc, 0.5 mM EDTA, at a concentration of 0.5 pmoles/µl. The primer was further diluted with 3 volumes of sterile distilled DEPC-treated H_2O. Labelled primer (0.5 pmoles) was then allowed to hybridize to 5 µg total liver RNA isolated from each of the three strains of mice previously studied (C57BL/6J, BALB/c, and 129/ReJ, section 2.2.2.1) in 12.5 µl total volume. Non-specific hybridization of the primer was assessed using 5 µg of yeast tRNA (control lane). The mixture was heated at 95°C for 90 seconds, transferred to a 47°C waterbath for 10 min., then room temperature for 15 min. to allow hybridization of the primer and the RNA. An equal volume of a solution containing 0.5 X MMLV reverse transcriptase buffer (supplied by BRL, Gaithersburg, Maryland), 20 mM DTT, 10 mM dATP,

10 mM dCTP, 10 mM dGTP, 10 mM dTTP (Boehringer, Laval, Quebec) and 10 U/µl MMLV reverse transcriptase (BRL) was added and the mixture incubated at 47°C for 40 min.. The enzyme transcribes a cDNA from the RNA template using the hybridized primer as a starting point. The transcription reaction continues until the 5' end of the RNA template has been reached. The length of the transcribed cDNA fragment, which is labelled with ^{32}P, therefore corresponds to the distance from the primer to the 5' end of the mRNA (Ausubel et al., 1987). The reaction was stopped with 5 volumes of 150 mM NaOAc, 125 mM EDTA, 0.1% SDS and 25 µg/ml tRNA after 40 min. to prevent non-specific reverse transcription and endonuclease activity. The mixture was extracted once with salt-saturated phenol/chloroform:isoamyl alcohol (24:1) and precipitated with 2.5 volumes of 100% ethanol at -20°C overnight.

The next morning the DNA was pelleted at 12,000 x g, 15 min. at 4°C in an RC5C centrifuge (DuPont, Markham, Ontario), rinsed with 80% ethanol, spun, and allowed to dry. The DNA was then dissolved in 2.5 µl of sterile H_2O and 7.5 µl of sequencing stop dye (Pharmacia T7 sequencing kit, Upsala, Sweden), denatured at 95°C for 5 min. and electrophoresed on an 8% sequencing gel (section 2.2.5). Sequencing reactions from DNA of a clone from the 5' region, obtained using the P4 primer, were electrophoresed on the gel at the same time. This allowed precise determination of the length of the labelled cDNA fragments and identification of the putative transcription initiation points (technique, personal communication from G. Mackie, Department of Biochemistry, University of Western Ontario).

2.2.9 *Transcription Run-Off Experiments*

Nuclei were extracted from the livers of control and ethanol-fed (section 2.2.1) C57BL/6J, BALB/c and 129/ReJ adult male mice as follows. Animals were sacrificed, the livers removed and rinsed in ice cold phosphate buffered saline and then homogenized in 5 volumes of 2 M sucrose/10% glycerol (2 M sucrose, 10 mM Hepes pH 7.6 [Boehringer Mannheim, Laval, Quebec], 15 mM KCl, 0.15 mM spermine, 0.5 M spermidine [Sigma Chemical, St. Louis Missouri], 2 mM EDTA, 10% glycerol, 0.5 mM DTT, 1% Trasylol, 0.7 µg/ml pepstatin A, 0.7 µg/ml leupeptin, 0.5 mM PMSF [Boehringer Mannheim], and 0.1 mM Benzamidine [Sigma]) with 5-10 strokes. This homogenate was then layered onto a 6 ml cushion of 2M sucrose-10% glycerol in a Beckman ultracentrifuge tube and spun at 22,000 rpm in an SW 41 Ti rotor in a pre-cooled 0°C Beckman L 8 M ultracentrifuge to pellet clean nuclei. The supernatant was then removed with a sterile 10 ml plastic pipette and the walls of the tubes flushed with sterile DEPC-treated H_2O. The nuclei were resuspended in 100 µl of 50% glycerol, 20 mM Tris pH 7.9, 7.5 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, 0.125 mM PMSF with 100 U/ml RNasin (Promega) and frozen at -80°C in aliquots. Nuclei concentration was determined by diluting 1 µl of nuclei into 4 µl of the above solution and 5 µl of trypan blue and counting with a hemocytometer.

The run-off reaction was performed by mixing 1 x 10^6 nuclei with 0.3 M $(NH_4)_2SO_4$, 100 mM Tris HCl pH 7.9, 4 mM $MnCl_2$, 200 mM NaCl, 400 µM EDTA, 0.1 mM PMSF, 1.2 µM DTT, 1 mM GTP, 1 mM ATP, 1 mM CTP (Pharmacia), 10 mM Creatine-P (Boehringer

Mannheim), 20 U/ml RNasin, 29% glycerol and 150 µCi ^{32}P UTP (ICN Biochemicals) and incubating at 26-28 °C for 30 min.. The reaction was stopped by adding 100 µg tRNA and 2 µl DNase (Pharmacia) and incubating for 30 min. at 37°C. The RNA was then extracted as previously described, section 2.2.2.1, then resuspended in 250 µl of Hepes pH 7.5, 5 mM EDTA, 200 mM NaOH and left on ice for 15 min.. The reaction was quenched by adding 280 mM Hepes free acid pH 5.5 and the RNA precipitated with 3 M NaOAc and 2 volumes of ethanol. After washing with 70% ethanol, the RNA was resuspended in 500 µl of pre-hybridization solution (50 mM Hepes pH 7, 0.75 M NaCl, 50% formamide, 0.5% SDS, 2 mM EDTA, 10 X Denhardt's, 200 µg/ml salmon sperm DNA and 10 µg/ml poly rA [Boehringer Mannheim]), and an aliquot counted using a Beckman Scintillation counter to determine c.p.m.

DNA dot blots were prepared by spotting 1.5 µg of λ DNA, 0.5, 1.0, 2.0 µg of whole ADH_{m16} plasmid and 0.125, 0.25, 0.5 µg of the pG121 insert (a 1164 bp *Eco* RI fragment) onto nitrocellulose membrane (Schleicher and Schuell, Keene, New Hampshire). The DNA was first denatured in 0.3 N NaOH at room temperature for 30 min., then quenched with 1.5 M ammonium acetate (NH_4OAc) and filtered through a BioRad dot blot apparatus. The membrane was then rinsed in 2 M NH_4OAc and baked at 80°C under vacuum for 60 min.. The strips of nitrocellulose were wet for 10 min. in 3 X SSC before prehybridization separately in 3 mls of prehybridization solution (see above) which had been heated to 90°C for 10 min. and cooled on ice. Prehybridization was allowed to proceed overnight at 42°C.

The next morning the prehybridization solution was removed and replaced with 1 ml of fresh solution and the labelled RNA for the three

strains, in separate hybridization bags. The same number of counts were used for all samples to allow comparative analysis. Hybridization was allowed to proceed for 48 hours at 42°C. The filters were then washed 5 times in 0.1 X SSC, 0.1% SDS, 20 min. each, wrapped in Saran wrap and exposed to Kodak X-OMAT AR film with intensifying screens as previously described (section 2.2.2.4). (based on technique from G. Shore, McGill University, Montreal, Quebec and modified from Ausubel et al., 1987).

2.2.10 Computer Analyses of DNA Sequences

GenBank (release 69) and EMBL (release 28) databases were searched for sequences similar to the obtained BALB/c *Ahd-2* coding region, the 369 nucleotide region of '*Ahd-2* related' and the *Ahd-2* 5' promoter region sequences. The software program DNA Inspector IIe (TEXTCO, West Lebanon, New Hampshire, version 3.13) was run on an Apple (Cupertino, CA) Macintosh II microcomputer (5 Megabytes of memory and operated under System software version 6.07) and used to edit the 5' regulatory and coding regions from genomic or cDNA databank sequences. Multiple sequence alignments and related genetic distance calculations were performed using the default parameters of The IntelliGenetics Software for Molecular Biology (Mountain View, California, version 5.1). The GENALIGN and REGION programs were used to obtain multiple sequence alignments and matrices of pair-wise genetic distance scores. GENALIGN scores were converted to represent relative percent identity between pairs of sequences based upon the known identity between the two sequences with the highest score. The Macintosh

software package Systat (version 5.1) was then used to generate dendograms based upon Euclidean distances. The promoter sequence was analyzed using Quick User-Directed Expression Search Tool (Quest: IntelliGenetics, release 5.4) to search the databanks "KeyTool 8" and "KeyBank 8". Analyses involving use of The IntelliGenetics Suite of Programs were performed by N. J. Schisler.

2.2.11 Statistical Analysis

Statistical analyses involving testing for significance of differences for all quantitative observations were performed using StatWorks (version 1.1), a Macintosh program from Cricket Software Inc. (Philadelphia, Pennsylvania).

Chapter 3

RESULTS

3.1 Ethanol Feeding Experiments

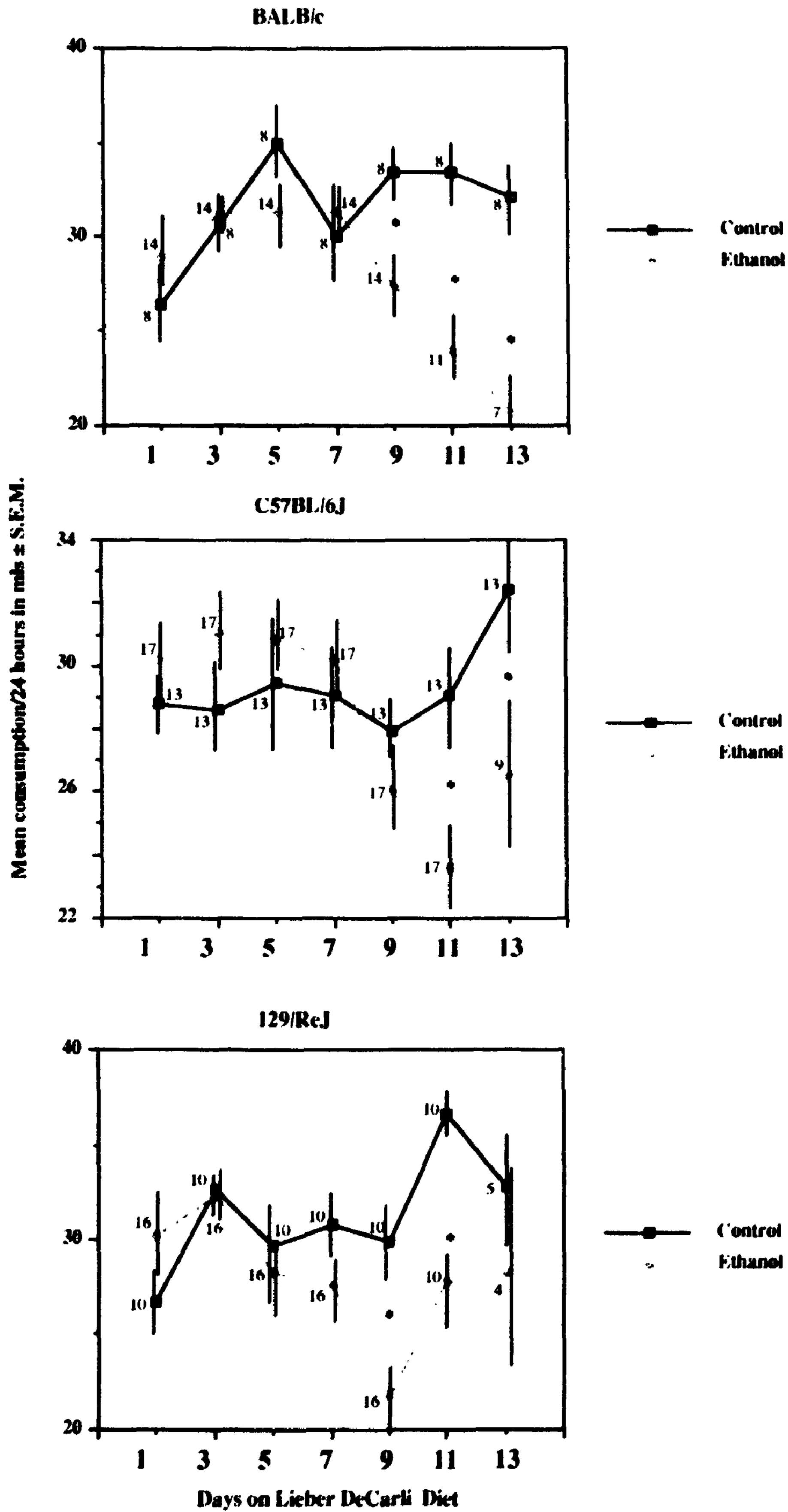
3.1.1 Consumption of the Lieber DeCarli Liquid Diet

Male mice approximately 6 to 10 weeks old from three inbred strains (C57BL/6J, BALB/c and 129/ReJ) were fed the Lieber DeCarli liquid diet (BioServ Inc., Frenchtown, New Jersey, U.S.A.) for two weeks (Rodgers, 1966; Bond et al., 1991). The experimental animals received 1% ethanol on days 1 to 3, 3% ethanol on days 4 to 7, and 5% ethanol on days 8 to 14. The 5% ethanol solution is equivalent to 35% of total caloric intake through ethanol (BioServ instruction manual, BioServ Inc., Frenchtown, New Jersey, U.S.A.). Weight-matched littermate controls received equivalent calories via the carbohydrate sources (maltose-dextran). Animals were placed in individual cages and body weights and approximate consumption of liquid diet from the preceeding 24 hours were recorded daily. In cases where mice died during the night, the consumption levels from the previous 24 hours were not recorded. Thus, the number of animals used to calculate the mean weight and the number used to calculate the mean daily consumption are not always identical. Measurements of diet consumption are only approximate in all cases due to the nature of the liquid diet. In addition to spillage by the animals, the

viscous liquid can cling to the sides of the tube and air is easily introduced into the solution. To facilitate measuring, the diet was made fresh daily with cold water and allowed to settle for approximately 30 minutes before feeding. Feeding tubes consisted of 50 ml disposable Falcon tubes (Falcon, Becton Dickinson, Lincoln Park, New Jersey, U.S.A.) with marked graduations fitted with standard animal care stainless steel and rubber tops. This eliminated the need to pour the liquid diet into a graduated cylinder to measure consumption. No other sources of food or water were available to the mice during the experiment.

Mice from all strains consumed roughly 25-30 mls/day of the diet during the initial stages of the experiment (days 1 to 7). During this period the intake of the control and the experimental diet was not significantly different (as shown by Student's t-tests) in animals of all three strains studied (Figure 3). Furthermore, there is no significant change in the consumption of control diet over the course of the experiment. The consumption of the experimental diet during the latter part of the experimental period (> day 9), however, is variable (Figure 3). On day 9, BALB/c and 129/ReJ both have significantly decreased average consumption of experimental diet as compared to the control ($p < 0.05$). This decrease in consumption coincides with the change from 3 to 5% ethanol solution, which occurs on day 8. BALB/c mice show a steady decline in the consumption of ethanol diet during the latter part of the experimental period. Reduced consumption of the ethanol diet during this time is also apparent for the 129/ReJ mice. C57BL/6J mice fed the experimental diet also have a decrease in consumption relative to controls on days 11 and 13 ($p < 0.05$). It may be pointed out that there was a

Figure 3. Consumption of the Lieber DeCarli liquid diet (control, ▫) and (ethanol, •) by male mice from three inbred strains (BALB/c, C57BL/6J, and 129/ReJ) during the previous 24 hour period (mean ± S.E.M.), measured in mls. Points represent mean consumption, bars represent S.E.M for each point, asterisks represent significantly different values between control and ethanol-fed animals (Student's t-test, $p<0.05$). The number of animals used to calculate each mean ± S.E.M. is given at each data point.


BALB/c
40
30
20
1
3
5
7
9
11
13
Control
Ethanol
C57BL/6J
34
30
26
22
Control
Ethanol
129/ReJ
40
30
20
Control
Ethanol
Mean consumption/24 hours in mls ± S.E.M.
Days on Lieber DeCarli Diet

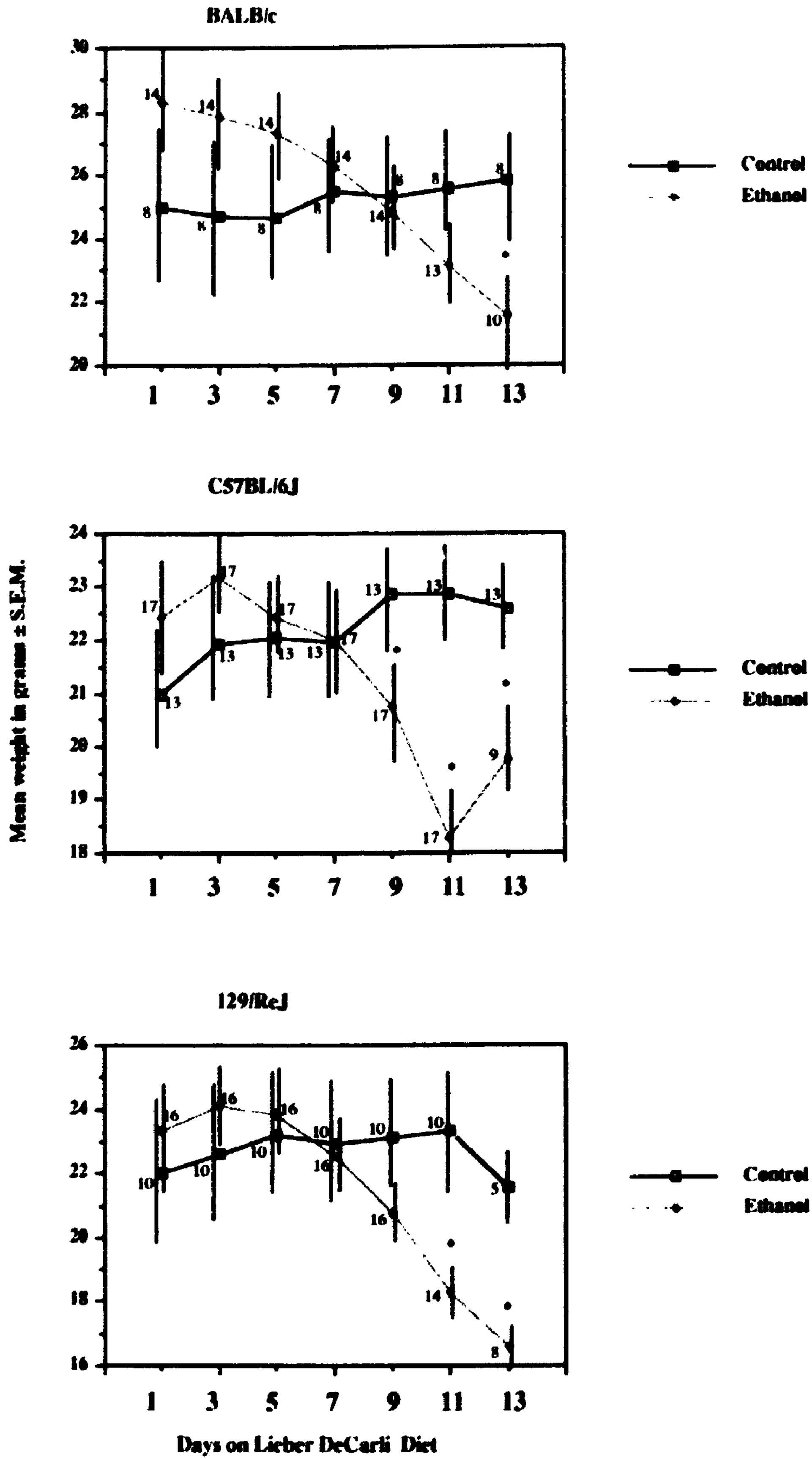
strain-specific selective mortality associated with the Lieber DeCarli liquid diet (see section 3.1.2) during the latter part of the experimental period.

3.1.2 Weight Gain/Loss and Other Effects of the Lieber DeCarli Liquid Diet

Body weights of the mice were recorded daily while on the Lieber DeCarli liquid diet. Since animals on the control and experimental diet regimens were weight-matched, there is no statistical difference between the two groups for the three strains at the start of the experiment. It is apparent from Figure 4 that for animals fed the control diet, there is no significant change in weight over the course of the experimental period. Animals fed the experimental diet, however, show a net weight loss during this time. Mice from the strain C57BL/6J weighed, on average, 12% less at the end of the experiment while mice from the strains BALB/c and 129/ReJ showed average weight losses of 24% and 29%, respectively. BALB/c and 129/ReJ mice fed an ethanol diet show an almost steady decline in weight over the time course of the experiment with weights on day 11 being statistically different from weights on day 1 (Student's t-test; $p< 0.01$).

The mortality rates in the three strains of mice on the Lieber DeCarli ethanol diet appear to be genotype-dependent. The rates are based on animals which either lived for the entire test period or which died during the course of the experiment. It does not include any animals which were sacrificed *in extremis* before day 15. Approximately 40% (6/15) of the C57BL/6J mice, 62% (8/13) of the BALB/c mice and 70% (7/10) of the 129/ReJ mice died while on the experimental diet. Thus overall, BALB/c

Figure 4. Weight in grams (mean ± S.E.M) for mice from three inbred strains (BALB/c, C57BL/6J, and 129/ReJ) fed the Lieber DeCarli liquid diet. Points represent the mean weights for the control (▫) and ethanol (•) fed mice. Bars represent standard error of the mean for each point, asterisks represent significantly different values between control and ethanol-fed animals (Student's t-test, $p<0.05$). The number of animals used to calculate each mean ± S.E.M. is given at each data point.


BALB/c
Control
Ethanol
C57BL/6J
Control
Ethanol
129/ReJ
Control
Ethanol
Mean weight in grams ± S.E.M.
Days on Lieber DeCarli Diet

and 129/ReJ mice appear to be more susceptible to the effects of ethanol than are C57BL/6J mice.

For a limited number of ethanol-fed animals, blood alcohol concentrations were obtained at the end of the test period. Experimental animals from the strains C57BL/6J and BALB/c had blood alcohol concentrations (mean % w/v ± S.E.M.) of 0.23 ± 0.04 (n = 4) and 0.18 (n = 1), respectively. Mice from the strain 129/ReJ had a blood alcohol concentration of 0.42 ± 0.02 (n = 4). Additional BALB/c mice were not available at the time of testing. Thus blood alcohol concentrations differed between the strains with ethanol feeding, but all three strains were considered to be 'grossly intoxicated' (Sigma Diagnostics Blood Alcohol Concentration Kit, procedure 332-UV, 1987).

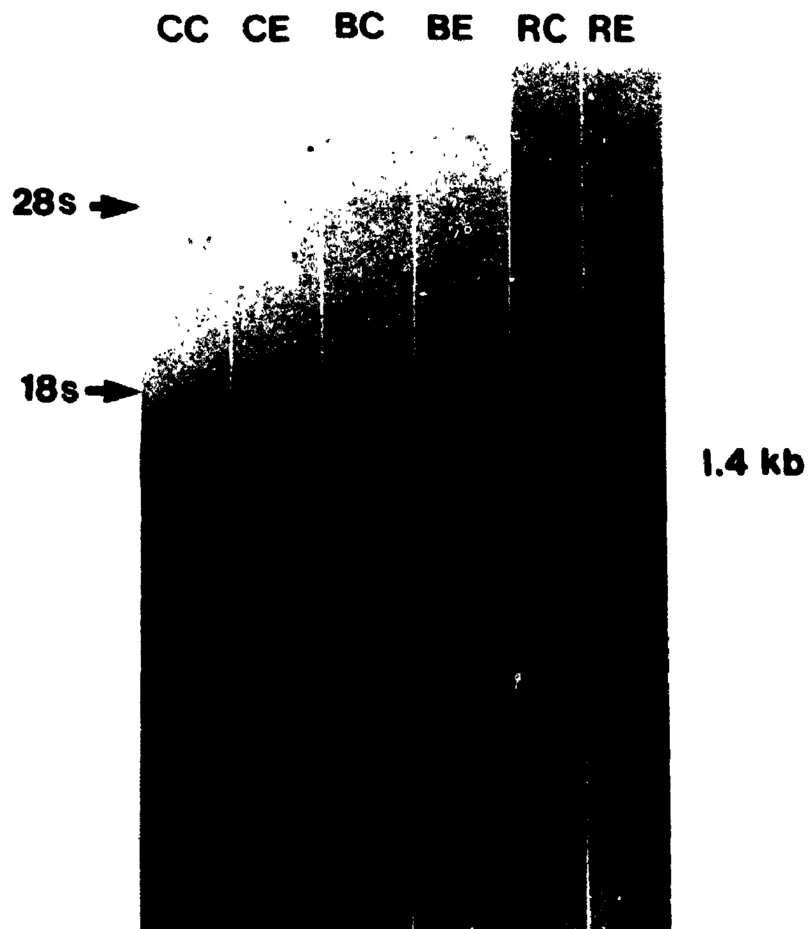
3.2 The Effect of Ethanol on Gene Specific mRNA

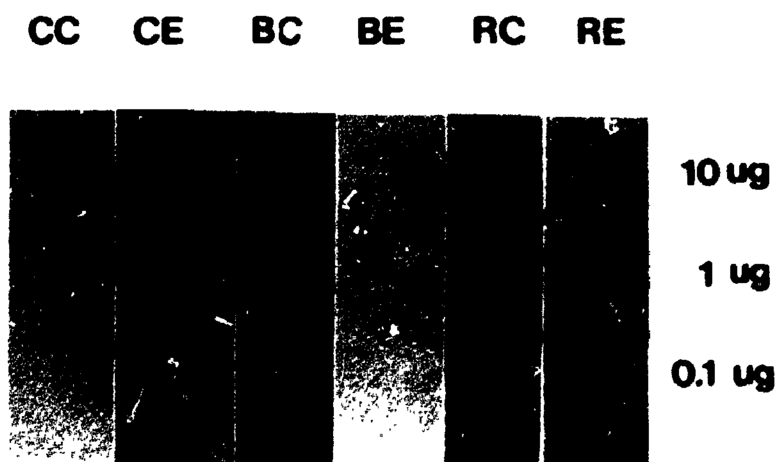
3.2.1 Alcohol Dehydrogenase (Adh-1)

Male mice approximately 6 to 10 weeks old from the three strains (C57BL/6J, BALB/c and 129/ReJ) were weight-matched and fed the Lieber DeCarli isocaloric liquid diet for two weeks, as previously described (see section 3.1.1). On day 15, the mice were sacrificed by cervical dislocation and the livers removed for RNA extraction. Total cellular liver RNA (30 µg) was electrophoresed, blotted onto BioTrace membranes and hybridized with nick-translated ^{32}P-pADH$_{m16}$ (section 2.2.2). This probe recognizes the mRNA produced by the mouse gene *Adh-1* (Ceci et al., 1986). As shown in the representative samples in Figure 5 (a), pADH$_{m16}$ hybridizes

Figure 5. (a) A representative composite Northern blot of total mouse liver RNA hybridized with pADH$_{m16}$ whole plasmid, showing the 1.4 kb *Adh-1* specific message in all samples studied. CC, C57BL/6J control-fed; CE, C57BL/6J ethanol-fed; BC, BALB/c control-fed; BE, BALB/c ethanol-fed; RC, 129/ReJ control-fed; RE, 129/ReJ ethanol-fed. The positions of the rRNA used to calculate size (28s = 5.1 kb and 18s = 2.0 kb, Davis et al., 1986) are indicated on the left side.

(b) Representative composite dot blot results on total mouse liver RNA from control and ethanol fed animals of the three strains. The amount of total RNA loaded is indicated on the right hand side of the figure.


a)
CC CE BC BE RC RE
28s
18s
1.4 kb


b)
CC CE BC BE RC RE
10 ug
1 ug
0.1 ug

to the RNA from all samples (all three strains, control and ethanol diet) as a single 1.4 kb message. This observation is consistent with earlier reports on the mouse *Adh-1* mRNA (Ceci et al., 1986, Patterson et al., 1987). The single mRNA signal on the Northern blots permitted quantification of the gene-specific message using dot blots.

Quantification of the *Adh-1* specific mRNA was done using replicated dot blots with different concentrations of RNA samples on each dot blot (Figure 5 [b]). The intensity of the hybridization signal, which is assumed to be proportional to the amount of *Adh-1* mRNA in the sample, was quantified by densitometry. Comparisons of the hybridization signal of RNA from control-fed mice between different strains are based on values within a single blot. The average value of the comparison between strains in a number of dot blots ± S.E.M is given in Table 2 (a). Comparisons of the hybridization signals between the RNA from control-fed animals and ethanol-fed animals were also made within a single blot. These numbers were used to calculate the relative change in *Adh-1* mRNA in ethanol-fed animals as compared to controls of the same genotype. The relative change was calculated for each strain present on the dot blot. The values presented in Table 2 (b) represent the mean of the relative change in a number of different dot blots ± S.E.M.and are given for each strain. It was not possible to compare hybridization signals between blots due to differences in the labelling of the probes and potential variations in the technique. The dataset, obtained as described above, was used to assess the effects of ethanol feeding on mRNA levels in the various strains of mice. As shown in Table 2 (a), the *Adh-1* mRNA levels are strain dependent. The control animals from the strain C57BL/6J have approximately 1.7 times the amount of *Adh-1* mRNA in the liver as compared to control mice

Table 2. Summary of dot blot analysis showing the relative levels of liver mRNA hybridizing to $pADH_{m16}$, a cDNA probe for mouse *Adh-1* in a) control mice from three different inbred strains and b) following ethanol feeding as compared to control-fed animals.

a)

	Relative levels ± S.E.M (number of animals)
C57BL/6J / BALB/c	1.71 ± 0.35 (4)
C57BL/6J / 129/ReJ	1.34 ± 0.52 (11)
BALB/c / 129/ReJ	1.22 ± 0.18 (6)

b)

Strain	**(n)**	**mean $\frac{[E - C]}{C}$ x 100% ± S.E.M.**
C57BL/6J	(8)	200.2 ± 86.97
BALB/c	(3)	21.98 ± 19.8
129/ReJ	(10)	-12.93 ± 14.06

E = ethanol-fed, C = control-fed, n = number of animals tested.

from the strain BALB/c. Strain-dependent differences in the level of *Adh-1* mRNA have been reported earlier and the results obtained here are similar to those of Patterson et al. (1987) and Zhang et al. (1987). The *Adh-1* specific mRNA values of the control-fed animals for each strain were also compared to the genotype-matched animals fed the ethanol diet. The effect of the ethanol feeding appears to be strain-specific (Table 2 [b]). In general, there is a large increase (approximately 200%) in the *Adh-1* mRNA of ethanol-fed C57BL/6J mice as compared to control-fed animals of the same strain. For BALB/c mice approximately a 20% increase is found and for 129/ReJ, there appears to be a reduction in *Adh-1* mRNA in ethanol-fed animals as compared to controls. It should be noted that, although the gene and strain-specific effect of ethanol is repeatable, values within a strain, (standard error of the mean, Table 2 [b]) also vary. These results, therefore, are representative of overall trends for changes in the *Adh-1* message associated with ethanol feeding.

3.2.2 Acetaldehyde Dehydrogenase (Ahd-2)

Figure 6 (a) shows a composite of typical Northern blot results on the hepatic RNA from the three strains of mice hybridized with a partial cDNA for the mouse *Ahd-2* gene (568 bp fragment of pG121, section 2.2.2.4). The hybridization produced a single major band at approximately 2.3 kb for both the control and ethanol-fed animals of all three strains. No other reports on the size of mouse *Ahd-2* mRNA have been published but it is comparable to the message size of human cytosolic acetaldehyde dehydrogenase mRNA of 2.1 kb (Hsu et al., 1989) and the 2.25 kb rat cytosolic aldehyde dehydrogenase (Dunn et al., 1989). Quantification of

Table 5. Summary of dot blot analysis showing the relative levels of liver mRNA hybridizing to pG121, a partial cDNA for mouse *Ahd-2* in a) control mice from three different inbred strains and b) following ethanol feeding as compared to control-fed animals.

a)

	Relative levels ± S.E.M (number of animals)
C57BL/6J / BALB/c	0.80 ± 0.24 (8)
C57BL/6J / 129/ReJ	0.90 ± 0.20 (12)
BALB/c / 129/ReJ	1.10 ± 0.12 (10)

b)

Strain	**(n)**	**mean $\frac{[E - C]}{C}$ x 100% ± S.E.M.**
C57BL/6J	(9)	11.04 ± 3.90
BALB/c	(7)	0.59 ± 11.74
129/ReJ	(9)	-35.57 ± 20.73

E = ethanol-fed, C = control-fed, n = number of animals tested.

Figure 6. (a) A representative composite Northern blot of total mouse liver RNA hybridized with the 568 bp internal *Pst* 1 fragment from pG121, showing the 2.3 kb *Ahd-2* specific message in all samples studied. CC, C57BL/6J control-fed; CE, C57BL/6J ethanol-fed; BC, BALB/c control-fed; BE, BALB/c ethanol-fed; RC, 129/ReJ control-fed; RE, 129/ReJ ethanol-fed. The positions of the rRNA used to calculate size (28s = 5.1 kb and 18s = 2.0 kb, Davis et al., 1986) are indicated on the left side.

(b) Representative composite dot blot results on total mouse liver RNA from control and ethanol fed animals of the three strains. The amount of total RNA loaded is indicated on the right hand side of the figure.

a)

b)

the mRNA hybridizing to the 568 bp fragment of pG121 was done using dot blots (Figure 6 [b], and calculated as described in section 3.2.1). These results suggest that, in general, control animals of the three strains do not differ significantly with respect to the *Ahd-2* hybridization signal (Table 3 [a]). However, when a comparison is made between control and experimental animals within a single strain, a different pattern emerges (Table 3 [b]). Here, ethanol-fed animals from the strain C57BL/6J have an apparent increase over control-fed animals (approximately 11%), BALB/c mice have no apparent change and mice from the strain 129/ReJ have an apparent decrease (approximately 36%) in the mRNA hybridizing to the probe. Again, large variability exists within strains but the trends seen in relative changes in mRNA levels with ethanol treatment are repeatable.

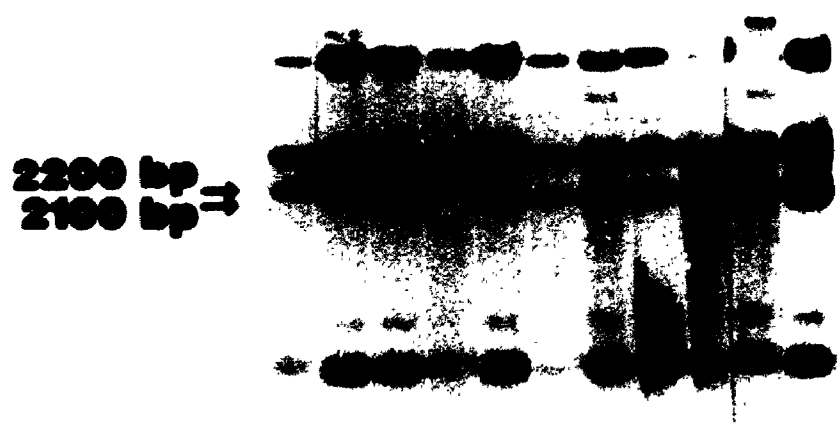
3.3 Southern Blot Analysis.

Representative Southern blots of mouse DNA cut with four restriction enzymes (*Hin*d III, *Rsa* I, *Eco* RI, and *Pst* I) and probed with the internal 568 bp *Pst* I fragment of pG121 (section 2.3) are shown in Figures 7 and 8. The bands shown in the figures were consistently observed in repeated experiments involving gels electrophoresed overnight (16 hours) and mini-gels electrophoresed for 4 hours. For each restriction enzyme, all DNA samples were electrophoresed on the same gel and repeated. The lanes were re-arranged in Figures 7 and 8 to facilitate comparison. A number of restriction enzymes tested in a preliminary study showed no differences in banding patterns for the strains examined (data not shown). It is apparent from Figures 7 and 8 that all strains yielded a number of

Figure 7. Banding patterns generated by DNA from 11 strains of mice digested with the restriction enzymes (a) *Hin*d III and (b) *Rsa* I and hybridized with the 568 bp internal *Pst* I fragment of pG121 (Figure 2). Fragment sizes, marked on the side, were calculated from λ DNA-*Hin*d III/ØX-174 RF DNA- *Hae* III (Pharmacia, Upsala, Sweden) molecular weight markers (not shown).

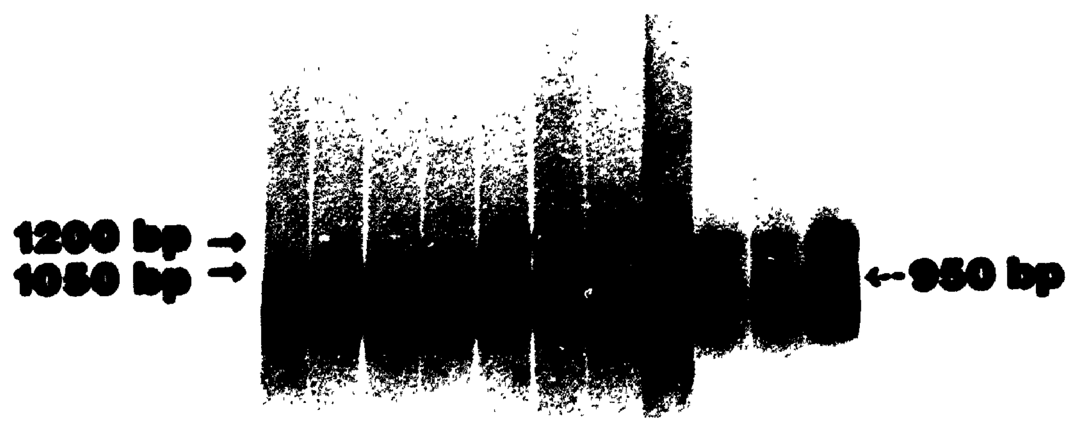
a)

C57BL/6J
C57BL/6J★
C57BL/10J
BALB/c
C57BL/KsJ
C3H/HeJ
C3H/HeSnJ
129/ReJ
Cs^{b}
SW
DBA/2J



Hind III

b)

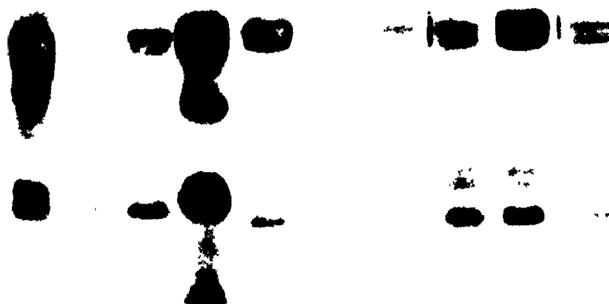


Rsa I

Figure 8. Banding patterns generated by DNA from 11 strains of mice digested with the restriction enzymes (a) *Eco* RI and (b) *Pst* I and hybridized with the 568 bp internal *Pst* I fragment of pG121 (Figure 2). Bands for Cs^b DNA digested with *Pst* I were detected but did not photograph clearly. Fragment sizes, marked on the side, were calculated from λ DNA-*Hin*d III/ØX-174 RF DNA- *Hae* III (Pharmacia, Upsala, Sweden) molecular weight markers (not shown).

a)

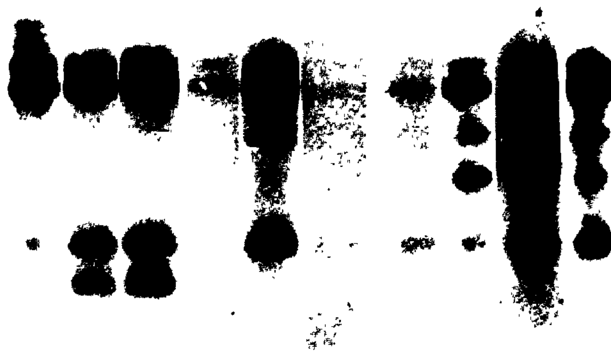
C57BL/6J
C57BL/6J★
C57BL/10J
BALB/c
C57BL/KsJ
C3H/HeJ
C3H/HeSnJ
129/ReJ
DBA/2J
SW
Cs^b



1600 bp
1450 bp
1360 bp
1300 bp

Eco RI

b)



← 850 bp

Pst I

restriction enzyme specific bands. A summary of the unique band patterns associated with the probe used, for the different mouse strains and the different restriction enzymes, is listed in Table 4.

A number of bands common to all strains were found for each of the four restriction enzymes used. However, a number of different restriction fragments hybridizing to the partial pG121 probe can be seen in each of the four polymorphic enzymes studied. When digested with *Hin*d III, DNA from the strains C57BL/6J and BALB/c (isolated from mice housed in the Department of Zoology Animal Quarters, The University of Western Ontario), C57BL/6J* and C57BL/10J (DNA obtained from the Jackson Laboratory Mouse DNA Resource) had a band at 2200 bp while DNA from C57BL/KsJ, C3H/HeJ, DBA/2J (Jackson Laboratory Mouse DNA Resource), C3H/HeSnJ, 129/ReJ, Cs^b, and SW mice (isolated from mice from the Department of Zoology Animal Quarters) had a band at 2100 bp. All eleven strains had several smaller bands and one large band at 6.8 kb in common, with the exception of SW which was missing the large fragment. Digestions with *Rsa* I produced two common fragments for all eleven strains at 800 bp and 650 bp. C57BL/6J, C57BL/6J*, C57BL/10J and BALB/c had one fragment at 1050 bp. This band was not present in the other strains which had bands at 1200 and 950 bp (Figure 7). For the restriction enzyme *Eco* RI, DNA from C57BL/6J, C57BL/6J* and C57BL/10J, BALB/c, had one band at 1360 bp while DNA from the strains C57BL/KsJ, C3H/HeJ, DBA/2J, C3H/HeSnJ, 129/ReJ, Cs^b, and SW had bands at 1600 bp and 1300 bp. A band at 1450 bp and two larger bands at approximately 4.2 and 5.5 kb were common to all strains (Figure 8). C57BL/6J, C57BL/6J*, C57BL/10J and BALB/c have an 850 bp band hybridizing to the probe when digested with *Pst* I. This band is not present

Table 4. Summary of the DNA banding patterns produced with the 4 enzymes (*Hin*d III, *Rsa* I, *Eco* RI, and *Pst* I) in 11 strains of mice shown in Figures 7 and 8. The approximate preference ratios (proportion of 10% ethanol consumed in total daily liquid intake) of the mice are also given (Rodgers, 1966; Tagliabracci and Singh, personal communication).

Table 4. DNA Banding Patterns and Preference Ratios for Various Mouse Strains.

Restriction Enzyme	Band Size (bp)	C57BL/6J	C57BL/6J*	C57BL/10J	BALB/c	C57BL/KsJ	C3H/HeJ	C3H/HeSnJ	129/ReJ	Csb	SW	DBA/2J
Hind III	2200	+	+	+	+							
	2100					+	+	+	+	+	+	+
Rsa I	1200					+	+	+	+	+	+	+
	1050	+	+	+	+							
	950					+	+	+	+	+	+	+
Eco R1	1600					+	+	+	+	+	+	+
	1360	+	+	+	+							
	1300					+	+	+	+	+	+	+
Pst I	850	+	+	+	+							
Approx. Preference Ratio		0.85	0.68	0.70	0.17	0.45	0.25	NA	0.20	0.19	0.16	0.05

in the other strains. Common bands at 7.3, 5.7 and 1.15 kb are found in all eleven strains studied. Other faintly hybridizing bands can also be seen on the autoradiograms in Figures 7 and 8. This may be due to cross-hybridization with the 568 bp internal *Pst* I fragment of pG121 and other related *Ahd* genes in the mouse genome.

Unique DNA banding patterns associated with the probe in the different mouse strains studied are summarized in Table 4. In addition, ethanol-preference ratios (the proportion of average daily total liquid intake consumed as 10% ethanol) are given for each strain (Rodgers, 1966 and Tagliabracci and Singh, personal communication). It was possible to categorize the strains into two different groups based on their DNA banding patterns. Mice from the strains C57BL/6J, C57BL/6J*, C57BL/10J and BALB/c consistently had similar bands generated by the four restriction enzymes. The strains C57BL/KsJ, C3H/HeJ, C3H/HeSnJ, 129/ReJ, Cs^b, SW, and DBA/2J also had similar banding patterns but had a different profile from the first group with all four polymorphic enzymes studied. With the exception of BALB/c, all strains of mice from the first group represent alcohol preferring mice (preference ratio > 0.68) while those from the second group are alcohol avoiding strains (preference ratio < 0.45) (Table 4). Although these results are useful and interesting, they do not offer a molecular basis for the relative sensitivity of the various mouse strains to ethanol. Additional experiments, therefore, concentrated on the sequencing of the *Ahd-2* cDNA and its 5' promoter region. Two different functional regions of *Ahd-2* (the promoter region and the putative Antabuse binding region) were sequenced in all three strains to look for strain-specific changes which could affect relative sensitivity to ethanol.

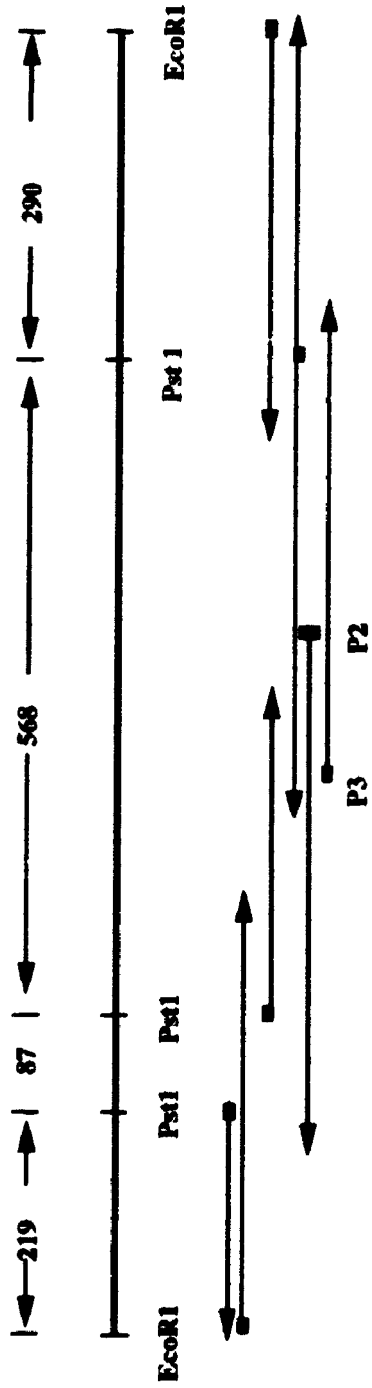
3.4 Sequencing of the BALB/c *Ahd-2* cDNA

3.4.1 Strategy for sequencing pG121

The probe pG121, used for Northern, RNA dot blot and Southern analysis, was a putative uncharacterized partial 3' cDNA for mouse cytosolic acetaldehyde dehydrogenase (*Ahd-2*). It was isolated from a BALB/c mouse liver expression library using monoclonal antibodies (Rongnoparut and Weaver, 1988). To ensure that the previous studies, which showed a genotype-dependent ethanol induced response in the amount of mRNA hybridizing to pG121 were, in fact, representative of changes in the *Ahd-2* mRNA levels, it was necessary to sequence pG121. There were no published reports on the amino acid or nucleotide sequence of mouse *Ahd-2* at the time.

Initially, pG121 was digested with *Hin*d III and *Pvu* II, which surround the *Eco* RI site in pGEM-1, to release the insert intact and to allow directional cloning into *Hin*d III/*Eco* RV cut Bluescript sequencing vector (Stratagene, La Jolla, California). Sequencing, using M13 and T3 sequencing primers and Pharmacia's T7 dideoxynucleotide sequencing kit was then performed. Subcloning of the pG121 insert was necessary to obtain the complete sequence. In addition two primers, P_2 and P_3, were synthesized (Table 1) to aid in sequencing those regions which could not be further subcloned. All regions were sequenced in both directions (Figure 9). Sequencing revealed that the pG121 insert was 1164 bp long including 129 bp of 3' untranslated region and 1035 bp coding for 345 amino acids. Comparison to human cytosolic acetaldehyde dehydrogenase (Hsu et al.,

Figure 9. The sequencing strategy for pG121 is shown. Arrows on bottom represent the direction of sequencing, P_2 and P_3 (Table 1) primer sites are marked. All fragments were subcloned into Bluescript for sequencing. Numbers denote fragment size in bp. Unless otherwise noted, the M13 universal sequencing primer or Bluescript T3 sequencing primer were used in the sequencing reaction.



219
87
568
290
EcoR1
Pst1
Pst1
Pst 1
EcoR1
P3
P2

1989) indicated that pG121 could not be a complete cDNA clone for *Ahd-2*.

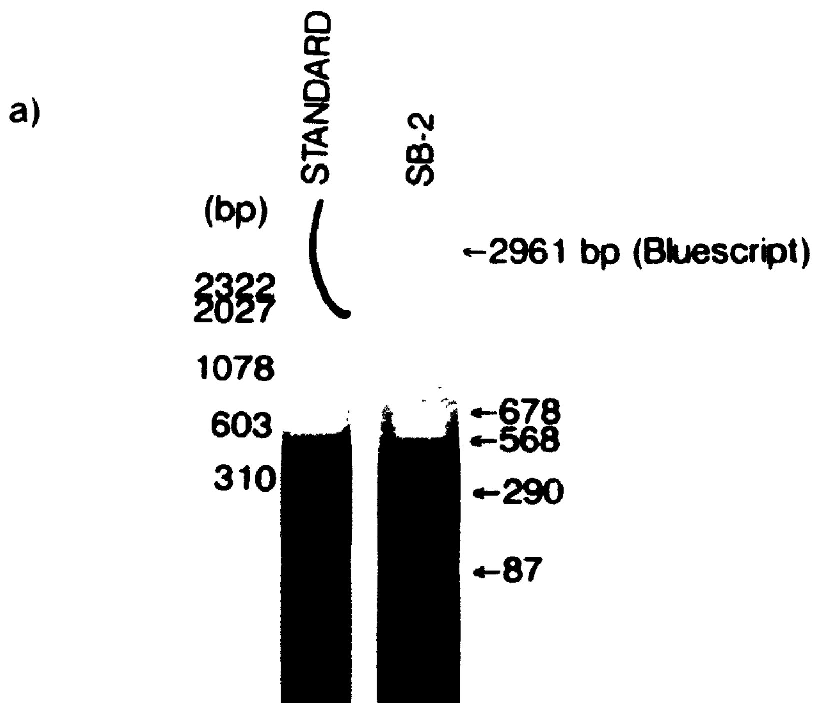
3.4.2 Screening and Sequencing of the complete Ahd-2 cDNA

An adult male BALB/c mouse liver cDNA library in λ gt 11 with an *E. coli* Y1090 host (Clonetech) was then screened with the 1164 bp *Eco* RI pG121 insert as described in section 2.2.4. Eighteen positive clones were obtained in the primary screening of this cDNA library. Twelve of these clones hybridized with the pG121 probe in the secondary and tertiary screens. DNA was then extracted from these twelve purified clones and digested with *Eco* RI to release the insert (section 2.2.4.5). The clone (SB-2) was determined to be the recombinant clone with the longest insert (approximately 1.7 kb). The other inserts ranged in size from 300 bp to 1.5 kb. SB-2 was then restriction mapped with *Eco* RI and *Pst* I and compared to pG121. Digestion of pG121 with *Eco* RI and *Pst* I gave insert fragments of 219, 87, 568 and 290 bp (5' to 3', Figure 2). Digestion of SB-2 with *Eco* RI and *Pst* I gave insert fragments of 678, 87, 568 and 290 bp (Figure 10 [a and b]). The 5' 678 bp *Eco* RI/*Pst* I fragment from SB-2, representing a 5' extension of pG121, was then subcloned into Bluescript and sequenced as previously described. This fragment was then further subcloned (*Mbo* I/*Pst* I fragment, Figure 10) to sequence internally.

Sequencing of the 678 bp *Eco* RI/*Pst* I fragment revealed that it was a 459 bp extension of the 219 bp fragment of pG121 coding for an extra 153 amino acids at the 5' end of the cDNA. However, the 'ATG' translation start codon and the 5' untranslated region of the cDNA were not found in the SB-2 clone. The 290 bp *Eco* RI/*Pst* I 3' fragment was also

Figure 10. (a) A restriction digest of SB-2 is shown. The recombinant cDNA clone SB-2 was subcloned into Bluescript, digested with *Eco* RI and *Pst* I and the DNA fragments run on a 0.8% agarose gel. Sizes were calculated using λ DNA-*Hin*d III/ØX-174 RF DNA-*Hae* III (Pharmacia, Upsala, Sweden) molecular weight markers as a standard.

(b) The restriction map and sequencing strategy for SB-2 is shown. Arrows on bottom represent the direction of sequencing, the P4 (Table 1) primer site is marked. Orientation is based upon the restriction map of pG121. All fragments were subcloned into Bluescript for sequencing. Numbers denote fragment size in bp. Unless otherwise noted, the M13 universal sequencing primer or Bluescript T3 sequencing primer were used in the sequencing reaction.


a)
STANDARD
SB-2
(bp)
←2961 bp (Bluescript)
2322
2027
1078
603
310
←678
←568
←290
←87

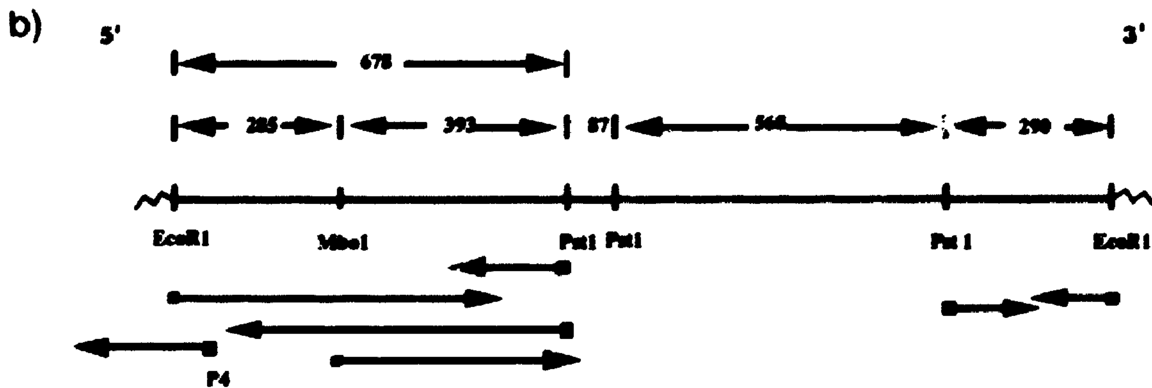

b)
5'
3'
678
285
393
87
568
290
EcoR1
Mbo1
Pst1
Pst1
Pst 1
EcoR1
P4

Figure 11. The complete nucleotide cDNA sequence for BALB/c *Ahd-2* is shown. Most of it (1632 bp) was obtained by sequencing pG121 and SB-2. Four internal PCR and sequencing primers used; P_1, P_2, P_3, and P_4, are noted with arrows (Table 1). The 9 most 5' nucleotides and amino acids of this cDNA (in bold) were obtained from SBg-5b, a recombinant genomic clone isolated from an adult male BALB/c liver genomic library (Clonetech). Numbering (nucleotides on left-hand side, amino acids on right-hand side) is in relation to 'ATG' translation start site. The 5' end of the clones pG121 and SB-2 are noted. The clone pG121 overlaps the clone SB-2 from nucleotide 469 to the 3' end. The boxed region represents the putative Antabuse binding region, as determined from human cytosolic acetaldehyde dehydrogenase (Hsu et al., 1985). The poly A' tail is not apparent in the 129 nucleotides of the 3' untranslated region sequenced.

5'of SB-2 ← P4

1 ATG-TCT-TCG-CCT-GCA-CAA-CCT-GCA-GTC-CCT-GCC-CC TTG-GCC-GAC-TTG-AAG-ATT-CAA-CAT-ACC-AAG-ATC
met-ser-ser-pro-ala-gln-pro-ala-val-pro-ala-pr leu-ala-asp-leu-lys-ile-gln-his-thr-lys-ile-23

70 TTC-ATA-AAC-AAT-GAA-TGG-CAC-AAT-TCA-GTG-AGC-GGC-AAG-AAA-TTT-CCA-GTT-CTT-AAC-CCT-GCA-AGT-GAG-
phe-ile-asn-asn-glu-trp-his-ile-ser-val-ser-gly-lys-lys-phe-pro-val-leu-asn-pro-ala-ser-glu 46

139 GAG-GTC-ATC-TGC-CAG-GTG-GAA-GAA-GGG-GAC-AAG-GCT-GAT-GTT-GAC-AAA-GCT-GTG-AAG-GCT-GCA-AGA-CAG-
glu-val-ile-cys-gln-val-glu-glu-gly-asp-lys-ala-asp-val-asp-lys-ala-val-lys-ala-ala-arg-gln-69

208 GCT-TTC-CAG-ATT-GGC-TCT-CCA-TGG-CGC-ACC-ATG-GAT-GCT-TCA-GAG-AGG-GGC-CGC-CTG-CTG-AAC-AAG-CTG
ala phe-gln-ile-gly-ser-pro-trp-arg-thr-met-asp-ala-ser-glu-arg-gly-arg-leu-leu-asn-lys-leu-92

277 GCT-GAC-TTA-ATG-GAG-AGA-GAT-CGT-CTG-CTG-CTA-GCT-ACA-ATG-GAG-GCA-CTC-AAT-GGT-GGG-AAA-GTC-TTT-
ala-asp-leu-met-glu-arg-asp-arg-leu-leu-leu-ala-thr-met-glu-ala-leu-asn-gly-gly-lys-val-phe-115

346 GCC-AAT-GCA-TAC-TTG-TCG-GAT-TTA-GGA-GGC-TGC-ATA-AAA-GCA-TTA-AAG-TAC-TGT-GCA-GGC-TGG-GCT-GAC
ala-asn-ala-tyr-leu-ser-asp-leu-gly-gly-cys-ile-lys-ala-leu-lys-tyr-cys-ala-gly-trp-ala-asp-138

5' of pG121

415 AAG-ATT-CAT-GGT-CAA-ACA-ATA-CCA-AGT-GAT-GGA-GAC-ATT-TTC-ACT-TAT-ACA-AGA-CGT-GAA-CCT-ATT-GGA-
lys-ile-his-gly-gln-thr-ile-pro-ser-asp-gly-asp-ile-phe-thr-tyr-thr-arg-arg-glu-pro-ile-gly-161

484 GTG-TGT-GGC-CAA-ATC-ATC-CCC-TGG-AAT-TTT-CCA-ATG-CTC-ATG-TTC-ATT-TGG-AAG-ATA-GGC-CCT-GCC-CTT-
val-cys-gly-gln-ile-ile-pro-trp-asn-phe-pro met-leu-met-phe-ile-trp-lys-ile-gly-pro-ala-leu-184

553 AGC-TGT-GGG-AAT-ACC-GTG-GTT-GTC-AAG-CCA-GCA-GAG-CAA-ACT-CCT-CTC-ACG GCT-CTT-CAC-CTG-GCA-TCT
ser-cys-gly-asn-thr-val-val-val-lys-pro-ala-glu-gln-thr-pro-leu-thr-ala-leu-his-leu-ala-ser-207

622 TTA-ATA-AAA-GAG-GCA-GGG-TTT-CCT-CCT-GGC-GTG-GTA-AAC-ATT-GTC-CCT-GGT-TAT-GGG-CCA-ACT-GCA-GGG-
leu-ile-lys-glu-ala-gly-phe-pro-pro-gly-val-val-asn-ile-val-pro-gly-tyr-gly-pro-thr-ala-gly-230

691 GCA-GCC-ATC-TCC-TCT-CAC-ATG-GAT-GTC-GAC-AAG-GTG-GCC-TTC-ACT-GGA-TCA-ACA-CAG-GTT-GGC-AAG-TTA
ala-ala-ile-ser-ser-his-met-asp-val-asp-lys-val-ala-phe-thr-gly-ser-thr-gln-val-gly-lys-leu-253

P1 →

760 ATC-AAG-GAA-GCT-GCA-GGG-AAA-AGC-AAT-CTG-AAG-AGA-GTC-ACC-CTG-GAG-CTG-GGG-GGA-AAG-AGC-CCT-TGC-
ile-lys-glu-ala-ala-gly-lys-ser-asn-leu-lys-arg-val-thr-leu-glu-leu-gly-gly-lys-ser-pro-cys-276

829 ATT-GTG-TTT-GCA-GAT-GCC-GAC-TTG-GAC-ATT-GCT-GTT-GAG-TTT-GCA-CAC-CAT-GGA-GTG-TTT-TAT-CAT-CAA-
ile-val-phe-ala-asp-ala-asp-leu-asp-ile-ala-val-glu-phe-ala-his-his-gly-val-phe-tyr-his-gln-299

P3

898 [GGC-CAA-TGT-TGT-GTC-GCA] GCA-TCC-CGG-ATT-TTT-GTT-GAG-GAG-TCA-GTT-TAT-GAT-GAG-TTT-GTG-AAA-AGG-
[gly-gln-cys-cys-val-ala] ala-ser-arg-ile-phe-val-glu-glu-ser-val-tyr-asp-glu phe-val-lys-arg-322

967 AGT-GTT-GAG-CGA-GCT-AAG-AAA-TAT-GTT-CTT-GGA-AAT-CCT-CTG-ACC-CCA-GGA-ATA-AAT-CAA-GGC CCT-CAG-
ser-val-glu-arg-ala-lys-lys-tyr-val-leu-gly-asn-pro-leu-thr-pro-gly-ile-asn-gln-gly-pro-gln-345

1036 ATT-GAC-AAG-GAA-CAA-CAT-GAT-AAA-ATA-CTC-GAT-CTC-ATT-GAG-AGT-GGG-AAG-AAA-GAA-GGA-GCC-AAA-CTG-
ile-asp-lys-glu-gln-his-asp-lys-ile-leu-asp-leu-ile-glu-ser-gly-lys-lys-glu-gly-ala-lys-leu-368

← P2

1105 GAG-TGT-GGT-GGA-GGA-CGC-TGG-GGG-AAC-AAA-GGC-TTC-TTT-GTG-CAG-CCC-ACA-GTG-TTC-TCC-AAC-GTG-ACT-
glu-cys-gly-gly-gly-arg-trp-gly-asn-lys-gly-phe-phe-val-gln-pro-thr-val-phe-ser-asn-val-thr-391

1174 GAT-GAG-ATG-CGC-ATT-GCC-AAA-GAG-GAG-ATA-TTT-GGA-CCA-GTG-CAA-CAA-ATC-ATG-AAG-TTT-AAG-TCT GTA-
asp-glu-met-arg-ile-ala-lys-glu-glu-ile-phe-gly-pro-val-gln-gln-ile-met-lys-phe-lys-ser-val-414

1243 GAT-GAT-GTG-ATC-AAG-AGA-GCA-AAC-AAT-ACT-ACC-TAT-GGT-TTA-GCA-GCA-GGA-CTC-TTC-ACT-AAA-GAC-CTG-
asp-asp-val-ile-lys-arg-ala-asn-asn-thr-thr-tyr-gly-leu-ala-ala-gly-leu-phe-thr-lys-asp-leu-437

1312 GAT-AAG-GCC-ATC-ACT-GTG-TCA-TCT-GCT-CTG-CAG-GCT-GGG-GTG-GTG-TGG-GTT-AAC-TGC-TAT-ATG-ATG-TTG-
asp-lys-ala-ile-thr-val-ser-ser-ala-leu-gln-ala-gly-val-val-trp-val-asn-cys-tyr-met-met-leu-460

1381 TCA-GCC-CAG-TGC-CCC-TTC-GGT-GGA-TTC-AAG-ATG-TCT-GGA-AAT-GGA-AGA-GAA-CTG-GGT-GAA-CAT-GGT-CTT-
ser-ala-gln-cys-pro-phe-gly-gly-phe-lys-met-ser-gly-asn-gly-arg-glu-leu-gly-glu-his-gly-leu-483

1450 TAT-GAA-TAC-ACT-GAG-CTC-AAG-ACA-GTC-GCA-ATG-AAG-ATA-TCT-CAG-AAG-AAC-TCC-TAAAGAAGCCAGCAGAGTA-
tyr-glu-tyr-thr-glu-leu-lys-thr-val-ala-met-lys-ile-ser-gln-lys-asn-ser-TER

1523 AAGAGAAACTCTCAGCAGTGGCTACACATCTCCTATAGTAACCAGCATAGTCCTGTTTTATTATAATTTCTTCTCCAGTTGATTTCTTAAG-

1614 CAAAAGTAATTCGATATCA

subcloned and sequenced to determine if there were any additional 3' untranslated sequences in SB-2 and to confirm that SB-2 and pG121 were representative of the same cDNA. The 290 bp fragments from both clones were determined to have identical sequences. In addition, the 219 most 3' nucleotides of the 678 bp fragment were identical to the 219 most 5' nucleotides of pG121.

The cDNA library was then re-screened with the 678 bp *Eco* RI/*Pst* I fragment of SB-2 in an attempt to isolate a full length cDNA clone. Ten positive clones were identified during the primary screening of the library. Secondary and tertiary screening gave 8 pure clones hybridizing to the 678 bp fragment. Sizing of the inserts by digestion of the recombinant phage DNA with *Eco* RI (section 2.2.4.5) revealed that none of the clones were larger than the clone SB-2 (sizes ranged from 1.3 to 1.6 kilobases) and did not, therefore, extend beyond SB-2 at the 5' end. The sequence of the 9 most 5' nucleotides of the BALB/c *Ahd-2* coding region and the 5' untranslated region was therefore determined from a BALB/c adult male mouse liver genomic DNA library clone (see section 3.5.1) using P4, a synthetic oligomer generated for this purpose (Table 1).

The complete cDNA sequence obtained for BALB/c *Ahd-2* is given in Figure 11. It represents a total of 1632 nucleotides including 1503 bp coding for a 501 amino acid long polypeptide with a molecular weight of 54,382. In addition, 129 nucleotides of the 3' untranslated region are given. This enzyme is known to be inhibited by Antabuse (Algar and Holmes, 1986). The putative site for the binding of Antabuse is specified by the six amino acids (gly-gln-cys-cys-ile-ala) in human cytosolic ALDH (Hsu et al., 1985). This region is relatively conserved between human cytosolic ALDH and BALB/c *Ahd-2*. Recently a similar study examining

the complete cDNA sequence for BALB/c *Ahd-2*, including the putative polyadenylation signal was published by Rongnoparut and Weaver (1991). Minor differences (5 nucleotide changes including one silent mutation at amino acid 271 and 3 amino acid changes at postions 8, 51, and 87) exist between the two sequences. Rongnoparut and Weaver (1991) also report a 1503 bp reading frame with an 87% identity to human cytosolic acetaldehyde dehydrogenase at the amino acid level.

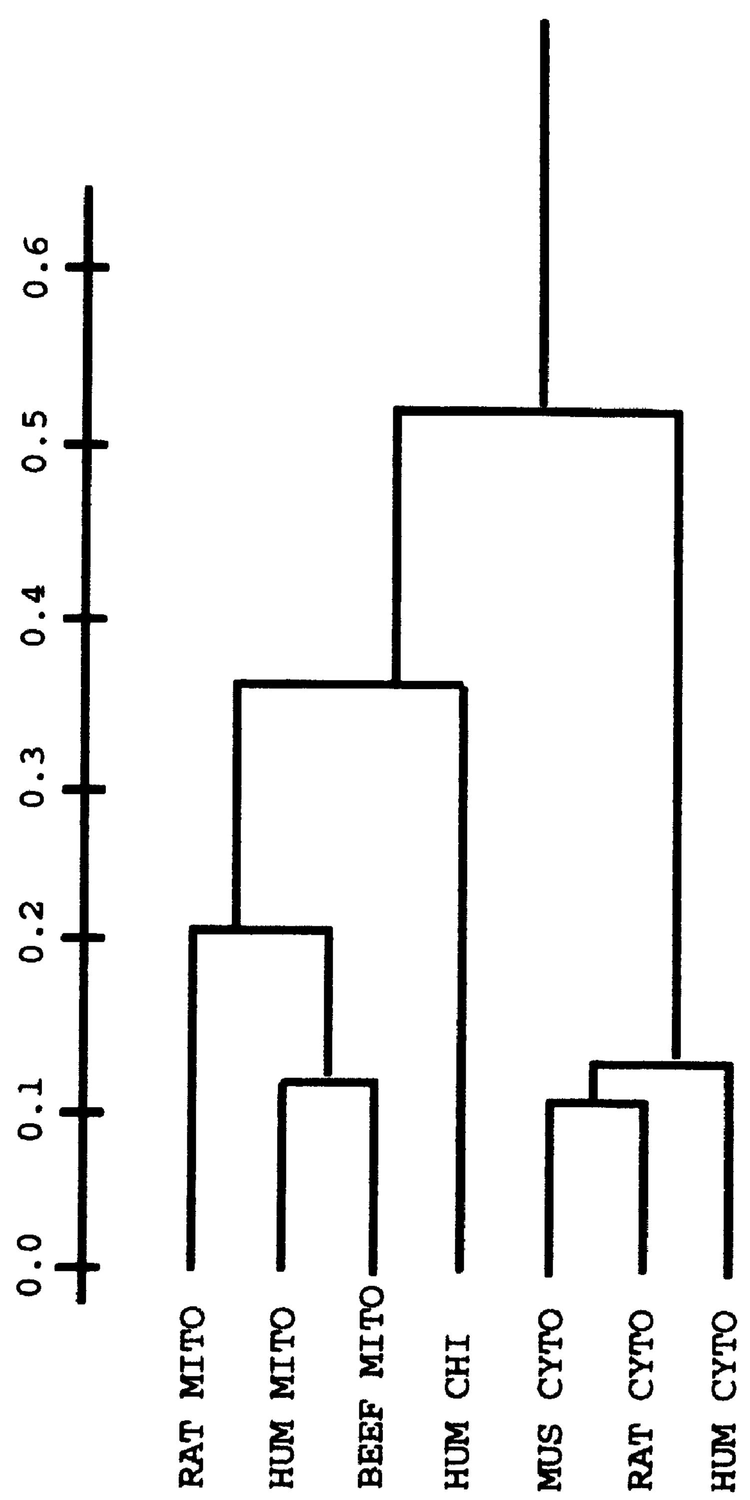
3.4.3 Evolutionary Analysis of the cDNA Sequence.

The BALB/c *Ahd-2* sequence was searched against the GenBank (release 69) and EMBL (release 28) sequence databanks. This search identified a number of sequences similar to BALB/c *Ahd-2*. These sequences belonged to a family of genes encoding the mitochondrial and cytosolic forms of the mammalian aldehyde dehydrogenases. Six sequences with a high percent identity to the BALB/c *Ahd-2* sequence were further evaluated using pair-wise comparisons of the coding regions. These include three ALDH genes from human, two from the rat, one from bovine, and the BALB/c *Ahd-2*. Multiple sequence alignments and genetic distance calculations (Table 5) were performed using the IntelliGenetics Software for Molecular Biology (release 5.1). The Macintosh software package Systat (version 5.1) was used to generate a dendogram (Figure 12) and to assess the similarity of the coding region sequences of the genes. Of the seven apparently related genes evaluated, the three cytosolic ALDH sequences of the human, mouse and rat cluster together, as do the three mitochondrial ALDH sequences of the human, rat, and bovine. Furthermore, the newly reported human ALDHχ sequence appears to

Table 5. A summary of pairwise Euclidean distance measurements calculated using multiple sequence alignments from the IntelliGenetics Software for Molecular Biology. RAT MITO, rat mitochondrial aldehyde dehydrogenase (Farres et al., 1989); HUM MITO, human mitochondrial acetaldehyde dehydrogenase (Hsu et al., 1988); BEEF MITO, bovine mitochondrial aldehyde dehydrogenase (Farres et al., 1989); HUM CHI, human ALDHχ (Hsu and Chang, 1991); HUM CYTO; human cytosolic acetaldehyde dehydrogenase (Hsu et al., 1989); MUS CYTO, BALB/c *Ahd-2*; and RAT CYTC, rat cytosolic aldehyde dehydrogenase (Dunn et al., 1989).

	RAT MITO	HUM MITO	BEEF MITO	HUM CHI	HUM CYTO	MUS CYTO	RAT CYTO
RAT MITO	0.000	0.124	0.127	0.323	0.533	0.535	0.554
HUM MITO	.	0.000	0.119	0.278	0.510	0.511	0.515
BEEF MITO	.	.	0.000	0.302	0.535	0.547	0.556
HUM CHI	.	.	.	0.000	0.557	0.551	0.537
HUM CYTO	.	.	.	.	0.000	0.154	0.173
MUS CYTO	.	.	.	.	.	0.000	0.109
RAT CYTO	.	.	.	.	.	.	0.000

Figure 12. Dendogram generated using the Macintosh program Systat, based upon Euclidean distances. An approximate scale is given. RAT MITO, rat mitochondrial aldehyde dehydrogenase (Farres et al., 1989); HUM MITO, human mitochondrial acetaldehyde dehydrogenase (Hsu et al., 1988); BEEF MITO, bovine mitochondrial aldehyde dehydrogenase (Farres et al., 1989); HUM CHI, human ALDHχ (Hsu and Chang, 1991); HUM CYTO; human cytosolic acetaldehyde dehydrogenase (Hsu et al., 1989); MUS CYTO, BALB/c *Ahd-2*; and RAT CYTO, rat cytosolic aldehyde dehydrogenase (Dunn et al., 1989).


0.0
0.1
0.2
0.3
0.4
0.5
0.6
RAT MITO
HUM MITO
BEEF MITO
HUM CHI
MUS CYTO
RAT CYTO
HUM CYTO

cluster with the mammalian mitochondrial ALDH sequences. These results suggest that the BALB/c cDNA cloned and sequenced here is likely mouse cytosolic ALDH(*Ahd-2*).

Identity among the three cytosolic genes (human, rat, and mouse), that clustered together in the dendogram based on the genetic distance estimates was further evaluated at the level of the DNA sequence and the deduced amino acid sequence. The results of such an alignment are given in Figure 13. All three genes encode for polypeptides with 501 amino acids. No gaps or re-arrangements were needed to align the sequences. All differences among the sequences are accounted for by substitutions. In quantitative terms, there is an identity of 89.1% and 84.7% between the BALB/c *Ahd-2* and the rat and human genes, respectively, at the nucleotide level, within the coding region. The corresponding identity at the deduced amino acid sequence level was 88.2% and 87.2%. Substitutions among these proteins does not appear to be randomly distributed along the length of the polypeptide, as large stretches of the sequence are highly conserved. Figure 13 also identifies the 13 exons of the human cytosolic ALDH gene as reported by Hsu et al., (1989). The authors identified 13 similar exons in the human mitochondrial ALDH gene and have suggested that the two genes must have evolved by means of duplication after incorporation of the introns. If this is so, and the duplication occurred before species divergence, then the three cDNAs shown in Figure 13 are expected to have a similar pattern of exons and introns. Confirmation of this hypothesis must await the study of genomic clones and their organization in the rat and the mouse.

Figure 13. A comparison of the obtained nucleotide sequence for the BALB/c *Ahd-2* cDNA coding region and its deduced amino acid sequence (mus.) with the nucleotide (bottom) and amino acid sequence (top) of rat cytosolic aldehyde dehydrogenase (rat; Dunn et al., 1989) and human cytosolic acetaldehyde dehydrogenase (hum; Hsu et al., 1989) is given in this figure. Dashes represent identical nucleotides or amino acids. Numbering (nucleotides on left-hand side, amino acids on right-hand side) is in relation to 'ATG' translation start site. Known exon-intron boundaries of the human gene (Hsu et al., 1989) are marked by *. The putative Antabuse binding region is underlined.

```
 1                                                                                          *       23
hum.------------ser-gly-thr----asp-leu----val-leu----thr--------------------------tyr------------
rat.-----------------------------------------------------asn-----------------------------------------
mus.met-ser-ser-pro-ala-gln-pro-ala-val-pro-ala-pro-leu-ala-asp-leu-lys-ile-gln-his-thr-lys-ile-
mus.ATG-TCT-TCG-CCT-GCA-CAA-CCT-GCA-GTC-CCT-GCC-CCA-CTG-GCC-GAC-TTG-AAG-ATT-CAA-CAT-ACC-AAG-ATC-
rat.----------C-----------G----------T---------------------A--------------------C-----------
hum.------A---C-T-A--GC-ACG---A--AC-T-A-----T---T---C-A----T-----------------T-----T--------
 70                                                                                                 46
hum.---------------------------asp-----------------------------------phe-------------thr-----
rat.---------------------------asn----leu-asn------------------------ile-------------thr-----
mus.phe-ile-asn-asn-glu-trp-his-ile-ser-val-ser-gly-lys-lys-phe-pro-val-leu-asn-pro-ala-ser-glu-
mus.TTC-ATA-AAC-AAT-GAA-TGG-CAC-AAT-TCA-GTG-AGC-GGC-AAG-AAA-TTT-CCA-GTT-CTT-AAC-CCT-GCA-AGT-GAG-
rat.--T--------------------------C-----T---AT-------------------T---C-A---------------C----A-
hum.-------------------------T-G------------T-------------------T---C-T----T----------C------
 139                                          *                                                     69
hum.----glu-leu-------------------------------glu-----------------------------------------------
rat.----------------his-----------------------------------------------------------------------
mus.glu-val-ile-cys-gln-val-glu-glu-gly-asp-lys-ala-asp-val-asp-lys-ala-val-lys-ala-ala-arg-gln-
mus.GAG-GTC-ATC-TGC-CAG-GTG-GAA-GAA-GGG-GAC-AAG-GCT-GAT-GTT-GAC-AAA-GCT-GTG-AAG-GCT-GCA-AGA-CAG-
rat.------------------T--------------------------A-------------------------------------------
hum.-----AG-C------------A-----------A---T-----AG---------------G---A-----------C-----------
 208                                                                                                92
hum.----------------------------------------------------------------------------------tyr---------
rat.--------------------------------------------------------------------cys--------------------
mus.ala-phe-gln-ile-gly-ser-pro-trp-arg-thr-met-asp-ala-ser-glu-arg-gly-arg-leu-leu-asn-lys-leu-
mus.GCT-TTC-CAG-ATT-GGC-TCT-CCA-TGG-CGC-ACC-ATG-GAT-GCT-TCA-GAG-AGG-GGC-CGC-CTG-CTG-AAC-AAG-CTG-
rat.----------------------C---C------------------------------------A---A-T-------------------
hum.------T-----------A-------G-------T---T-----------------C-----------G---A---A-T-A-T-------T---
 277                                                *                                               115
hum.-------------ile-----------------------------------------------ser-met----------------leu-tyr-
rat.-----------------------------val-----------------------------ser-met----ala----------ile-----
mus.ala-asp-leu-met-glu-arg-asp-arg-leu-leu-leu-ala-thr-met-glu-ala-leu-asn-gly-gly-lys-val-phe-
mus.GCT-GAC-TTA-ATG-GAG-AGA-GAT-CGT-CTG-CTG-CTA-GCT-ACA-ATG-GAG-GCA-CTC-AAT-GGT-GGG-AAA-GTC-TTT-
rat.-----------------------------C-G---------G---------------A-T---A-G-----C----A-----A------
hum.------T-------C---A------------------------G---G--------------T---A-G-----------A-----C----A--
 346                                                                                                138
hum.ser-------------------asn-----------------------------thr-----arg-------------------------------
rat.thr-his-------------leu-----thr-glu-val-ser--------------------------phe--------------------
mus.ala-asn-ala-tyr-leu-ser-asp-leu-gly-ggc-cys-ile-lys-ala-leu-lys-tyr-cys-ala-gly-trp-ala-asp-
mus.GCC-AAT-GCA-TAC-TTG-TCG-GAT-TTA-CGA-GGC-TGC-ATA-AAA-GCA-TTA-AAG-TAC-TGT-GCA-GGC-TGG-GCT-GAC-
rat.A-T-C-----------C-T--T-----AC---AG--T--A--------------C-----------------T----------------A-----
hum.T-------------T-C---AAT----------C-------------C-----A-----G-CGC---------------T-------------
 415                                     *                                                          161
hum.--------gln-----arg-------------ile----------asn-phe----------------------his-----------------
rat.----------------------------------------------val---------------------------------------------
mus.lys-ile-his-gly-gln-thr-ile-pro-ser-asp-gly-asp-ile-phe-thr-tyr-thr-arg-arg-glu-pro-ile-gly-
mus.AAG-ATT-CAT-GGT-CAA-ACA-ATA-CCA-AGT-GAT-GGA-GAC-ATT-TTC-ACT-TAT-ACA-AGA-CGT-GAA-CCT-ATT-GGA-
rat.--------------C-----------T----------------------T-G-----------------------------------G-
hum.------C---G---C--TG---------------T-----------A-T-T-----T---A---------------A---------------T-
 484                             *                                                                  184
hum.------------------------------------------leu-val-----leu-----------------------------------
rat.-------------------------------------gly-----leu-ile-leu------------------------ala---------
mus.val-cys-gly-gln-ile-ile-pro-trp-asn-phe-pro-met-leu-met-phe-ile-trp-lys-ile-gly-pro-ala-leu-
mus.GTG-TGT-GGC-CAA-ATC-ATC-CCC-TGG-AAT-TTT-CCA-ATG-CTC-ATG-TTC-ATT-TGG-AAG-ATA-GGC-CCT-GCC-CTT-
rat.---------------------T---T---------GG----G-T---A-T-T-A----------------------------G----------
hum.--A--------------------T---T-----------C---G-T---G-T-----C--------------------G--------A---G-
 553                                                                                                207
hum.------------------------------------------------------------------------------------val---------
rat.------------------------ile------------------------------------------------tyr-met---------
mus.ser-cys-gly-asn-thr-val-val-val-lys-pro-ala-glu-gln-thr-pro-leu-thr-ala-leu-his-leu-ala-ser-
mus.AGC-TGT-GGG-AAT-ACC-GTG-GTT-GTC-AAG-CCA-GCA-GAG-CAA-ACT-CCT-CTC-ACG-GCT-CTT-CAC-CTG-GCA-TCT-
rat.-------------C---T-----A-----G--------------------------------------A---------T---A------------
hum.----------A---C---A----------------A-----------------------------------T-------C-----G------------
 622        *                                                                                       230
hum.-------------------------------------------------------------------------------------------
rat.-----------------------------------------------val-------------------------ser---------------
mus.leu-ile-lys-glu-ala-gly-phe-pro-pro-gly-val-val-asn-ile-val-pro-gly-tyr-gly-pro-thr-ala-gly-
mus.TTA-ATA-AAA-GAG-GCA-GGG-TTT-CCT-CCT-GGC-GTG-GTA-AAC-ATT-GTC-CCT-GGT-TAT-GGG-CCA-ACT-GCA-GGG-
rat.-----------------------------------T-------G-----G------------------------A-T--------------
hum.-----------------------------------A---A---G---T-------T--------------------T---A-----------
 691                                                                           *                    253
hum.---------------------------------ile-------------------------------------glu----------------
rat.---------------------------------ile----------------ser--------------------glu----------------
mus.ala-ala-ile-ser-ser-his-met-asp-val-asp-lys-val-ala-phe-thr-gly-ser-thr-gln-val-gly-lys-leu-
mus.GCA-GCC-ATC-TCC-TCT-CAC-ATG-GAT-GTC-GAC-AAG-GTG-GCC-TTC-ACT-GGA-TCA-ACA-CAG-GTT-GGC-AAG-TTA-
rat.--------------T------------------C-A-A--------------T-T-------A-------------G--------------A-----
hum.----------T---T-------------------A-A-------A---A-----------A-------------G-----------------G-
```

```
760                                                                                     276
hum.-------------------------------------------------------------------------------------
rat.-------------------------------------------------------------------------------------
mus.ile-lys-glu-ala-ala-gly-lys-ser-asn-leu-lys-arg-val-thr-leu-glu-leu-gly-gly-lys-ser-pro-cys
mus.ATC-AAG-GAA-GCT-GCA-GGG-AAA-AGC-AAT-CTG-AAG-AGA-GTC-ACC-CTG-GAG-CTG-GGG-GGA-AAG-AGC-CCT-TGC-
rat.------A---------------------------------G-----------------T--------------------------
hum.------A-----------C-------------------------G---G-----------T---A--------------------
829                                   *                                                 299
rat.----------------------------------ser-------------------------gln--------phe---
hum.--------leu-----------------------asn----------------------------------------------
mus.ile-val-phe-ala-asp-ala-asp-leu-asp-ile-ala-val-glu-phe-ala-his-his-gly-val-phe-tyr-his-gln
mus.ATT-GTG-TTT-GCA-GAT-GCC-GAC-TTG-GAC-ATT-GCT-GTT-GAG-TTT-GCA-CAC-CAT-GGA-GTG TTT-TAT-CAT-CAA
rat.--------------------T----------T--G-------------------------A-------A---C--TC---C---G-
hum.----------A---T---------------------A-----------A----------------------G--A---C---C---C--G
898                                                                                     322
hum.----------------ile-------------------------------------------ile-----------------arg---
rat.--------ile-------------------------leu---------------------ile-------------------arg----
mus.gly-gln-cys-cys-val-ala-ala-ser-arg-ile-phe-val-glu-glu-ser-val-tyr-asp-glu-phe-val-lys arg-
mus.GGC-CAA-TGT-TGT-GTC-GCA-GCA-TCC-CGG-ATT-TTT-GTT-GAG-GAG-TCA-GTT-TAT-GAT GAG-TTT-GTG-AAA AGG
rat.--T---G-ATT----------------------A-A-C------------------------C-A-----C-------A------T--GG--
hum.------G---------A-A---C-------------A-----------------G---A---A-----A-----------------T-CG
967                                                                                     *345
hum.--------------------------------ile-----------------------------------val-thr---------------
rat.-------------------------------------------------------------asp-ser--------ser--------------
mus.ser-val-glu-arg-ala-lys-lys-tyr-val-leu-gly-asn-pro-leu-thr-pro-gly-ile-asn-gln-gly-pro-gln-
mus.AGT-GTT-GAG-CGA-GCT-AAG-AAA-TAT-GTT-CTT-GGA-AAT-CCT-CTG-ACC-CCA-GGA-ATA-AAT CAA-GGC-CCT CAG-
rat.------G-------G---------------C-------A-------------------GA--T-------------G---------T-------
hum.---------------G-----------G-----A-C---------------------------------------G-C--C---------------
1036                                                                                    368
hum.----------------------tyr----------------------------------------------------------
rat.--------------------------ala-----------------------------------------------------
mus.ile-asp-lys-glu-gln-his-asp-lys-ile-leu-asp-leu-ile-glu-ser-gly-lys-lys-glu-gly-ala-lys-leu-
mus.ATT-GAC-AAG-GAA-CAA-CAT-GAT-AAA-ATA-CTC-GAT-CTC-ATT-GAG-AGT-GGG-AAG-AAA-GAA-GGA-GCC AAA-CTG
rat.---------------G---------C--------C---T-------------------------------------------C---------
hum.---------------------T-----------------T---C--------------------------------------G---------
1105                                                                                    391
hum.----------------------pro-----------------------tyr------------------------------------
rat.-------------------------------------------------------------------------------------
mus.glu-cys-gly-gly-gly-arg-trp-gly-asn-lys-gly-phe-phe-val-gln-pro-thr-val-phe-ser-asn-val thr-
mus.GAG-TGT-GGT-GGA-GGA-?GC-TGG-GGG-AAC-AAA-GGC-TTC-TTT-GTG-CAG-CCC-ACA-GTG-TTC-TCC-AAC-GTG ACT-
rat.-----------------------------------------------------C-------T-------C-----------T------C
hum.--A-------A-----------CG-----------T---------A--------C---------------------T---T---T--A-
1174                                    *                                               414
hum.-------------------------------------------------------------------------------leu-
rat.-------------------------------------------------------------------------------ile-
mus.asp-glu-met-arg-ile-ala-lys-glu-glu-ile-phe-gly-pro-val-gln-gln-ile-met lys-phe-lys-ser-val-
mus.GAT-GAG-ATG-CGC-ATT-GCC-AAA-GAG-GAG-ATA-TTT-GGA-CCA-GTG-CAA-CAA-ATC-ATG-AAG-TTT-AAG-TCT GTA
rat.---------------------------------------------------------------------------------C-A-
hum.--------------------------------------T-----------------------------------A----T---
1243                                                                                    437
hum.-------------------------------------phe--------------ser----------val-------------------ile-
rat.----glu------------------------------pro------------------------------val------------------ile-
mus.asp-asp-val-ile-lys-arg-ala-asn-asn-thr-thr-tyr-gly-leu-ala-ala-gly-leu-phe-thr-lys-asp-leu-
mus.GAT-GAT-GTG-ATC-AAG-AGA-GCA-AAC-AAT-ACT-ACC-TAT-GGT-TTA-GCA-GCA-GGA-CTC-TTC-ACT-AAA-GAC-CTG-
rat.------G-------------------C-----------C------------C-------------------G--------A-----------
hum.------C-----------A-------------------TT--------C-----T-------------G-G---T---C---------A-T-
1312                                                        *                           460
hum.----------------------ile----------------------------thr----------------------------gly-val-val-
rat.----arg------------------------------------------------thr----------------------leu-thr---
mus.asp-lys-ala-ile-thr-val-ser-ser-ala-leu-gln-ala-gly-val-val-trp-val-asn-cys-tyr-met-met-leu-
mus.GAT-AAG-GCC-ATC-ACT-GTG-TCA-TCT-GCT-CTG-CAG-GCT-GGG-GTG-GTG-TGG-GTT-AAC-TGC-TAT-ATG-ATG-TTG-
rat.--C--G--------------------T--------------------C-----ACA-----------G---T---T-----T----CT-C-C-
hum.------A ------A---A-A C---C-------------------------A---A-ACA-----------G---T---------GGC-G---G-A-
1381                                                                      *             483
hum.--------------------------------------------------------------------------tyr----phe-
rat.----val---------------------------------------------------------met----------gln-----val
mus.ser-ala-gln-cys-pro-phe-gly-gly-phe-lys-met-ser-gly-asn-gly-arg-glu-leu-gly-glu-his-gly-leu
mus.TCA-GCC-CAG-TGC-CCC-TTC-GGT-GGA-TTC-AAG-ATG-TCT-GGA-AAT-GGA-AGA-GAA-CTG-GGT-GAA CAT-GGT-CTT
rat.---T--T--------------A---T-------G----------------------------G-C--------A------------G-----G---
hum.AGT----------------------T---------------------------------------------------A---G-T-C-----T-C-
1450                                                                   501
hum.his-------------------val--------------thr-val-----------------------------------
rat.---------------------------------------------------------------------------------
mus.tyr-glu-tyr-thr-glu-leu-lys-thr-val-ala-met-lys-ile-ser-gln-lys-asn-ser-term
mus.TAT-GAA-TAC-ACT-GAG-CTC-AAG-ACA-GTC-GCA-ATG-AAG-ATA-TCT-CAG-AAG-AAC-TCC-TAA
rat.---------------------------------------------------------------------------------
hum.C---------T---A-----G-----A----------A---G-----A---C-------------------A----
```

3.5 Analysis of the 5' Region of *Ahd-2*

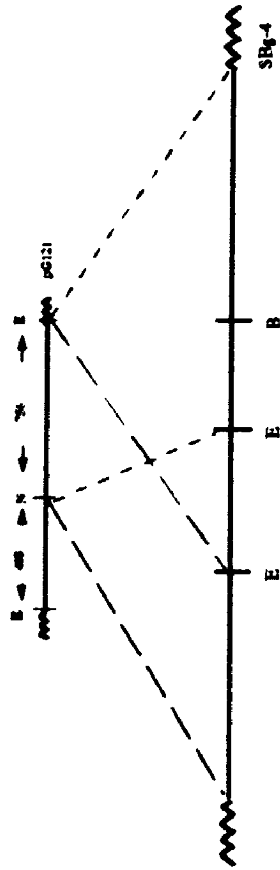
3.5.1 Mapping of the gDNA clones and re-screening of the gDNA library

An adult male BALB/c genomic library in EMBL-3 SP6/T7 (Clonetech) was screened with the 1164 bp *Eco* RI fragment of pG121 (section 2.2.4.2). Fourteen positively hybridizing clones were identified on the primary screen. The secondary and tertiary screenings of these clones gave 6 pure gDNA clones (SBg-3, SBg-4, SBg-5, SBg-7, SBg-8 and SBg-9). Insert size in these six clones ranged from approximately 13 to 16 kb. Three of the clones, SBg-3, SBg-4 and SBg-8, were partially mapped using restriction enzyme digests and Southern blot analysis (section 2.2.4.5). The recombinant gDNA clones were digested with *Xho* I to release the insert from the vector (Clonetech protocol manual for EMBL-3 SP6/T7 genomic libraries, Palo Alto California). Additional enzymes (*Eco* RI and *Bam* HI) were then used to map the inserts. Southern blots of the digests were hybridized with the entire pG121 insert (1164 bp *Eco* RI fragment) , the 5' end (408 bp *Eco* RI/*Nco* I fragment) and the 3' end (756 bp *Nco* I/*Eco* RI fragment) of pG121 (Figure 2). This allowed orientation of the clones and identified overlapping fragments between the clones (Figure 14).

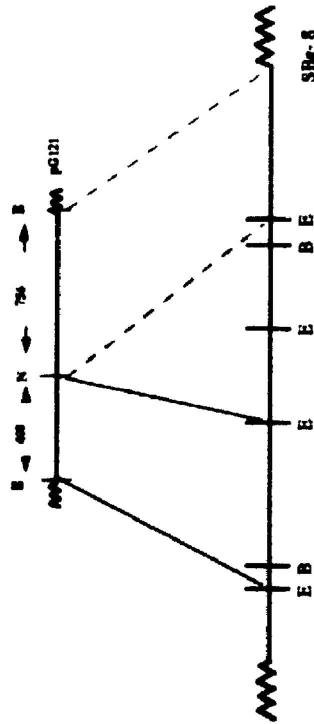
After sequencing of the cDNA for BALB/c *Ahd-2* (section 3.4) was completed, the genomic library was re-screened with the 678 bp *Eco* RI/*Pst* I fragment from SB-2 (Figure 10). This cDNA fragment, which codes for amino acids 4 to 232 in BALB/c *Ahd-2*, hybridized to six recombinant clones in the primary screening. Purification by secondary and tertiary

Figure 14 A partial restriction map of three genomic clones (a) SBg-4, (b) SBg-8 and (c) SBg-3 isolated from a BALB/c EMBL-3 SP6/T7 library is shown. Southern blots were hybridized with a 408 bp *Eco* RI/*Nco* I fragment and 756 bp *Nco* I/ *Eco* RI fragment of pG121, a 3' partial *Ahd-2* cDNA clone (see Figure 2 for details) to determine orientation. Positive hybridization of the cDNA probes to fragments from the gDNA clones is indicated by dashed (756 bp) or plain (408 bp) lines. Inserts were released from the EMBL-3 SP6/T7 vector (represented by the wavy lines) by digestion with *Xho* I. The insert from (a) SBg-4 is approximately 13 kb long, (b) SBg-8, 13 kb and (c) SBg-3, 15 kb. Representation is not to scale. E, *Eco* RI; B, *Bam* HI; N, *Nco* I.

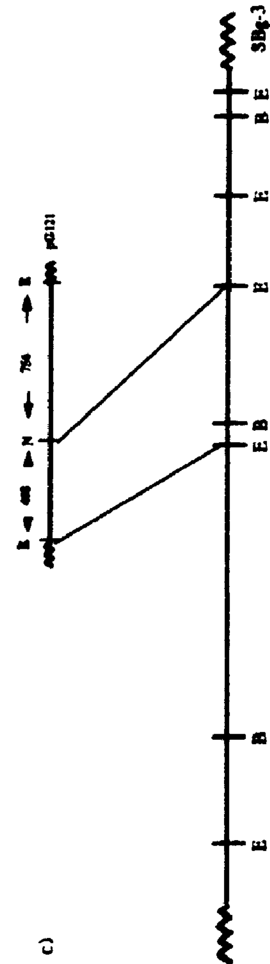
a)



b)



c)



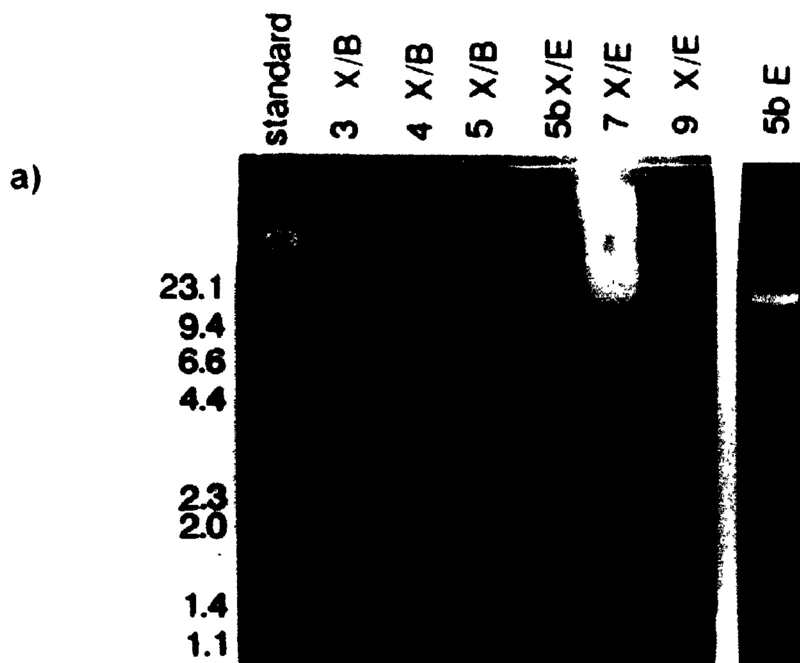
screens yielded one clone, SBg-5b, with an insert approximately 15 kb long, which hybridized to the 678 bp SB-2 cDNA probe. The 5' untranslated region of the *Ahd-2* gene was further isolated using olignucleotide probes and the polymerase chain reaction.

3.5.2 Screening of gDNA Clones with P4, a synthetic oligonucleotide probe

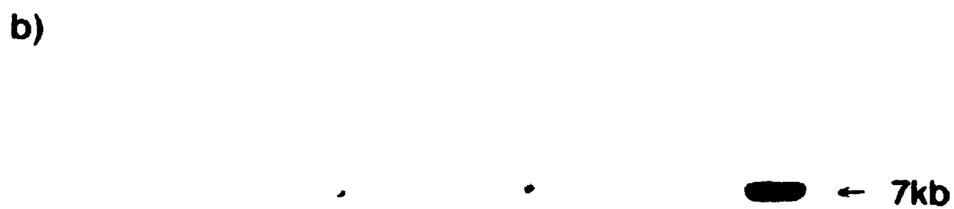
Five recombinant clones from the initial genomic library screening plus clone SBg-5b were then screened with P4, a synthetic oligonucleotide primer corresponding to amino acids 12 to 17 in BALB/c *Ahd-2* (Table 1, Figure 11). Both dot blots and Southern blots (section 2.2.4.6) were hybridized. As shown in Figure 15 (c), clone SBg-5b hybridized to P4, indicating that only clone SBg-5b contains sequences corresponding to the most 5' region of *Ahd-2*. The SBg-5b fragment (cut with *Xho* I and *Eco* RI) hybridizing to P4 is approximately 7 kb long (see Figure 15 [b]). To determine the restriction ends of this 7 kb fragment, clone SBg-5b was digested with *Eco* RI only and re-hybridized to P4. As shown, again a 7 kb fragment hybridizes to the P4 oligonucleotide. There is no *Eco* RI site in EMBL-3 SP6/T7 *Xho* I cut cloning vector and the left and right arm of the vector are 21 and 8 kb, respectively (Clonetech protocol manual for EMBL-3 SP6/T7 genomic libraries, Palo Alto California). This indicates that the 7 kb fragment from SBg-5b hybridizing to P4 represents a fragment arising from the *Ahd-2* insert and has *Eco* RI sites at both ends.

Figure 15. A restriction digest of six recombinant gDNA clones is shown in (a). Lane (1), *Hind* III cut λ /*Hae* III cut ØX 174 DNA standard used to calculate band size; (2), SBg-3 digested with *Xho* I and *Bam* HI; (3), SBg-4 digested with *Xho* I and *Bam* HI; (4) SBg-5 digested with *Xho* I and *Bam* HI; (5) SBg-5b digested with *Xho* I and *Eco* RI; (6), SBg-7 digested with *Xho* I and *Eco* RI; (7), SBg-9 digested with *Xho* I and *Eco* RI; (8) SBg-5b digested with *Eco* RI. The corresponding Southern blot (b) and dot blot (c, spotted with DNA from the clones SB-3, SB-4, SB-5, SB-5b, SB-7, and SB-9) and hybridized to P4 are also shown (c). The 678 bp *Eco* RI/*Pst* I fragment from cDNA clone SB-2 (Figure 10) was included as a positive control in (c).

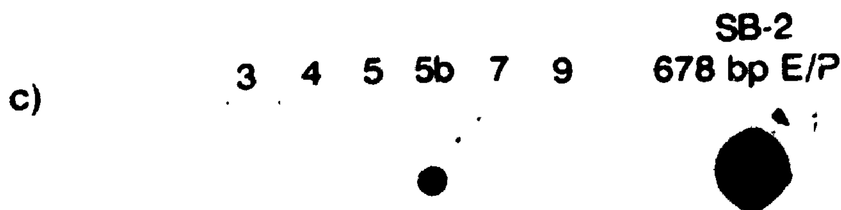
a)



b)



c)



3.5.3 Subcloning and Sequencing of the 5' Region of Ahd-2 in BALB/c

The 7 kb *Eco* RI fragment of SBg-5b was subcloned into *Eco* RI cut dephosphorylated Bluescript sequencing vector as previously described (section 2.2.5). The 5' region of BALB/c *Ahd-2* was then sequenced using a T7 polymerase dideoxynucleotide sequencing kit (section 2.2.5) with P4 as the sequencing primer. This allowed sequencing of the 5' end of the BALB/c *Ahd-2* coding region (amino acids 1 to 12) as well as the 5' region of the gene without sequencing the entire 7 kb fragment. A second primer, P8 a 24-mer located 482 nucleotides upstream from the ATG start site (Table 1), was synthesized and used to confirm the sequence in the opposite direction.

The 448 bp BALB/c *Ahd-2* 5' sequence from the 7 kb fragment of SBg-5b is shown in Figure 16. An extended 545 nucleotide sequence section 3.5.5), which also included 16 nucleotides of the coding region and the 5' untranslated region was used in a Fast Pairwise Comparison of Sequences (Fast DB IntelliGenetics release 5.1) with the EMBL and GenBank sequence data banks (Figure 18). This comparison identified the corresponding 5' region of human cytosolic acetaldehyde dehydrogenase (*Aldh-1*) as having a residue identity of 72% to this sequence. No other sequences in the sequence data banks showed a comparable identity. The BALB/c *Ahd-2* 5' region sequence published by Rongnoparut and Weaver (1991) has one minor difference, a missing 'c' at position -144, from the sequence shown in Figure 16.

The 5' *Ahd-2* sequence was also analysed using Quest, Quick User-directed Expression Search Tool, IntelliGenetics (release 5.4). Quest is

Figure 16. A 448 bp sequence corresponding to the 5' end of BALB/c *Ahd-2* sequenced from SBg-5b, a recombinant clone from a BALB/c EMBL-3 SP6/T7 genomic library (Clonetech, Palo Alto California) is shown. The putative CCAAT boxes, -46 to -42 and -116 to -112 and TATA box, -74 to -68 (numbers in relation to the 'ATG' translation start site) are underlined. The triplet nucleotide sequences in bold represent the *Ahd-2* coding region, capital letters represent probable transcribed sequence, and small letters represent untranscribed sequence.

tcctcttcatttcaatgcctttgttccggagtctgttagagaagaaaaagttacacagtagcataacaaagca -360

ggaaaagggaatggaaaaaaaaaataataactggcttccagtgtctggagcagctgcacacacacccttagca -287

caggtttggcttggtaaattaattcatctgtaaatagtgagcagtctccagatagaacttcagggctgaggtg -214

caggttccttaaggatttacatgtaaaggcaaaggctttcagccctaggtgttacaagtgagtggtgcccttc -141

atgccctgccctgagtctgcccatccaatcatatcccgatatgcaaatgacccttagtgcatgcagataaaaa -68

ggagcaagggctctttcagaaccaatttgctgagcCTGTCACCTGTGTTCCAGGAGCCAAACCAGC**AATG-TC** +5

T-TCG-CCT-GCA-C

useful in identifying sequences important in regulatory functions. This extensive search located a nuclear factor binding site reported as the Moloney Murine Leukemia viral enhancer sequence (LVa, GAACAG) at position -445 to -452 (Figure 18) (Speck and Baltimore, 1987). Four locations for the Moloney Murine Leukemia viral enhancer sequence LVc (CCTGC) were also detected at positions -132 to -136 and at positions +10 to +14, +19 to +23, and +28 to +32 in the coding region (Figure 18). The biological significance of these sequences remains unknown.

Two putative CCAAT boxes were also identified and are underlined in Figure 16. The putative TATA box was identified by examining the 5' promoter region of similar genes. The relative position of these consensus sequences is identical to those identified for .LB/c *Ahd-2* by Rongnoparut and Weaver (1991) and similar to those for human cytosolic acetaldehyde dehydrogenase (ALDH-1, Hsu et al., 1989) and rat cytosolic aldehyde dehydrogenase (Dunn et al., 1989). The same sequence is found for the putative TATA box for all three species (ATAAAAA).

Analysis of the BALB/c *Ahd-2* 5' region using the DNA Inspector IIe revealed eight sequences which share identity (8 of 9 nucleotides) with the C/EBP binding consensus sequence (T[T/G]NNG[C/T]AA[T/G]; Ryden and Beemon, 1989). Summaries of these sequences, their relative positions and similar sequences in the 5' regions of human cytosolic ALDH, human mitochondrial ALDH, human ALDHχ, and the rat cytosolic ALDH are given in Tables 6 and 7. C/EBP is a rat liver transcription factor known to bind to the human *Adh-2* promoter at two sites surrounding the TATA box (Stewart et al., 1990). The sequences CTTAGTGCA (position -88 to -80) and AGGAGCAAG (position -68 to -60), in the BALB/c *Ahd-2* promoter also surround the TATA box (-74 to -68), (Table 6 and Figure

Table 6. A summary of the sequences similar to the C/EBP binding consensus sequence (Ryden and Beemon, 1989) found in the BALB/c *Ahd-2* 5' region using the DNA Inspector IIe. Location is given relative to the ATG translation start site. Coding or non-coding strand is indicated.

Gene	Sequence (5' to 3')	Location	Strand
BALB/c *Ahd-2*	TTCCTTAAG	-209 to -201	coding
	TGTTACAAG	-165 to -156	coding
	AGGAGCAAG	-68 to - 60	coding
	CTTAGCACA	-293 to -285	non-coding
	CTTGGTAAA	-277 to -268	non-coding
	ATTAATTCA	-269 to -261	non-coding
	CTTAGTGCA	-88 to -80	non-coding
	CTTTCAGAA	-55 to -47	non-coding

Table 7. A summary of the sequences similar to the C/EBP binding consensus sequence (Ryden and Beemon, 1989) found in the human *Aldh-1*, human *Aldh-2*, human *ALDH*χ and the rat cytosolic aldehyde dehydrogenase 5' region using the DNA Inspector IIe.

Gene	Sequence (5' to 3')	Reference
Human *Aldh-1*	TGGAGCACT	Hsu et al., 1989
	TTTCTTAAG	
	CTTATCACA	
	ATTAATTCA	
	ATTGCTGAG	
Human *Aldh-2*	CTTAATAAA	Hsu et al., 1988
	CTTTCCGCA	
	CTAGCGCCA	
	CTTGCATCA	
Human ALDHχ	TTTTCCAAG	Hsu and Chang, 1991
	TGCGGCCAG	
	CTTGCAGCC	
	CTTGCGGAG	
	CCTGCTGCA	
Rat cytosolic aldehyde dehydrogenase	TGGTGTAGG	Dunn et al., 1989
	TTCAGGAAG	
	TTTACCAAG	
	AGGAGCAAG	
	CTTAGCACA	
	ATTAATTCA	
	CTTGCAGAT	
	CTTCAAGAA	

16). Regions with similar sequences binding mouse liver nuclear extracts have also been located in the mouse *Adh-1* gene (Stewart et al, 1990, Zhang et al., 1987, Carr et al., 1989). The sequence AGGAGCAAG is found in both the BALB/c *Ahd-2* promoter region and in the rat cytosolic aldehyde dehydrogenase promoter. In both cases it is directly downstream from the TATA box. In all instances, except for one perfect match (CTTGCATCA, found in the human mitochondrial gene), 8 of the 9 nucleotides are the consensus C/EBP sequence (Tables 6 and 7).

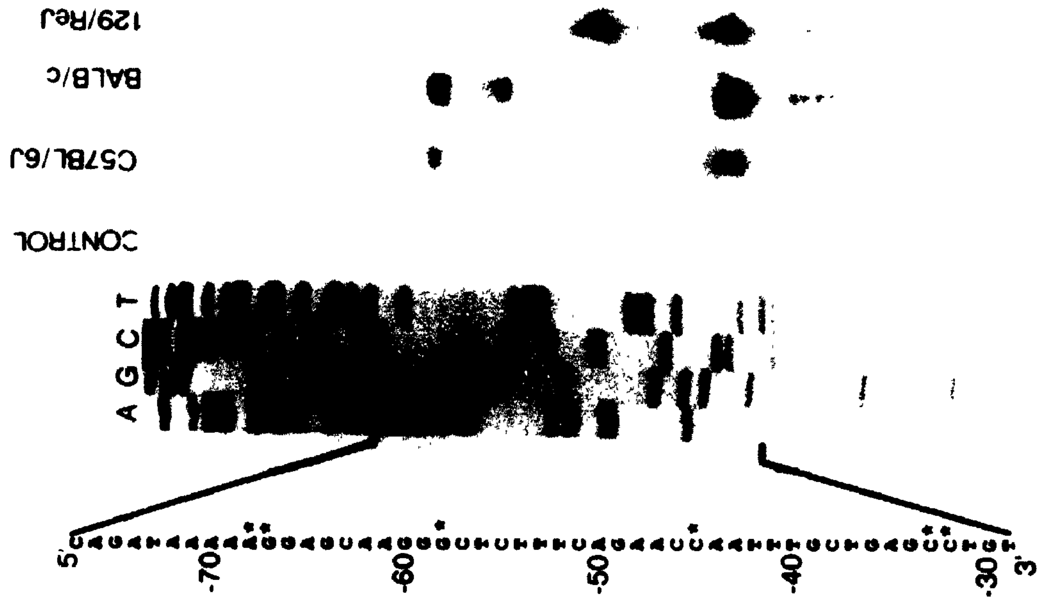
3.5.4 Primer Extension Analysis of Ahd-2 in Three Strains of Mice

Primer extension analysis permits localization of putative transcription start points (tsps) for transcribed genes. To determine the tsps for mouse *Ahd-2*, P4, a 5' oligonucleotide primer (Table 1) was labelled with γ ^{32}P and hybridized to 5 µg of total RNA isolated from the livers of three strains of mice (BALB/c, C57BL/6J and 129/ReJ) along with 5 µg of yeast tRNA as a negative control. The hybridized primer was then extended using MMLV reverse transcriptase to give a labelled cDNA (section 2.2.8). This DNA fragment was electrophoresed on an 8% sequencing gel along with the BALB/c 5' *Ahd-2* sequence using the 7 kb *Eco* RI fragment from SBg-5b and the same P4 primer.

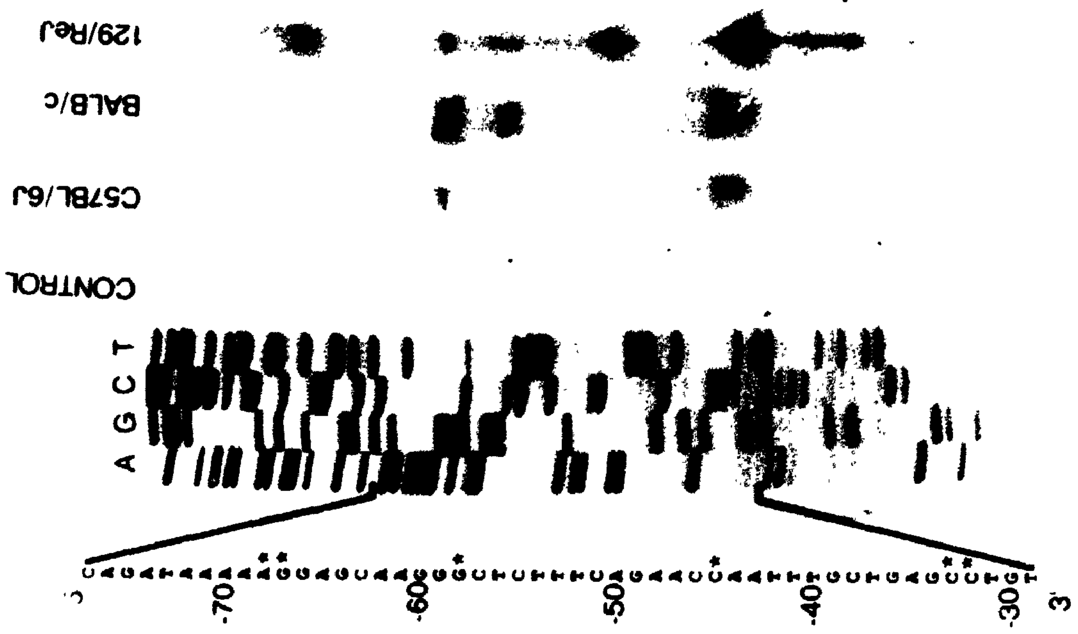
A number of possible tsps were found for *Ahd- 2* in the three mouse strains. No bands were visible with the negative control, yeast tRNA (Figure 17 [a and b]). Major bands were found in all three strains indicating possible tsps 32 and 33 nucleotides upstream from the ATG start site (Figure 17 [a]). This would place the TATA and CCAAT boxes at 35

Figure 17. (a) Primer extension analysis of *Ahd-2* mRNA in three inbred strains of mice. Primer extension products were generated using 5 μg of total liver RNA isolated from C57BL/6J (lane 2), BALB/c (lane 3) 129/ReJ (lane 4) or 5 μg yeast tRNA (control, lane 1) and resolved on an 8% sequencing gel are shown. Also shown are the four dideoxynucleotide sequencing lanes generated using the same primer and the 7 kb *Eco* RI fragment of SBg-5b. The sequences to the left of the sequencing reactions are written 5' to 3'. Possible transcription start sites (tsps) are marked with asterisks. Numbering corresponds to the position relative to the 'ATG' translation start site. No bands were visible with the negative control (yeast tRNA).

(b) Results of a repeated experiment using different RNA samples.



b)



a)

and 79 nucleotides upstream from the transcription start site, around the expected consensus range. The location of the TATA box varies in eukaryotic genes, generally falling 26 to 34 nucleotides upstream from the transcription start site (Breathnach and Chambon, 1981). Similarly CCAAT boxes have been detected 70 to 80 nucleotides upstream. RNA from the strain 129/ReJ also produced a minor band indicating a tsp 45 nucleotides upstream from the ATG start site. This is the same as the distance between the tsp and the ATG translation start site in rat cytosolic aldehyde dehydrogenase (Dunn et al., 1989). Another study examining possible tsps in BALB/c *Ahd-2* reported bands at positions -43 and -44 (Rongnoparut and Weaver, 1991). These positions were not indicated in any of the samples examined in this study (see Figure 17). In addition, bands were also found indicating tsps at position -67 and -68 (relative to ATG). These bands were found in all three strains. Primer extended RNA from BALB/c and C57BL/6J mice but not 129/ReJ, had a band indicating nucleotide -58 as a possible tsp. This experiment was repeated with similar results. In the second experiment RNA from the strain 129/ReJ did not produce a band at -67, all other bands remained the same (Figure 17 [b]). The multiple bands seen in the primer extension may be due to binding of P4 to mRNA other than that of *Ahd-2* or secondary structure in the mRNA which causes shorter than expected extended products (Hsu et al., 1988; Ausubel et al., 1987).

3.5.5 PCR Amplification and Sequencing of the 5' Region of Ahd-2 in Three Strains of Mice

The 5' region of *Ahd-2* was PCR amplified from genomic DNA of C57BL/6J, BALB/c and 129/ReJ mice using P4 and P8 (Table 1), the primers previously used for sequencing of the 5' region (section 2.2.6.1). A fragment 545 nucleotides in length was obtained for all three strains following PCR amplification. This fragment was then subcloned into Bluescript. Individual recombinant clones for each strain were picked, the DNA isolated, and sequenced in both directions. Figure 18 shows the complete genomic sequence of this region in the three strains It is evident from this figure that there are no differences in the transcribed region of the gene, which represents approximately 80 bases in this amplification, 51 of which encode for the first 17 amino acids. There are only two minor differences among the three strains in the remaining untranscribed nucleotides sequenced. Unlike BALB/c and C57BL/6J, 129/ReJ has an additional 'ac' dinucleotide at position -364 to -365. This deletion and a missing 'a' at position -326 seen in the BALB/c amplified sequence, however, were not observed in the BALB/c *Ahd-2* promoter sequenced from the EMBL-3 SP/T7 genomic library clone SBg-5b (Figure 16). It is therefore concluded that the 5' region of this gene is extremely conserved among the strains. It may be pointed out that the gene pool of the three strains studied has remained separated for over 70 years or approximately 400 to 500 generations (Green and Witham, 1990) and the identical sequences observed among the strains at this region of the *Ahd-2* may be due to some still uncharacterized selective regime. Furthermore, the

Figure 18. A 545 bp fragment corresponding to the 5' region of *Ahd-2*, was PCR amplified using two primers (P4 and P8, Table 1, marked with arrows) from genomic DNA isolated from three strains of mice (BALB/c, C57BL/6J and 129/ReJ). Alignment of this sequence from the three strains is shown in this figure. The triplet nucleotide sequences represent the *Ahd-2* coding region, capital letters represent probable transcribed sequence, and small letters represent untranscribed sequence. Dots represent identical nucleotides, dashes represent gaps inserted to ensure alignment. Numbering is in relation to the 'ATG' translation start site of longest sequence.

```
         P8 ------------->
129/REJ    .........................................................................................
BALB/c     gttaggcagatttgtggttctctgctccgtatctcctcgtgagaacagaggtctctttagactcctcttcatttcaatgcctttgttcc   -406
C57BL/6J   .........................................................................................

129/REJ    ...........................ac........................................a...................
BALB/c     ggagtctgttagagaagaaaaagttac--agtagcataacaaagcaggaaaagggaatggaaaaaaaaa-taataactggcttccagtg   -317
C57BL/6J   ...........................--........................................a...................

129/REJ    .........................................................................................
BALB/c     tctggagcagctgcacacacacccttagcacaggtttggcttggtaaattaattcatctgtaaatagtgagcagtctccagatagaact   -228
C57BL/6J   .........................................................................................

129/REJ    .........................................................................................
BALB/c     tcagggctgaggtgcaggttccttaaggatttacatgtaaagacaaaggctttcagccctaggtgttacaagtgagtggtgcccttcat   -139
C57BL/6J   .........................................................................................

129/REJ    .........................................................................................
BALB/c     gccctgccctgagtctgcccatccaatcatatcccgatatgcaaatgacccttagtgcatgcagataaaaaggagcaaggctctttca   -50
C57BL/6J   .........................................................................................

129/REJ    ................. ...................................-...-...-...-...-...-...-...-...-...
BALB/c     gaaccaatttgctgagc CTGTCACCTGTGTTCCAGGAGCCAAACCAGCAATG-TCT-TCG-CCT-GCA-CAA-CCT-GCA-GTC-CCT   +30
C57BL/6J   ................. ...................................-...-...-...-...-...-...-...-...-...

                <------------- P4
129/REJ    ...-...-...-...-...-...-...
BALB/c     GCC-CCA-CTG-GCC-GAC-TTG-AAG   +51
C57BL/6J   ...-...-...-...-...-...-...
```

apparent differences observed among the three strains for ethanol sensitivity cannot be accounted for by differences in the sequence of the 5' region of *Ahd-2*.

3.5.6 Evolutionary Analysis of the Promoter Sequence

Euclidean distance measurements among the promoter regions (494 bp upstream from the ATG translation start site) of BALB/c *Ahd-2*, the human cytosolic acetaldehyde dehydrogenase (Hsu, 1989), the human ALDHχ (Hsu and Chang, 1991), and the human mitochondrial aldehyde dehydrogenase (Hsu et al., 1988) were determined by computer analysis (Table 8). Multiple sequence alignments and related genetic distance calculations were performed using The IntelliGenetics Software for Molecular Biology. The Macintosh software package Systat was used to generate dendograms based upon Euclidean distances. As shown in Figure 19, the 5' sequence of the BALB/c *Ahd-2* gene clusters most closely with the 5' sequence of the human cytosolic aldehyde dehydrogenase gene. Unlike the coding region, however, the BALB/c *Ahd-2* 5'sequence does not show a high percent identity to rat cytosolic aldehyde dehydrogenase (data not shown; Dunn et al., 1989).

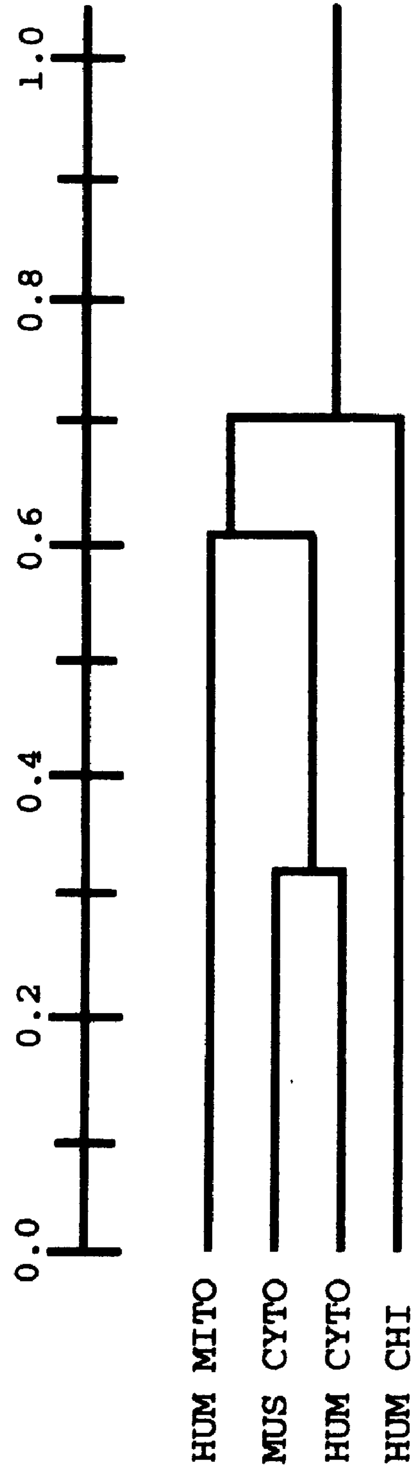
3.5.7 The Effect of Ethanol Feeding on Gene Transcription

To determine if differences in the levels of *Adh-1* and *Ahd-2* mRNA in ethanol-fed mice as compared to the genotype, sex and age-matched control-fed mice (section 3.2) were due to differences in transcription following ethanol feeding or differential stability of the message,

Table 8. A summary of Euclidean distance measurements calculated for the 494 bp of the 5' promoter region using multiple sequence alignments from the IntelliGenetics Software for Molecular Biology. HUM CYTO; human cytosolic acetaldehyde dehydrogenase (Hsu et al., 1989); MUS CYTO, BALB/c *Ahd-2*; HUM MITO, human mitochondrial acetaldehyde dehydrogenase (Hsu et al., 1988); and HUM CHI, human ALDHχ (Hsu and Chang, 1991).

	HUM CYTO	MUS CYTO	HUM MITO	HUM CHI
HUM CYTO	0.000	0.328	0.551	0.996
MUS CYTO	.	0.000	0.996	0.587
HUM MITO	.	.	0.000	0.996
HUM CHI	.	.	.	0.000

Figure 19. A dendogram generated using the Macintosh program Systat based upon Euclidean distances between the 5' region of four different ALDH genes. An approximate scale is given. HUM CYTO; human cytosolic acetaldehyde dehydrogenase (Hsu et al., 1989); MUS CYTO, BALB/c *Ahd-2*; HUM MITO, human mitochondrial acetaldehyde dehydrogenase (Hsu et al., 1988); and HUM CHI, human ALDHχ (Hsu and Chang, 1991).


0.0
0.2
0.4
0.6
0.8
1.0
HUM MITO
MUS CYTO
HUM CYTO
HUM CHI

transcription run-off experiments were attempted. In this procedure (see 2.2.9 for experimental details) nuclei were isolated from the livers of ethanol-fed and control mice of the three strains (BALB/c, C57BL/6J, and 129/ReJ). The nuclei were then incubated for a short period of time with ribonucleotides and ^{32}P labelled UTP and the RNA then extracted from the mixture. It is hypothesized that new RNA transcripts are not initiated *in vitro* during this protocol, but those which were initiated at the time of nuclei extraction are faithfully elongated (Ausubel et al., 1987). Thus, a reasonable assessment of the level of transcription occuring in the cell at the time of isolation can be made.

It was possible to obtain an average of 1.1×10^8 nuclei per mouse liver ($1.1 \times 10^8 \pm 1.2 \times 10^7$, mean ± S.E.M., n = 12), with no significant difference between the number of nuclei obtained for control-fed animals ($9.4 \times 10^7 \pm 1.4 \times 10^7$, mean ± S.E.M., n = 7) and ethanol-fed animals ($1.3 \times 10^8 \pm 1.7 \times 10^7$, mean ± S.E.M., n = 5), as assessed by a Student's t-test. No strain dependent differences were apparent. When equal numbers of nuclei from control and experimental animals were used in the RNA elongation mixture, differences in the level of labelled RNA, however, were found. The c.p.m. for labelled RNA isolated from the nuclei of control-fed animals ranged from 4.2×10^5 to 5.7×10^6 c.p.m. ($2.8 \times 10^6 \pm 1.1 \times 10^6$, mean ± S.E.M., n = 4) while that for ethanol-fed animals ranged from 1.5×10^4 to 1.8×10^6 c.p.m. ($1.0 \times 10^6 \pm 3.4 \times 10^5$, mean ± S.E.M., n = 5). Again, no strain dependent differences were apparent.

The c.p.ms. of the RNA isolated from the nuclei of control and ethanol-fed mice hybridized to the dot blots of *Adh-1* and *Ahd-2* cDNA clones must be equal in order to compare gene-specific transcription levels in the samples. Thus a low number of counts, needed to equilibrate the two

test groups, and subsequently a very small amount of labelled RNA was used in the hybridizations. This resulted in autoradiograms with little hybridization signal apparent above the background. Other recent studies have also shown that overall RNA transcription is significantly reduced in various tissues of alcohol-fed animals (Preedy and Peters, 1990). The results obtained during this research indicated an apparent reduction in RNA synthesis in ethanol-fed animals. However, it was not possible to quantitate the effect of ethanol on the transcription of specific genes, particularly those involved in ethanol metabolism (*Adh-1* and *Ahd-2*), in the three strains of mice.

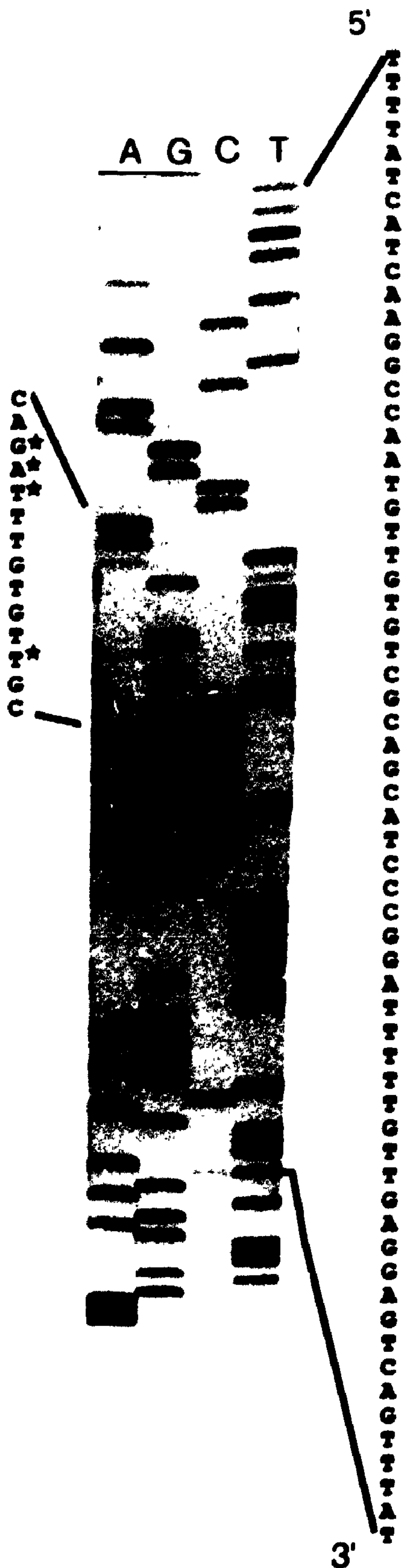
3.6 Molecular Analysis of the Putative Antabuse Binding Region

3.6.1 PCR Amplification and Sequencing

Sequencing of the BALB/c *Ahd-2* cDNA (section 3.4) revealed a single amino acid difference in the putative Antabuse binding region between human and mouse cytosolic acetaldehyde dehydrogenase (gly-gln-cys-cys-ile-ala in human and gly-gln-cys-val-ala in BALB/c, amino acid positions 300 to 305, Figure 13). The isoleucine-valine alteration, however, is a conservative one, with the amino acids being very similar in charge and size (Lehninger, 1975). In order to examine the possibility that amino acid changes in this region of the *Ahd-2* gene may contribute to the relative ethanol sensitivity of different strains of mice, a 369 nucleotide fragment, surrounding this region was PCR amplified and sequenced.

Total liver RNA from two C57BL/6J mice, two BALB/c mice, a 129/ReJ mouse, two mice from C57BL/6J x BALB/c recombinant inbred lines, #2 and #13 (in $^{+}$40 generations of brother-sister matings and apparently homozygous at almost all loci) was first reverse transcribed using MMLV and then PCR amplified (RT-PCR) using P_1 and P_2 (see Table 1). The resulting DNA fragment, 369 nucleotides in length, was then subcloned into Bluescript and transformed as previously described (section 3.5.5). However, instead of plating the transformation mixtures and selecting a single colony for DNA isolation and sequencing, the entire transformation mixture was used (section 2.2.7.2) for DNA isolation. The DNA was sequenced using P_1 and P_2 as sequencing primers. The reactions were then resolved on an 8% polyacrylamide sequencing gel, as previously described (section 2.2.5). As shown in Figure 20, both major and minor bands are apparent on the sequencing gels. In all cases the major bands in the sequence were identical to the original BALB/c *Ahd-2* cDNA sequence (except for a silent mutation in the third position of codon 302 from 'T' to 'C' in 129/ReJ and the two recombinant inbred lines, Figure 21). Approximately 180 nucleotides surrounding the Antabuse binding region were read for all genotypes. In some instances it was not possible to read the sequences in both directions due to the background caused by the minor bands. The five diverse genotypes of mice having an almost identical sequence in this region of the gene suggests that there is a selective pressure on this sequence. The minor bands visible on the sequencing gel indicate the presence of a sequence other than *Ahd-2* in the PCR reaction. Thus, the PCR fragments derived from the RT-PCR amplification of the total liver RNA from these mice contained at least two different but related sequences. This indicates that there are at least two related genes

Figure 20. A photograph showing the major and minor bands visible in sequencing reactions from the transformation mixture of the 369 nucleotide RT PCR fragments from (a) C57BL/6J and (b) BALB/c total liver RNA. Sequences on the right are written 5' to 3'. A partial sequence is read using the minor bands on the left in (a). Differences between the sequences of the major and minor bands are noted with asterisks. Arrows indicated the presence of more than one minor band at a particular site Note that the minor bands were clearly readable in (a), but not in (b).


a)
A G C T
5'
3'
TTTATCATCAAGGCCAATGTTGTGTCGCAGCATCCCGGATTTTTGTTGAGGAGTCAGTTTAT
CAGATTTGTGTTGC

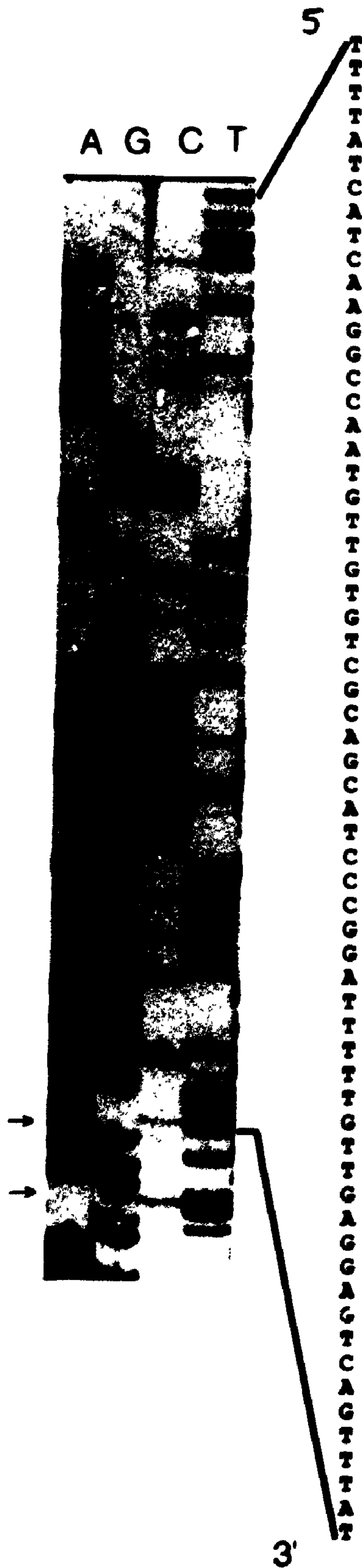

b)
A G C T
5'
3'
TTTATCATCAAGGCCAATGTTGTGTCGCAGCATCCCGGATTTTTGTTGAGGAGTCAGTTTAT

Figure 21. The sequence (major bands) of the putative Antabuse binding region following RT-PCR amplification from two C57BL/6J mice (C57Bl/6J-1, C57BL/6J-2), two BALB/c mice (BALB/c-1, BALB/c-2), a 129/ReJ mouse, a C57BL/6J x BALB/c recombinant inbred line #2 (RI # 2) and a recombinant inbred line #13 (RI # 13) mouse is given in this figure. Dashes represent identical nucleotides. The putative Antabuse binding region is underlined. Numbering is in relation to the 'ATG' translation start site. Note the silent mutation in 129/ReJ, RI #2, and RI #13.

```
             856
C57BL/6J-1   ATT-GCT-GTT-GAG-TTT-GCA-CAC-CAT-GGA-GTG-TTT-TAT-CAT-
C57BL/6J-2   ----------------------------------------------------
BALB/c-1     ----------------------------------------------------
BALB/c-2     ----------------------------------------------------
129/ReJ      ----------------------------------------------------
RI#2         ----------------------------------------------------
RI#1         ----------------------------------------------------

             895
C57BL/6J-1   CAA-GGC-CAA-TGT-TGT-GTC-GCA-GCA-TCC-CGG-ATT-TTT-GTT-
C57BL/6J-2   ----------------------------------------------------
BALB/c-1     ----------------------------------------------------
BALB/c-2     ----------------------------------------------------
129/ReJ      --------------C-------------------------------------
RI#2         --------------C-------------------------------------
RI#13        --------------C-------------------------------------

             934
C57BL/6J-1   GAG-GAG-TCA-GTT-TAT-GAT-GAG-TTT-GTG-AAA-AGG-AGT-GTT-
C57BL/6J-2   ----------------------------------------------------
BALB/c-1     ----------------------------------------------------
BALB/c-2     ----------------------------------------------------
129/ReJ      ----------------------------------------------------
RI#2         ----------------------------------------------------
RI#13        ----------------------------------------------------

             973
C57BL/6J-1   GAG-CGA-GCT-AAG-AAA-TAT-GTT-CTT-GGA-AAT-CCT-CTG-ACC-
C57BL/6J-2   ----------------------------------------------------
BALB/c-1     ----------------------------------------------------
BALB/c-2     ----------------------------------------------------
129/ReJ      ----------------------------------------------------
RI#2         ----------------------------------------------------
RI#13        ----------------------------------------------------

            1012
C57BL/6J-1   CCA-GGA-ATA-AAT-CAA-GGC-CCT-CAG-ATT-GAC-AAG-GAA-
C57BL/6J-2   ------------------------------------------------
BALB/c-1     ------------------------------------------------
BALB/c-2     ------------------------------------------------
129/ReJ      ------------------------------------------------
RI#2         ------------------------------------------------
RI#13        ------------------------------------------------
```

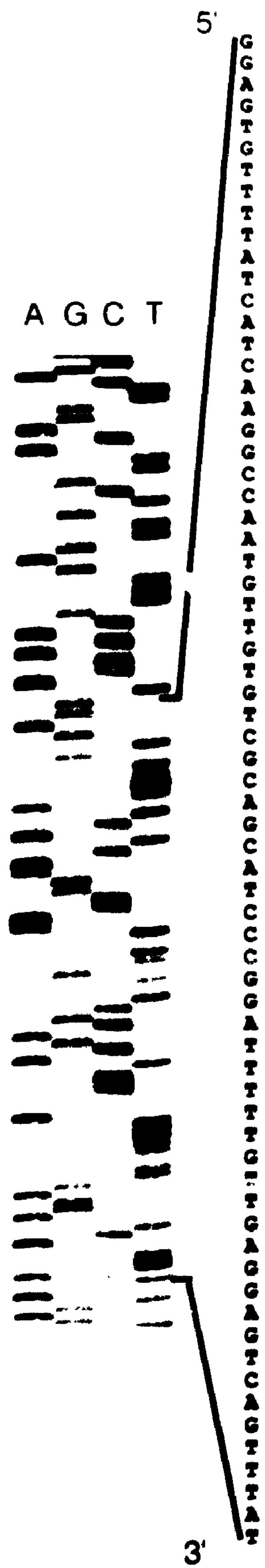
containing sequences complementary to P_1 and P_2, being transcribed in the mouse liver. The presence of additional sequences in the reaction mixture was not expected as the other major aldehyde dehydrogenase product in the mouse liver (*Ahd-5* transcript) is not believed to have a large sequence identity to *Ahd-2* (Manthey and Sladek., 1989; Jornvall et al., 1987). The experiments were repeated, with similar results, to rule out the possibility of PCR induced errors.

3.6.2. Partial Sequence of a cDNA Related to Ahd-2.

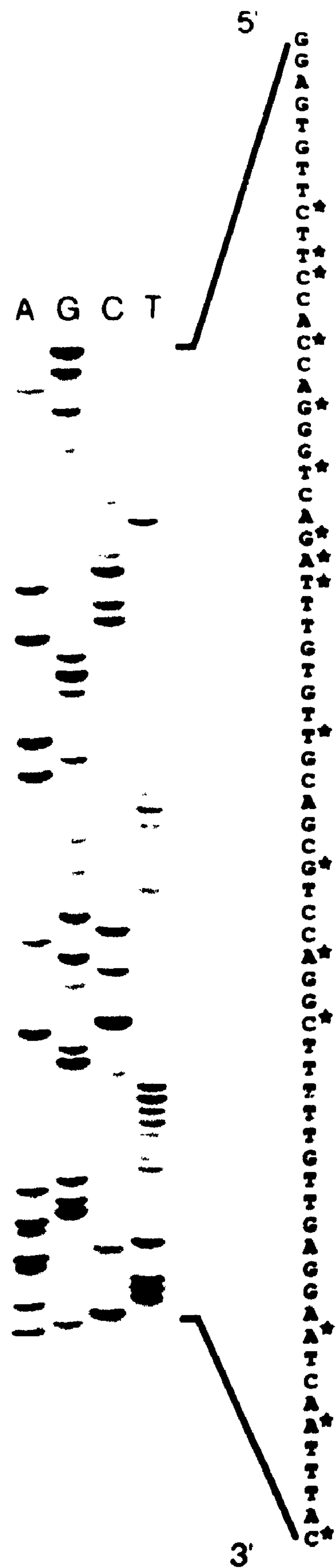
In order to examine the sequence of the transcripts related to *Ahd-2* detected in the liver it was necessary to use a different sequencing procedure. Total liver RNA from a C57BL/6J mouse was reverse transcribed, a 369 nucleotide fragment PCR amplified (RT-PCR) using P_1 and P_2, and transformed as above (3.6.1). The transformation mixture was then plated, single recombinant colonies picked, and the DNA isolated for sequencing (section 2.2.7.2).

One of the clones (referred to as '*Ahd-2* related') was determined to have a sequence similar, but not identical, to the sequence of BALB/c *Ahd-2* for this region (section 3.6.1). Figure 22 shows an example of the differences seen in approximately 70 nucleotides of the 369 bp amplified product. All 369 nucleotides of this recombinant clone were sequenced in both directions and are shown in Figure 23, along with the corresponding region of BALB/c *Ahd-2*. The '*Ahd-2* related' sequence has a 90.8% identity to the BALB/c *Ahd-2* sequence at the nucleotide level and a 91% identity at the deduced amino acid level (Table 9). A difference in the putative Antabuse binding region in amino acid 302, (isoleucine in '*Ahd-2*

Figure 22. A portion (approximately 70 nucleotides) of the sequencing reactions for the region surrounding the putative Antabuse binding site from (a) *Ahd-2* sequence (obtained from pG121) and (b) '*Ahd-2* related' sequence (obtained from a 369 nucleotide RT-PCR fragment) is shown in this figure. Sequences on the right are written 5' to 3'. Differences between the two sequences are noted with asterisks.



a)
A G C T
5'
GGAGTGTTTATCATCAGGCCAATGTTGTGTCGCAGCATCCCGGATTTTGTTGAGGAGTCAGTTAT
3'



b)
A G C T
5'
GGAGTGTTC*TT*C*CAC*CAG*GGT*CAG*A*T*TTGTGTT*GCAGCG*TCCA*GGC*TTTTGTTGAGGA*TCAA*TTAC*
3'

Figure 23. A comparison between the 369 nucleotide BALB/c *Ahd-2* sequence (Mus. *Ahd-2*) obtained from pG121 and the '*Ahd-2* related' sequence (obtained by RT-PCR amplification from total liver RNA). The deduced amino acid sequences are also given. Numbering is in relation to the 'ATG' translation start site, see Figure 11. Dashes represent identical nucleotides or amino acids. The putative Antabuse binding site is underlined.

```
                                                                                      279
Mus Ahd-2 ...-----------------------------------------------------------------------------
Ahd-2 rel ...-asn-leu-lys-arg-val-thr-leu-glu-leu-gly-gly-lys-ser-pro-cys-ile-val-phe
Ahd-2 rel .GC-AAT-CTG-AAG-AGA-GTC-ACC-CTG-GAG-CTG-GGG-GAA-AAG-AGC-CCT-TGC-ATT-GTG-TTT
Mus Ahd-2 .-------------------------------------------G-----------------------------------
                                                                                      837

                                                                                      298
Mus Ahd-2 -----------------------ile-----------------------his------------tyr---
Ahd-2 rel ala-asp-ala-asp-leu-asp-ser-ala-val-glu-phe-ala-his-gln-gly-val-phe-phe his
Ahd-2 rel GCA-GAT-GCC-GAC-TTG-GAC-AGT-GCT-GTT-GAG-TTT-GCA-CAC-CAA-GGA-GTG-TTC TTC CAC-
Mus Ahd-2 ------------------------T-------------------------T------------T- AT-- T-
                                                                                      894

                                                                                      317
Mus Ahd-2 -----------cys-----------------------ile-------------------val-------
Ahd-2 rel gln-gly-gln-ile-cys-val-ala-ala-ser-arg-leu-phe-val-glu-glu ser-ile-tyr-asp
Ahd-2 rel CAG-GGT-CAG-ATT-TGT-GTT-GCA-GCG-TCC-AGG-CTT-TTT-GTT-GAG-GAA TCA-ATT-TAC GAT-
Mus Ahd-2 --A---C---A-TG--------C-------A-----C---A----------------G-TCA-G---- T ---
                                                                                      951

                                                                                      336
Mus Ahd-2 -----------lys------------------------------------val-----------------
Ahd-2 rel glu-phe-val-arg-arg-ser-val-glu-arg-ala-lys-lys-tyr-ile-leu-gly-asn-pro leu-
Ahd-2 rel GAG-TTT-GTG-AGG-AGG-AGT-GTG-GAG-CGG-GCT-AAG-AAA-TAC-ATT-CTA-GGA-AAT CCT-CTG-
Mus Ahd-2 ------------AA-----------T-------A--------------T-G----T------------------
                                                                                      1008

                                                                                      355
Mus Ahd-2 thr-pro------------------------------------------------asp-------------
Ahd-2 rel asn-ser-gly-ile-asn-gln-gly-pro-gln-ile-asp-lys-glu-gln-his-asn-lys ile leu-
Ahd-2 rel AAC-TCC-GGA-ATA-AAT-CAA-GGT-CCT-CAG-ATT-GAC-AAG-GAA-CAA-CAC-AAT-AAA-ATA CTC-
Mus Ahd-2 -C--C-A-----------------C---------------------------T-G-------------
                                                                                      1065

                                                                                      374
Mus Ahd-2 ------------------------------------------------------------------------------
Ahd-2 rel asp-leu-ile-glu-ser-gly-lys-lys-glu-gly-ala-lys-leu-glu-cys-gly-gly-gly-arg
Ahd-2 rel GAT-CTC-ATT-GAG-AGT-GGG-AAG-AAA-GAA-GGA-GCC-AAG-CTG-GAG-TGT-GGT-GGA-GGT-CGC
Mus Ahd-2 -----------------------------------------A------------------------A --
                                                                                      1122

                                  383
Mus Ahd-2 ----------------------------------.
Ahd-2 rel trp-gly-asn-lys-gly-phe-phe-val-gln-.
Ahd-2 rel TGG-GGG-AAC-AAA-GGC-TTC-TTT-GTG-CAG-C
Mus Ahd-2 -----------------------------------
                                  1150
```

Table 9. The percent identity between pairwise comparisons of sequences from BALB/c *Ahd-2* (Ahd-2), '*Ahd-2* Related' (Ahd-2 Rel), Rat cytosolic aldehyde dehydrogenase (Rat Cyto), Human cytosolic acetaldehyde dehydrogenase (Hum Cyto), Human mitochondrial acetaldehyde dehydrogenase (Hum Mito), Bovine mitochondrial aldehyde dehydrogenase (Bovine Mito), Rat mitochondrial aldehyde dehydrogenase (Rat Mito) and Human ALDHχ for the 369 nucleotides surrounding the putative Antabuse binding site at the nucleotide (upper) and amino acid (lower) level.

nucl / a.a.	Ahd-2	Ahd-2 Rel	Rat Cyto	Hum Cyto	Hum Mito	Bovine Mito	Rat Mito	Hum ALDHx
Ahd-2	—	90.8	88.3	88.1	60.2	59.9	60.7	61.0
Ahd-2 Rel	91.0	—	92.9	86.2	51.8	59.6	61.0	62.1
Rat Cyto	91.0	96.7	—	85.9	61.5	59.6	60.7	62.3
Hum Cyto	89.3	88.5	86.1	—	59.9	59.3	59.9	62.6
Hum Mito	60.7	63.1	64.8	62.3	—	85.9	86.7	71.2
Bovine Mito	59.0	60.7	61.5	59.0	91.0	—	95.1	67.2
Rat Mito	61.5	61.5	63.9	60.7	93.4	87.5	—	68.0
Hum ALDHx	61.5	61.5	61.5	59.8	68.0	67.8	67.2	—

related' and cysteine in *Ahd-2*) was also noted. Careful examination of the minor bands in the sequencing reactions of the 369 nucleotide RT-PCR fragments (section 3.6.1) showed that in the sequences with clear, readable minor bands (i.e. Figure 20 [a]), the minor bands corresponded to the sequence of '*Ahd-2* related'.

Analysis of the '*Ahd-2* related' sequence with the IntelliGenetics Fast DB pairwise comparison with the EMBL and GenBank sequence data banks revealed that the sequence is most similar to rat cytosolic aldehyde dehydrogenase, for the 369 nucleotides examined (Dunn et al., 1989). The BALB/c *Ahd-2* cDNA sequence is not present in the data banks and is therefore not found by the computer search. As shown in Table 9, the '*Ahd-2* related' sequence has a 92.9% identity at the nucleotide level and a 96.7% identity in the deduced amino acid composition to rat cytosolic aldehyde dehydrogenase in this region. As indicated above, the '*Ahd-2* related' sequence is 90.8% identical to the BALB/c *Ahd-2* cDNA in this 369 nucleotide region and 91% identical at the deduced amino acid level. Comparison to human cytosolic acetaldehyde dehydrogenase (*Aldh-1*) cDNA sequence in this region gives comparable values, 86.2% and 88.5%, respectively. In contrast, the identity of the sequence at the nucleotide and deduced amino acid level to human, bovine, and rat mitochondrial ALDH sequences is reduced to approximately 60% (Table 9, Figures 24 and 25). BALB/c *Ahd-2*, rat cytosolic aldehyde dehydrogenase, and human cytosolic acetaldehyde dehydrogenase also show approximately 60% identity to the mitochondrial sequences. In addition, the '*Ahd-2* related' sequence was compared to human ALHDχ, (Hsu and Chang, 1991). '*Ahd-2* related' and ALHDχ are 62.1% identical at the nucleotide level and 61.5% identical at

Figure 24. A comparison involving the '*Ahd-2* related' sequence (*Ahd-2* rel.), BALB/c *Ahd-2* cDNA sequence (Mus *Ahd-2*), rat cytosolic aldehyde dehydrogenase (Rat cyto.; Dunn et al., 1989), human cytosolic acetaldehyde dehydrogenase (H. cyto.; Hsu et al., 1989), human mitochondrial acetaldehyde dehydrogenase (H. mito.; Hsu et al., 1988), bovine and rat mitochondrial aldehyde dehydrogenase (Bov mito and Rat mito.; Farres et al., 1989) and human ALDHχ (Hum. ALDHχ; Hsu and Chang, 1991) for the 369 nucleotides surrounding the putative Antabuse binding region. Numbering in the figure is relative to the 'ATG' translation start site. Dashes represent identical nucleotides.

```
                                                                                       837
Ahd-2 rel .GC-AAT-CTG-AAG-AGA-GTC-ACC-CTG-GAG-CTG-GGG-GAA-AAG-AGC-CCT-TGC-ATT-GTG-TTT-
Mus Ahd-2 .-------------------------------------------G------------------------------
Rat cyto. .-----------------G-------------------T------G------------------------------
H. cyto.  .-----------------G-------------------T-------------------------------T-----
H. mito.  .-----C---C-----------G-----T----------------GG-----------C-AA---C-A-C-A-G-
Bov.mito. .-T---C---C-----------G-------------A-A---A--G--------T---C-AAT---C-A-C-A-G-
Rat mito. .---------C-----------A-----------A----------G------------C-AAT---C-A-C-A-G-
Hum ALDHχ..C-----C---C-------------------------------T--GT-----------C-A-----C-----C-G-

                                                                                       894
Ahd-2 rel GCA-GAT-GCC-GAC-TTG-GAC-AGT-GCT-GTT-GAG-TTT-GCA-CAC-CAA-GGA-GTG-TTC-TTC-CAC-
Mus Ahd-2 -------------------------T----------------------------T-----------T--AT---T-
Rat cyto. ----------T-----------T---------------------------------------A-------------
H. cyto.  --T----------------------A------------A---------------T---G---A------A------
H. mito.  T-------------T-A-----T-T-G---C---G---A-CAG---C-----TTC--CC-C-----------A---
Bov.mito. T-----C---T-----A-------T-G-T-------T-A-CAG---C-----TTT--CC-C-----------A---
Rat mito. T-----C---T-----A-------T-G-------G---A-CAG---C-----TTT--CC-C-----------A---
Hum ALDHχ.--C-------T-----A-----G-CA----C---G------AG-TGC-----G-----CC-C-----------A---

                                                                                       951
Ahd-2 rel CAG-GGT-CAG-ATT-TGT-GTT-GCA-GCG-TCC-AGG-CTT-TTT-GTT-GAG-GAA-TCA-ATT-TAC-GAT-
Mus Ahd-2 --A-- C---A-TG--------C-------A-----C---A-----------------G-TCA-G-----T-----
Rat cyto. -------  -------------G-------A-------A-------------------G---C-------------
H. cyto.  --A---C-----TG------A-A---C---A---------A---------G---A---------------T-----
H. mito.  ------C-----TGC---C-TGC---C---C-----C---ACC---C---G-C-----G-GAC---C---T-----
Bov.mito. ------C-----TGC---C-TG-------GC---G-C---ACC-------G-C-----G-GAC-----------CC-
Rat mito. ------C-----TGC-----TG----G--GC-----C---ACC---C---G-C-----G-GAT-G-G---T-----
Hum ALDHχ.ATG---C-----TGC---C-TG----T--GC-----C---ACC---C---G---A-------C---C-----A---

                                                                                      1008
Ahd-2 rel GAG-TTT-GTG-AGG-AGG-AGT-GTG-GAG-CGG-GCT-AAG-AAA-TAC-ATT-CTA-GGA-AAT-CCT-CTG-
Mus Ahd-2 -------------AA-----------T-------A---------------T-G-----T-----------------
Rat cyto. --A-------T-----------------------------------------G-----------------------
H. cyto.  ----------T-C-A-----------T-------------------G---T---C---T-----------------
H. mito.  ------------GT--C-----C---T--CC-------C-----TCT-CGG-G-C-G-C---G---C---C-T-T-
Bov.mito. ------------GAA-C---------T--CC-------C-----TCT-CGT-G-G-G-C---G---C---C-T-T-
Rat mito. --A---C-----GAA-C-C----------CC-------C-----TCT-CGG-G-G-G-C---G---C-----T-C-
Hum ALDHχ.--------C-C-GA----A--CC---------AAA---A-----C-G-AGG--AA-G-G---G---C---C-T-T-

                                                                                      1065
Ahd-2 rel AAC-TCC-GGA-ATA-AAT-CAA-GGT-CCT-CAG-ATT-GAC-AAG-GAA-CAA-CAC-AAT-AAA-ATA-CTC-
Mus Ahd-2 -C--C-A-------------------C-------------------------------T-G---------------
Rat cyto. G-----A----------G--------------------------------G-------T-GC---------C---T-
H. cyto.  ----C-A-----G-C--C--------C-----------------------------T-T-G-------------T-
H. mito.  G-T-AG--AAG--CC-G-G---G---G---G-----G-G---T-G-A-ACT---G-TTT---G---G---C-----
Bov.mito. G---AG--C-G--CG-G-G---G---G---G-----G-G---T-G-A-ACG---G-TTT---G---G-G-C---T-
Rat mito. G---AG--C-G--CG-G-G---G---G---G-----G-G---T-G---ACT---G-TTT---G---G---C---G-
Hum ALDHχ.G-G-CTG--AC--CC-C-G---G---G---------G-G-----------G---G-TTT-G-A-CG--G-C---A-

                                                                                      1122
Ahd-2 rel GAT-CTC-ATT-GAG-AGT-GGG-AAG-AAA-GAA-GGA-GCC-AAG-CTG-GAG-TGT-GGT-GGA-GGT-CGC-
Mus Ahd-2 ----------------------------------------------A-----------------------A-----
Rat cyto. - ------------------------------------C-------A-----------------------A-----
H. cyto.  --C-----------------------------------G-------A-------A-------A-------C--CG-
H. mito.  -GC-TA----C-A-C--CG---------C---C-G---G---G---------CT-----------G---C-ATT-
Bov.mito. -GC-TAT---C-A-A-TC----------G-G---G---G-CTT---------CT------------------G-GC--
Rat mito. -GC-TAT---C-A-G-TCA---A-C-A-C---------G---G---------CT---C-------G---C-GC--
Hum ALDHχ.-GC-TA----C-C---CT----C-C-----G---G---C---A---A---C-CTC-------C------AG---T-

                                 1150
Ahd-2 rel TGG-GGG-AAC-AAA-GGC-TTC-TTT-GTG-CAG-C
Mus Ahd-2 -------------------------------------
Rat cyto. ------------------------------C------
H. cyto.  ----------T----------A--------C------
H. mito.  GCT--CT-G---CGT---T--A----C-A-C------
Bov.mito. GCT--CT-G---CGT---T--A----C-A-C------
Rat mito. GCC--CA-G---CGT---T--A----C-A-C------
Hum ALDHχ.-TC-----G-G-CGT---T-------C-A-C-A----
```

Figure 25. A comparison involving the deduced amino acid sequence surrounding the putative Antabuse binding region for '*Ahd-2* related' (*Ahd-2* rel.), BALB/c *Ahd-2* (Mus *Ahd-2*), rat cytosolic aldehyde dehydrogenase (Rat cyto.; Dunn et al., 1989), human cytosolic acetaldehyde dehydrogenase (H. cyto.; Hsu et al., 1989), human mitochondrial acetaldehyde dehydrogenase (H. mito.; Hsu et al., 1988), bovine and rat mitochondrial aldehyde dehydrogenase (Bov mito and Rat mito.; Farres et al., 1989) and human ALDHχ (Hum. ALDHχ; Hsu and Chang, 1991). The putative Antabuse binding site is underlined. Numbering in the figure is relative to the 'ATG' translation start site. Dashes represent identical amino acids.

280
Ahd-2 rel asn-leu-lys-arg-val-thr-leu-glu-leu-gly-gly-lys-ser-pro-cys-ile-val-phe-ala-
Mus Ahd-2 --
Rat cyto. --
H. cyto. --glu------------------------leu-----
H. mito. --glu-----------------asn-----ile-met-ser-
Bov.mito. --------------------------------ile-----------------------asn-----ile-met-ser-
Rat mito. --asn-----ile-met-ser-
Hum ALDHx.--ser-----------leu-----

299
Ahd-2 rel asp-ala-asp-leu-asp-ser-ala-val-glu-phe-ala-his-gln-gly-val-phe-phe-his-gln-
Mus Ahd-2 --------------------ile--------------------------his---------------tyr--------
Rat cyto. --
H. cyto. --------------------asn--------------------------his---------------tyr--------
H. mito. ------------met-----trp---------------gln-----------phe-ala-leu-----------asn-----
Bov.mito. ------------met-----trp---------------gln-----------phe-ala-leu-----------asn-----
Rat mito. ------------met-----trp---------------gln-----------phe-ala-leu-----------asn-----
Hum ALDHx.------------met-glu-his---------------gln-cys-----glu-ala-leu-----------asn-met-

318
Ahd-2 rel gly-gln-ile-cys-val-ala-ala-ser-arg-leu-phe-val-glu-glu-ser-ile-tyr-asp-glu-
Mus Ahd-2 --------cys-------------------------ile-----------------------val---------------
Rat cyto. --
H. cyto. --------cys-----ile-----------------ile---------------------------------------
H. mito. --------cys-----cys-----gly-----------thr-----------gln-----asp------------------
Bov.mito. --------cys-----cys-----gly-----------thr-----------gln-----asp-----------ala-----
Rat mito. --------cys-----cys-----gly-----------thr-----------gln-----asp-val---------------
Hum ALDHx.--------cys-----cys-----gly-----------thr-----------------------------asn-----

337
Ahd-2 rel phe-val-arg-arg-ser-val-glu-arg-ala-lys-lys-tyr-ile-leu-gly-asn-pro-leu-asn-
Mus Ahd-2 --------lys-------------------------------------val-----------------------thr-
Rat cyto. --val-----------------------asp-
H. cyto. --thr-
H. mito. --------val-------------ala---------------ser-arg-val-val---------------phe-asp-
Bov.mito. --------glu-------------ala---------------ser-arg-val-val---------------phe-asp-
Rat mito. --------glu-------------ala---------------ser-arg-val-val---------------phe-asp-
Hum ALDHx.----leu-glu-----thr-----------lys-----------gln-arg-lys-val---------------phe-glu-

356
Ahd-2 rel ser-gly-ile-asn-gln-gly-pro-gln-ile-asp-lys-glu-gln-his-asn-lys-ile-leu-asp-
Mus Ahd-2 pro---asp----------------
Rat cyto. ------------ser---ala----------------
H. cyto. pro-----val-thr-----------------------------------tyr--------------------
H. mito. ----lys-thr-glu-------------------val-----glu-thr-----phe-lys---------------gly-
Bov.mito. ----arg-thr-glu-------------------val-----glu-thr-----phe-lys-----val-----gly-
Rat mito. ----arg-thr-glu-------------------val-----glu-thr-----phe-lys---------------gly-
Hum ALDHx.leu-asp-thr-gln-------------------val-------------------phe-glu-arg-val-----gly-

375
Ahd-2 rel leu-ile-glu-ser-gly-lys-lys-glu-gly-ala-lys-leu-glu-cys-gly-gly-gly-arg-trp-
Mus Ahd-2 --
Rat cyto. --
H. cyto. --pro-----
H. mito. tyr-----asn-thr-----------gln-----------------------leu-------------------ile-ala-
Bov.mito. tyr-----lys---------------glu-----------leu-----------leu-------------------ala-ala-
Rat mito. tyr-----ly -----------gln-gln-----------------------leu-------------------ala-ala-
Hum ALDHx.--------gln-leu-----gln---------------------------leu---------------glu-----phe-

383
Ahd-2 rel gly-asn-lys-gly-phe-phe-val-gln-
Mus Ahd-2 --------------------------------
Rat cyto. --------------------------------
H. cyto. ----------------tyr-------------
H. mito. ala-asp-arg-----tyr-----ile-----
Bov.mito. ala-asp-arg-----tyr-----ile-----
Rat mito. ala-asp-arg-----tyr-----ile-----
Hum ALDHx.----glu-arg-------------ile-lys-

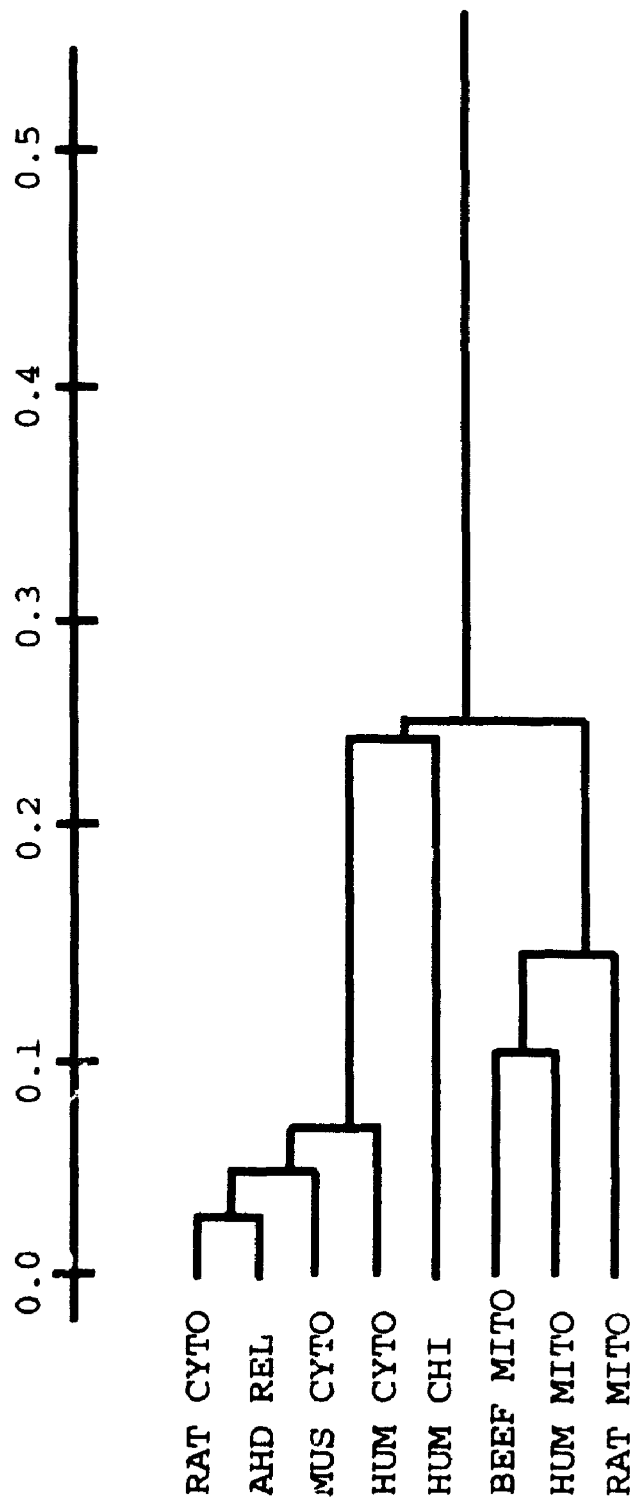
the deduced amino acid level. These results indicate that '*Ahd-2* related' is most likely not a ALDHχ-related or a mitochondrial ALDH in the mouse.

Euclidean distance measurements between all possible pairs of '*Ahd-2* related', BALB/c *Ahd-2*, human cytosolic acetaldehyde dehydrogenase, rat cytosolic aldehyde dehydrogenase, human, bovine and rat mitochondrial aldehyde dehydrogenase, and human ALDHχ for the 369 nucleotides surrounding the putative Antabuse binding region were determined by computer analysis and are given in Table 10. As shown in Figure 26, the '*Ahd-2* related' sequence clusters with rat cytosolic aldehyde dehydrogenase. All of the cytosolic sequences (BALB/c *Ahd-2*, human, and rat) and '*Ahd-2* related' also cluster together. Futhermore, the three mitochondrial ALDH sequences (human, bovine, and rat) also cluster together, but separate from the cytosolic cluster. Again, human ALDHχ does not directly cluster with either group. These results support the hypothesis that the '*Ahd-2* related' sequence is from a gene related to the cytosolic acetaldehyde dehydrogenase gene and is likely not from mouse mitochondrial ALDH or an ALDHχ-related enzyme. The identity and function of '*Ahd-2* related' gene awaits detailed analysis including sequencing of the entire cDNA for this transcript.

Table 10. A summary of Euclidean distance measurements calculated using multiple sequence alignments from the IntelliGenetics Software for Molecular Biology. AHD REL, '*Ahd-2* related'; RAT CYTO, rat cytosolic aldehyde dehydrogenase (Dunn et al., 1989); MUS CYTO, BALB/c *Ahd-2*; HUM CYTO, human cytosolic acetaldehyde dehydrogenase (Hsu et al., 1989); HUM CHI, human ALDHχ (Hsu and Chang, 1991); BEEF MITO, bovine mitochondrial aldehyde dehydrogenase (Farres et al., 1989); HUM MITO, human mitochondrial acctaldehyde dehydrogenase (Hsu et al., 1988); and RAT MITO, rat mitochondrial aldehyde dehydrogenase (Farres et al., 1989).

	AHD REL	RAT CYTO	MUS CYTO	HUM CYTO	HUM CHI	BEEF MITO	HUM MITO	RAT MITO
AHD REL	0.000	0.071	0.090	0.144	0.375	0.397	0.399	0.386
RAT CYTO	.	0.000	0.109	0.142	0.373	0.400	0.402	0.394
MUS CYTO	.	.	0.000	0.120	0.383	0.400	0.397	0.386
HUM CYTO	.	.	.	0.000	0.373	0.394	0.394	0.394
HUM CHI	.	.	.	.	0.000	0.302	0.277	0.313
BEEF MITO	.	.	.	.	.	0.000	0.120	0.144
HUM MITO	.	.	.	.	.	.	0.000	0.123
RAT MITO	.	.	.	.	.	.	.	0.000

Figure 26. Dendogram generated using the Macintosh program Systat based on Euclidean distances between 8 genes for a 369 nucleotide region surrounding the putative Antabuse binding region. An approximate scale is given. AHD REL, '*Ahd-2* related'; RAT CYTO, rat cytosolic aldehyde dehydrogenase (Dunn et al., 1989); MUS CYTO, BALB/c *Ahd-2*; HUM CYTO, human cytosolic acetaldehyde dehydrogenase (Hsu et al., 1989); HUM CHI, human ALDHχ (Hsu and Chang, 1991); BEEF MITO, bovine mitochondrial aldehyde dehydrogenase (Farres et al., 1989); HUM MITO, human mitochondrial acetaldehyde dehydrogenase (Hsu et al., 1988); and RAT MITO, rat mitochondrial aldehyde dehydrogenase (Farres et al., 1989).


0.0
0.1
0.2
0.3
0.4
0.5
RAT CYTO
AHD REL
MUS CYTO
HUM CYTO
HUM CHI
BEEF MITO
HUM MITO
RAT MITO

Chapter 4

DISCUSSION

Alcohol abuse causes a number of serious and potentially fatal health problems including cirrhosis of the liver and neurological disorders. In addition, society carries a large economic burden caused by alcohol abuse. Absenteeism, traffic accidents, suicide, crime, and job loss all contribute to an estimated $100 billion cost, per annum, in the United States (Leo, 1982; Lieber, 1982; Chambers, 1990). The ability to metabolize ethanol and its associated physical and psychological effects are highly variable among individuals. Racial and familial patterns of alcohol metabolism suggest the involvement of genetic determinants in the ability to detoxify alcohols and the expression of physiological and behavioural responses. The nature of these genetic determinants appears complex and multifactorial and is not understood. In general, the adverse effects of ethanol are attributed to its metabolite, the highly toxic acetaldehyde (Lieber, 1988; Goedde and Agarwal, 1990). Furthermore, a number of genetic determinants regulating the steady state level of acetaldehyde may contribute to individual and racial differences in ethanol sensitivity. In this context, the genes controlling alcohol metabolism remain the focus of attention towards the understanding of the genetic determinants of relative sensitivity to ethanol. The range of variation in the rate of alcohol metabolism is almost three-fold in the general population, including dizygotic twins, while the rates in monozygotic twins are much more similar. In order to understand its genetic basis, a number of studies have attempted to correlate

polymorphisms in the primary genes of ethanol metabolism (*Adh-1*, *Adh-2*, *Adh-3*, *Aldh-1* and *Aldh-2* in humans) to alcoholism and ethanol sensitivity. Thomasson et al., (1991) reported a possible association between the absence of ADH β_2 and γ_1 alleles in male Orientals and alcoholism. A strong correlation also exists between ethanol sensitivity and an $ALDH_2$ polymorphism. One half of all Orientals exhibit a severe physiological reaction to ethanol including facial flushing, nausea, and tachycardia (Agarwal and Goedde, 1987). A single amino acid substitution in these individuals ($ALDH_2$ position 487 glu to lys) inactivates the enzyme. It is believed that the build up of acetaldehyde following ethanol consumption in these individuals is caused by this inactive allele (Harada, 1989). This build up produces severe sensitivity to alcohol, discouraging drinking in these individuals. This aversion has been exploited as a treatment for alcoholism. The drug Antabuse (disulfiram) mimics the effects of the inactive $ALDH_2$ allele and causes the same unpleasant reaction after alcohol consumption. Antabuse irreversibly inhibits $ALDH_1$ activity in patients causing increased acetaldehyde levels and therefore increased sensitivity to alcohol. Alcoholics have significantly lower ADH and ALDH enzyme levels than healthy controls. Jenkins (1984) observed that the reduced enzyme levels return to normal once patients abstain from alcohol, thus suggesting that this depression in enzyme activity may be a secondary response to ethanol rather than an inherent trait. However, the ALDH enzyme levels in other studies on alcoholics remained at a low level even after abstinence, thus suggesting that this is a primary abnormality found in some alcoholics (Thomas et al., 1982). Although these results are important and interesting, their biological basis remains speculative.

Ethical considerations limit even controlled experimentation in humans, so a number of animal models have been exploited. Ethanol consumption and preference levels are highly heritable in inbred mouse and rat strains supporting the influence of genetic factors. The two primary enzymes controlling ethanol metabolism (ADH-A2, alcohol dehydrogenase; AHD-2 and AHD-5, acetaldehyde dehydrogenase) have been well characterized in the mouse (Algar et al., 1983). Several studies have examined the effect of ethanol on ADH and ALDH activity in mice. Wang and Singh (1985) reported genotype-specific induction and repression of ADH activity in inbred strains of mice given 15% (v/v) ethanol in drinking water over a 6 week period. The follow-up study showed a decrease or no change in ADH enzyme activity in the alcohol avoiding (BALB/c and 129/ReJ) and the alcohol preferring (C57BL/6J) strains fed the Lieber DeCarli liquid diet (Wei and Singh, 1988), respectively. Liver cytosolic ALDH enzyme activity, on the other hand, was found to be significantly increased in C57BL/6J mice but was decreased, or showed no apparent change, in BALB/c and 129/ReJ mice (Wei and Singh, 1988). These results suggest that the relative sensitivity of the mouse genotypes to ethanol may depend on the alteration of ALDH activity following ethanol challenge (Singh, 1986). Although this correlation holds true for a limited number of mouse genotypes, its relevance, and particularly the molecular basis for this phenomenon is not known.

This research project was undertaken to fulfill three primary objectives: 1) To determine if the effect of ethanol *in vivo* is also apparent at the mRNA or transcript level, which could contribute to the alterations found in the strain specific enzyme activities; 2) To provide the complete

cDNA sequence for the mouse cytosolic acetaldehyde dehydrogenase (*Ahd-2*) gene and 3) To provide a molecular characterization of the mouse *Ahd-2* gene, in relevant strains particularly at (a) the 5' untranscribed and the 5' transcribed but untranslated regions and (b) a physiologically relevant (Antabuse binding) region of the AHD-2 polypeptide. In doing so, an additional partial sequence ('*Ahd-2* related') was identified.

4.1 Ethanol Feeding Experiments

Male mice 6 to 10 weeks old from 3 inbred strains (C57BL/6J, BALB/c and 129/ReJ) known for their relative ethanol preference were used in the experiments. Given free choice between water and 10% ethanol, C57BL/6J mice consume 85% of their average daily liquid intake as the ethanol solution. On the other hand, BALB/c and 129/ReJ mice consume only 17 and 20%, respectively, of their daily total liquid intake as 10% ethanol. As a result, C57BL/6J are considered to be an 'alcohol preferring' or an 'alcohol resistant' strain while BALB/c and 129/ReJ are 'alcohol avoiding or 'alcohol sensitive' strains (McClearn and Rodgers, 1979 and Rodgers, 1966). This phenotypic difference is transmitted to subsequent generations and segregates in F_2 and recombinant inbred lines. Tagliabracci and Singh (personal communication, 1990) have suggested that a minimum of two genes may be involved in determining strain-specific differences in ethanol preference between C57BL/6J and BALB/c mice. In order to assess the effects of ethanol on ADH and ALDH hepatic mRNA, mice from these 3 strains were fed the Lieber DeCarli liquid diet for 2 weeks. Since no other sources of food or water were provided to the animals, aversion to ethanol is quickly overcome (Lieber and DeCarli,

1989). The response of both alcohol preferring and alcohol avoiding mice can therefore be examined using this technique.

The results presented in section 3.1.1 and Figure 3 indicate that no initial preference for ethanol diet or control diet is apparent under the experimental conditions used. On days 1 to 7, while the ethanol concentration is low, the consumption between animals on the ethanol diet and animals on the control diet remains comparable, for all 3 strains. In contrast, on day 9 when the ethanol level has been raised to 5%, all three strains show a reduction in the consumption of the ethanol diet as compared to matched animals on the control diet. The average consumption of the mice on the control diet did not change over the 2 week experimental period.

Body weights in animals fed the ethanol diet followed a similar pattern (3.1.2 and Figure 4). Control and ethanol diet animals, for all 3 strains, were weight-matched on day 1 of the experiment. The control-fed mice showed no apparent change in average weight over the two week experimental period. In contrast, ethanol-fed mice from all three strains (BALB/c, 129/ReJ and C57BL/6J) had an almost constant weight loss over the test period. The net weight calculations revealed that C57BL/6J mice had an average weight loss of 12% over the 2 week time period. On the other hand, the BALB/c and 129/ReJ mice had an average weight loss of 24% and 29%, respectively. This suggests that the weight loss in ethanol-fed mice may be strain dependent. In other studies, C57BL/6J mice gained weight on the ethanol diet, while BALB/c and 129/ReJ mice did not (Wei and Singh, 1988). Wei and Singh (1988) also indicated that mice from the strain 129/ReJ were among the most sensitive to the effects of ethanol, as seen by net weight loss at the end of the experimental treatment.

Mice fed the ethanol diet had no apparent physical effects due to the alcohol during the first 7 days, when fed the 1% and the 3% ethanol solutions. The ethanol-fed animals which died during the course of the experiment did so after day 8, when the ethanol level was raised to 5% from 3%. This was also the time frame when consumption levels decreased, weight loss became apparent, the animals appeared intoxicated and had a deterioration in physical condition. The mortality rates of the 3 strains fed the ethanol diet were also apparently strain dependent. Seventy percent of the 129/ReJ mice and 62% of the BALB/c mice died before completing 14 days on the Lieber DeCarli ethanol diet. In comparison, approximately 40% of the C57BL/6J mice died. Lowering the alcohol level in the Lieber DeCarli diet would decrease the mortality rate in the experiments but would not provide clinically relevant blood alcohol concentrations (Ward, 1987). Blood alcohol concentrations are negligible with even a slight decrease in the alcohol level as the capacity to oxidize ingested alcohol is directly related to its percentage of the total caloric energy in the diet (Lieber and DeCarli, 1989). This point is illustrated by the minimal effect of the lower ethanol concentrations (1% and 3%) in the experimental diet. Blood alcohol concentrations at the time of sacrifice (after 7 days on 5% ethanol) varied among the strains examined (0.23% w/v for C57BL/6J, 0.18% w/v for BALB/c and 0.42% w/v for 129/ReJ; section 3.1.2). However, all the strains were considered to be 'grossly intoxicated' and within the range of human blood alcohol concentration (Sigma Diagnositics Blood Concentration Kit, protocol manual, 1987) following completion of the feeding regime used in this experimental design.

A causal relationship between weight loss and a decrease in liquid diet consumption cannot be established from this data set. However, administration of the Lieber DeCarli diet and the subsequent weight loss and deteriorating physical condition of the ethanol-fed mice accurately reflects the effects of long-term chronic alcohol abuse in humans (Lieber, 1982). Alcohol is known to suppress food intake and to depress growth, even when alcoholics receive adequate nutrition (Lieber, 1982 and Lieber and DeCarli, 1989). It has been postulated that induction of MEOS (membrane-associated electron transport system) by alcohol use may be, at least in part, responsible for this phenomonen (Lieber and DeCarli, 1989). The cofactor NADPH is consumed when ethanol is oxidized by MEOS and heat is generated. However, the amount of heat generated far exceeds the normal needs for thermoregulation and the energy is thus 'wasted'. Damage to the liver and other organs caused by the build-up of acetaldehyde and inhibition of the protein metabolism and enzyme inactivation may also cause the severe physical deterioration seen in these animals (Lieber, 1982 and Lieber and DeCarli, 1989).

4.2 The Effect of Ethanol Feeding on *Adh-1* and *Ahd-2* mRNA.

The mouse ADH-A_2 specific cDNA probe (pADH$_{m16}$) identified a single 1.4 kb mRNA in the three strains (C57BL/6J, BALB/c and 129/ReJ) of mice in both ethanol and control-fed animals (Figure 5[a]). This size is comparable to other reports (Ceci et al., 1986 and Patterson et al., 1987). Quantification of this signal in control-fed animals showed that C57BL/6J mice had approximately 1.7 times more *Adh-1* specific mRNA than

BALB/c and 129/ReJ mice (Table 2 [a]). These results are similar to the those of Patterson et al. (1987) who also noted higher *Adh-1* mRNA levels in the C57BL/6J mice. In both studies, relative mRNA levels between strains can be correlated to relative ADH-A2 enzyme activity levels in control animals. That is, strains which showed higher *Adh-1* mRNA levels also showed higher ADH-A2 activity levels (Patterson et al., 1987; Wei and Singh, 1988). Sequencing of the first intron of *Adh-1* has revealed that the low activity strains have a 101 bp deletion in this region (Zhang et al., 1987). The authors hypothesized that the deletion, which is part of an alternating purine-pyrimidine sequence, may function as an enhancer element. The deletion may cause a change in the conformation of the DNA which may, in turn, cause a decrease in the rate of constitutive transcription.

The Lieber DeCarli ethanol diet caused genotype-specific changes in *Adh-1* mRNA levels (Table 2 [b]). In general, there was a large increase in the level of *Adh-1* mRNA in ethanol-fed C57BL/6J mice as compared to the matched controls. For BALB/c mice, there was a 20% increase, and for 129/ReJ, there was an apparent reduction in *Adh-1* mRNA in ethanol-fed animals as compared to genotype matched controls. No direct correlation between the change in ADH-A2 enzyme activity with ethanol feeding (C57BL/6J, -26.15%; BALB/c, -39.42%; 129/ReJ, -36.65%; Wei and Singh, 1988) and the change in *Adh-1* mRNA levels observed during this experiment appears to exist. Other authors have reported an increase in ADH-A2 enzyme activity in C57BL/6J mice (McClearn et al., 1964). However, these results were obtained by forced alcohol feeding. It would be necessary to quantify liver *Adh-1* mRNA levels after duplicating the forced feeding procedure to draw any firm conclusions. The mechanisms

of alteration of strain-specific ADH enzyme activity and mRNA levels following ethanol feeding is not evident and remains to be established.

The effect of ethanol feeding is also apparent as strain-dependent changes in mRNA levels identified by the partial cDNA probe pG121. This probe was previously not sequenced and was uncharacterized. The probe was determined to represent a partial cDNA for mouse *Ahd-2* (see results). Hybridization of pG121 to Northern blots of total cellular liver RNA from control and ethanol-fed mice (C57BL/6J, BALB/c and 129/ReJ) produced a single band of approximately 2.3 kb. This is comparable to the 2.1 kb human cytosolic acetaldehyde dehydrogenase mRNA (Hsu et al., 1989) and the 2.25 kb rat cytosolic aldehyde dehydrogenase mRNA (Dunn et al., 1989). The cDNA probe pG121 appeared to detect a single mRNA species specific to the mouse *Ahd-2* gene at the hybridization and experimental conditions used in these experiments. Levels of the *Ahd-2* specific message in the total RNA were quantified from dot blots using replicated experiments and a series of RNA concentrations for each sample. The results suggest that the *Ahd-2* mRNA levels in control-fed animals of the three strains did not differ significantly (Table 3 [a]). Control-fed animals were also shown to have similar levels of AHD-2 enzyme activity (Wei and Singh, 1988). However, when such a comparison is made between the control and experimental animals within a strain, a genotype-dependent pattern emerges. Ethanol-fed C57BL/6J mice had an apparent increase in the mRNA hybridizing to pG121, BALB/c mice had no apparent change, and 129/ReJ mice had an apparent decrease as compared to matched controls. AHD-2 enzyme activity was also found to increase in C57BL/6J mice with ethanol feeding (+29.07%; Wei and Singh, 1988). In comparison, BALB/c and 129/ReJ mice both exhibited a slight decrease or

no change in AHD-2 enzyme activity as compared to control fed mice in separate and earlier experiments (Wei and Singh, 1988).

The results presented here showing changes in the gene-specific hepatic mRNA levels are unique in the *in vivo* literature and suggest that chronic ethanol feeding may affect the steady-state levels of ADH-A2 and AHD-2 specific mature transcripts available for translation. Other factors such as alterations in amino acid metabolism and enzyme inactivation by ethanol may affect translation of these mRNAs and enzyme activity levels during chronic alcohol levels (Lieber, 1982). The changes observed in the levels of specific mRNAs may be the result of changes in transcription of the genes following ethanol treatment, or from changes in mRNA stability. Other authors have shown that the Lieber DeCarli liquid diet decreases both total RNA content and RNA synthesis in the rat intestine and the gastrocnemius muscle (Preedy and Peters, 1990 and Marway et al., 1990). A decrease in brain mRNA with ethanol treatment was also seen in rats (Tewari et al, 1975). These studies, however, examined total RNA and did not focus on the possible induction or reduction of the gene-specific mRNA species associated with ethanol metabolism. Additional studies focusing on poly A+ selected (messanger) RNA from ethanol and control-fed animals as well as an appropriate control mRNA, which does not change with ethnaol treatment, could lead to further insight into this phenomenon.

C57BL/6J mice are relatively resistant to the effects of ethanol feeding and show a preference for 10% alcohol over water (Section 3.1 and Rodgers, 1966). At the level of *Adh-1* and *Ahd-2* mRNA, they responded to ethanol feeding in a unique way by showing increased levels of both message types. C57BL/6J mice also have an increase in AHD-2 enzyme activity after ethanol feeding (Wei and Singh, 1988). BALB/c and

129/ReJ mice, which are both ethanol sensitive strains, showed little change or a decrease in *Adh-1* and *Ahd-2* mRNA levels following ethanol feeding. They also exhibited a slight decrease or no change in AHD-2 enzyme activity with the ethanol diet (Wei and Singh, 1988). The above results are compatible with the hypothesis that efficient removal of the toxic metabolite acetaldehyde by acetaldehyde dehydrogenase may be associated with the relative resistance of C57BL/6J mice to the effects of ethanol (Singh, 1986). Also, the molecular regulation of this phenomenon may be at the level of the gene-specific mRNA identified by the probe pG121.

4.3 DNA Banding Patterns in Mouse Strains with Variable Ethanol Preferences

The results presented here suggest that level of the mRNA identified by pG121 is affected by ethanol challenge in mice in a strain dependent manner, however no qualitative differences in this mRNA were apparent among the different strains in the Northern blot analysis (Figure 6). Genomic DNA differences associated with the pG121 among mouse strains can be evaluated using Southern blots. Data on DNA banding patterns among mouse strains which had been previously studied for ethanol preference was generated. The results presented in section 3.3 indicate that extensive differences exist among mouse strains at the genomic DNA level. The strains studied (C57BL/6J, C57BL/6J*, C57BL/10J, BALB/c, C57BL/KsJ, C3H/HeJ, C3H/HeSnJ, 129/ReJ, Cs^b, SW, and DBA/2J) showed bands detected by pG121 for the restriction enzymes *Eco* RI, *Hin*d III, *Pst* I and *Rsa* I. The DNA from the mouse strains included in this study could

be categorized into two groups based on their banding patterns. DNA from the strains C57BL/6J, C57BL/6J*, C57BL/10J, and BALB/c formed group 1 and strains C57BL/KsJ, C3H/HeJ, C3H/HeSnJ, 129/ReJ, Cs^{b}, SW, and DBA/2J formed group 2. Group 1 DNA, with the exception of BALB/c, represent alcohol preferring strains (preference ratio > 0.68) while group 2 are alcohol avoiding strains (preference ratio < 0.45) (Table 4). Although the DNA banding pattern groups BALB/c with the alcohol preferring strains, their preference ratio for 10% ethanol solution is only 0.17 (Rodgers, 1966 and Tagliabracci and Singh, personal communication, 1990). This incongruency may indicate that a number of factors contribute to ethanol avoidance in BALB/c and suggest that the observed banding patterns do not correlate to ethanol preference in all strains of mice. Alcohol preference in mice must be considered multifactorial. It is also interesting to note that the alcohol preference ratio of C57BL/KsJ is 0.45. This is lower than the other C57BL sublines. As shown in Table 4 and Figures 7 and 8, the DNA banding patterns for C57BL/KsJ placed these mice in group 2 and not in group 1 with the other C57BL sublines. This difference may be the result of genetic contamination which has occurred in C57BL/KsJ (Festing, 1979). In general, the DNA banding patterns detected by pG121 hybridization are associated with alcohol preference and alcohol avoidance in different strains of mice. A cause and effect association between the observed patterns and relative sensitivity to ethanol cannot be concluded from these results. Additional experiments using appropriate recombinant inbred lines with distinct ethanol preferences could elucidate a possible causal relationship between the DNA banding patterns and relative sensitivity to ethanol of the mouse strains. The results presented so far, however, suggest that ALDH must play a major role in

the clearance of acetaldehyde following ethanol challenge and contribute to relative ethanol preference and sensitivity. The remaining effort in this research, therefore, was directed towards sequencing and molecular characterization of the *Ahd-2* gene in mice.

4.4 Sequencing of BALB/c Ahd-2 cDNA.

The partial cDNA probe pG121 was determined to be 1.16 kb long. A complete sequence of this probe was obtained in both directions. It contained 129 bp of 3' untranslated region and 1035 bp coding for 345 amino acids. The cytosolic aldehyde dehydrogenases sequenced to date from other species have 501 amino acids (Dunn et al., 1989 and Hsu et al., 1989). The 3' end of human cytosolic acetaldehyde dehydrogenase, a well characterized enzyme which is believed to be similar in form and function to mouse *Ahd-2* (Algar and Holmes, 1986), was aligned with the obtained pG121 sequence. A 90% identity at the deduced amino acid level and an 83% identity at the nucleotide level was found between pG121 and the corresponding human sequence. Since the percent identity between cytosolic ALDHs in two different species is higher than the percent identity between two different forms of ALDH within the same species (68% identity between human cytosolic and human mitochondrial ALDH), it further confirmed that pG121 was a partial cDNA for mouse cytosolic acetaldehyde dehydrogenase. The complete cDNA sequence for mouse *Ahd-2* was obtained by screening BALB/c cDNA and gDNA libraries (section 3.4.2) and is given in Figure 11. It represents a total of 1632 nucleotides including 1503 bp coding for a 501 amino acid long

polypeptide with a molecular weight of 54,382 and 129 nucleotides of the 3' untranslated region. The sequence shown is similar, but not identical, to that of BALB/c *Ahd-2* given by Rongnoparut and Weaver, 1991. Analysis of the complete *Ahd-2* cDNA sequence showed an 89.1% and an 84.7% identity to rat cytosolic aldehyde dehydrogenase (Dunn at al., 1989) and human cytosolic acetaldehyde dehydrogenase (Hsu et al., 1989), respectively, within the coding region at the nucleotide level. Comparison at the deduced amino acid level reveals identities of 88.2% and 87.2%, respectively to the two sequences. No gaps or deletions were necessary to align the three sequences. In addition, all three cDNAs code for proteins 501 amino acids in length with a molecular weight of approximately 54,000 daltons (Dunn et al., 1989 and Hsu et al., 1989). The eight residues believed to be functionally important in ALDH enzyme activity (Cys 50, 163, 303, 370; Glu 269; Gly 246; His 236; and Ser 75; Lindahl and Hempel, 1991; and Weiner et al., 1991) are also conserved in the BALB/c *Ahd-2* cDNA (Figure 13). One conservative amino acid change from the human cytosolic acetaldehyde dehydrogenase sequence in the putative Antabuse binding region was noted in *Ahd-2*. Mouse cytosolic acetaldehyde dehydrogenase is known to be inhibited by Antabuse (Algar and Holmes, 1986). The putative site for the binding of Antabuse is specified by the six amino acids (gly-gln-cys-cys-ile-ala) in human cytosolic ALDH (Hsu et al., 1985). This region is relatively conserved between human cytosolic ALDH and BALB/c *Ahd-2* (gly-gln-cys-cys-val-ala; Figure 13). This conservation lead to the hypothesis that the region may have a functional significance in acetaldehyde metabolism and changes in this region may affect ethanol sensitivity.

The BALB/c cDNA sequence was also compared to sequences in the GenBank (release 62) and EMBL (release 26) sequence data banks. Data bank sequences with the highest percent identity to the BALB/c *Ahd-2* sequence were aligned and a matrix of pair-wise genetic distance scores was obtained (section 2.2.9) using IntelliGenetics Software for Molecular Biology. Systat (version 5.1), a Macintosh software package, was then used to generate a dendogram based on the Euclidean distances between the sequences (Figure 12). Dendograms are generated by first clustering the two sequences which are most similar, then a third sequence, most similar to the first two sequences is added and its relationship to the first cluster determined. This continues until all the sequences have been included in the comparison. Dendograms, therefore, are pictorial representations of the degree of genetic differentiation or similarites among DNA sequences. In order for the dendogram to accurately reflect the genetic distances among the sequences, the number of changes at the nucleotide level between the sequences must be proportional to the time of their last common ancestral sequence. The dendogram shown in Figure 12 is based on the minimum number of nucleotide differences between the sequences and may, therefore, underestimate the genetic distance between the sequences.

The BALB/c cDNA sequence clusters with rat cytosolic aldehyde dehydrogenase and human cytosolic acetaldehyde dehydrogenase. Also the human, bovine, and rat mitochondrial aldehyde dehydrogenases cluster, but separately from the cytosolic enzymes (Figure 12). Furthermore, human ALDHχ, a novel ALDH sequence recently reported by Hsu and Chang (1991), clusters more closely to the mitochondrial enzymes than to the cytosolic enzymes but is not directly clustered with either group. The

ALDHχ cDNA encodes 517 amino acid residues, the same as the mitochondrial enzymes, however its function remains unknown.

The comparative analysis indicates that the cDNA sequence included here is that of mouse cytosolic acetaldehyde dehydrogenase, *Ahd-2*. Also the cDNA probe (pG121) used in the Northern, RNA dot blot, and Southern analyses is a 3' partial cDNA for the mouse *Ahd-2*. More importantly, the strain specific associations between ethanol preference/resistance and DNA and RNA signals following pG121 hybridization are likely due to differences in the *Ahd-2* gene and its mRNA product. In particular, the RFLP patterns and the strain specific alterations in the *Ahd-2* mRNA levels following ethanol challenge are of interest in the understanding of the molecular basis for relative sensitivity to alcohols. Further analysis attempted included sequencing and characterization of a) the 5' region of the *Ahd-2* gene to assess possible transcriptional signals and b) a putative functional domain of the polypeptide (the Antabuse binding region) to provide a possible molecular basis for the observed differences in ethanol sensitivity..

4.5 Isolation and Characterization of the 5' Region of *Ahd-2*.

The 5' promoter region of *Ahd-2* was isolated by screening a BALB/c gDNA library first with pG121 and then with a more 5' *Ahd-2* cDNA clone (SB-2, section 3.5.1). P4, a primer corresponding to amino acids 12 to 17 of the coding region was then used to sequence upstream into the 5' untranslated region and the putative promoter of this gene. The 5' sequence analyzed represents 448 bp, including transcribed and untranscribed regions (Figure 16). An extended 545 bp sequence was

analyzed by Quest, Quick User-directed Expression Search Tool, IntelliGenetics Release 5.4. Two putative CCAAT boxes were identified. A putative TATA-like sequence (ATAAAAA) was identified by examining the similar regions from human cytosolic acetaldehyde dehydrogenase (Hsu et al., 1989) rat cytosolic aldehyde dehydrogenase (Dunn et al., 1989) and BALB/c *Ahd-2* (Rongnoparut and Weaver, 1991). This TATA-like sequence appears to be conserved in all three species. The relative positions of these three consensus sequences are also similar in the cytosolic ALDH genes of the three species. Both CCAAT and TATA boxes have been noted in the promoter region of other genes and have been shown to be required for efficient transcription (Takiguchi et al., 1987 and Lawn et al., 1980).

A number of regions with comparable similarity to known enhancer sequences were also recognized in the DNA sequences of the mouse *Ahd-2* 5' region. This included five binding sites for Moloney Murine Leukemia Viral enhancer elements (LVa and LVc; section 3.5.3). Viral enhancer elements have also been reported in the 5' region of other mammalian genes, such as the immunoglobin κ and μ genes (Speck and Baltimore, 1987). Enhancers are thought to contain binding sites for specific proteins which, when bound to the DNA, enhance transcription (Speck and Baltimore, 1987). The specific proteins binding to the LVa and LVc DNA enhancer sequences have been found in a number of different mouse cell lines (Speck and Baltimore, 1987), even though their biological significance is not always determined or apparent.

Analysis of the BALB/c *Ahd-2* 5' region using the DNA Inspector IIe, a Macintosh software program, detected 8 sequences similar to the C/EBP consensus binding sequence. C/EBP is a DNA binding protein

which can regulate gene transcription (Friedman et al., 1989). Originally purified from rat liver nuclei, it has been found in fully differentiated liver, fat, and lung tissue in the mouse. C/EBP has been shown to bind to the promoter region of transthyretin, α-1-antitrypsin, and albumin, three genes selectively expressed in the liver (Costa et al., 1988). Stewart et al. (1990) located two C/EBP protected sites in the human *Adh-2* promoter and in the mouse *Adh-1* gene. These two sites surround the TATA box and C/EBP binding is believed to either create a pocket to enhance RNA polymerase II interaction with the TATA box or to stimulate transcription by another factor (Stewart et al., 1990). Eight regions similar to the C/EBP binding sequence (8 of 9 nucleotides) are located in the BALB/c *Ahd-2* promoter (section 3.5.3). Two of these sequences CTTAGTGCA (-88 to -80) and AGGAGCAAG (-68 to -60) also surround the putative TATA box. One of these sequences (AGGAGCAAG) is also located directly downstream from the TATA box in the rat cytosolic aldehyde dehydrogenase promoter (Dunn et al., 1989). It has not been determined if these sites in the BALB/c *Ahd-2* 5' region bind C/EBP *in vivo* or *in vitro*. Thus the C/EBP-*Ahd-2* 5' sequence interaction and its role in regulating the transcription of this gene remains to be established. It does, however, seem to be possible given the fact that C/EBP is found in differentiated liver cells and the position of two of the C/EBP binding sequences matches that of human *Adh-2*. Alcohol dehydrogenase and acetaldehyde dehydrogenase are both expressed in the liver and are both involved in the ethanol metabolism pathway. They do not, however, have sequence similarity but their co-regulation in alcohol metabolism may be physiologically significant. Other authors have shown that a perfect match is not essential for binding of, and trancriptional regulation by, C/EBP

(Ryden and Beeman, 1989). Examination of the promoter regions of human cytosolic and mitochondrial acetaldehyde dehydrogenase and ALDHχ genes also revealed the presence of C/EBP consensus binding sequences (Hsu et al., 1988; Hsu et al., 1989 and Hsu and Chang, 1991). The C/EBP consensus sequence, therefore, may be important in the regulation of the genes of alcohol metabolism.

The 5' region of *Ahd-2* was PCR amplified and sequenced from genomic DNA from mouse strains with different alcohol responses (C57BL/6J, BALB/c, and 129/ReJ) in order to identify and characterize possible genotype-dependent differences in the promoter. It is evident from Figure 18 that there are no differences in the transcribed region of this amplification, which represents approximately 80 bases, 51 of which encode for the first 17 amino acids. There are only two minor differences between the three strains in the remaining untranscribed 5' nucleotides sequenced. In addition, the differences seen in the BALB/c sequence (missing 'a' at position -326 and missing 'ac' at position -365, -364) were not found in the BALB/c *Ahd-2* promoter sequenced from the EMBL-3 SP/T7 genomic library clone SBg-5b (Figure 16). The region was found to be extremely conserved amongst the strains. A number of studies have examined differences in protein isozymes, immune loci, endogenous viral loci, and RFLP differences among inbred strains of mice (Atchley and Fitch, 1991 and Zhang et al., 1987), however, the promoter region of the same gene in divergent inbred strains has not been published. Conservation of sequence in the coding region of genes often implies a functional constraint (Dobzhansky et al., 1977). Although the implications of sequence similarity in the promoter region among the strains remains undetermined, it must be viewed as significant given the approximate 100%

identity of this sequence in the three strains from variable origins representing different gene pools and separated from each other for 50 to 70 years (Green and Witham, 1990). Sequence information on the 5' region of a variety of genes is necessary for an accurate assessment of the significance of these findings, in particular, and the significance of the conservation of 5' sequences, in general. In the context of the overall objective of this study, it was concluded that differences in the 5' *Ahd-2* sequences from these three strains do not explain their variable preference and sensitivity to ethanol.

Primer extension analysis was performed to determine possible transcription initiation start points (tsps) for the mouse *Ahd-2*. Total RNA from the livers of 3 inbred strains (C57BL/6J, BALB/c and 129/ReJ) of mice was used in this experiment. A number of possible start sites were found in repeated experiments. Major bands caused by termination 32 and 33 nucleotides upstream from the ATG translation start site (Figure 17) were found in all 3 strains, indicating this as the region of the transcription start site. RNA from the strain 129/ReJ also produced a minor band indicating a tsp 45 nucleotides upstream from the ATG start site in repeated experiments. This site corresponds to the distance between the tsp and the ATG translation start site in rat cytosolic aldehyde dehydrogenase (Dunn et al., 1989). It may be pointed out that GC clusters cause hairpin loops in RNA and are known to prematurely terminate reverse transcriptase (Farres et al., 1989). The tsps indicated for 32 and 33 nucleotides upstream from the ATG site may be caused by such a premature termination, as the region (GCTGAGCCTGTCACC) is G/C rich and may form such a loop by pairing of G-TGA with TCA-C. Thus a tsp at position -45 in BALB/c and C57BL/6J may not be apparent due to

this premature termination. Location of the tsp at -45, -32 or -33 would place the putative CCAAT and TATA boxes around their expected consensus range of distance from the RNA transcription start site (Breathnach and Chambon, 1981), with the distances corresponding best to position -45. This is not, however, the case for possible tsps located at 58, 67 and 68 nucleotides upstream from the ATG start site. It is hypothesized that the bands indicating these nucleotides as possible tsps may be caused by binding of the primer to similar sequences in the mRNAs of related genes. This hypothesis is supported by the fact that the band indicating nucleotide -67 was not present in the RNA from 129/ReJ in the repeated experiment (section 3.5.4). It was concluded that nucleotide -32, -33 or -45 likely represents the tsp for the *Ahd-2* gene in all three strains of mice. A definitive transcription start point cannot be determined from this experiment but could, perhaps, be determined by S1 nuclease mapping. Differences in the observed tsps among the strains cannot be explained by differences in the sequence in this region.

Transcription run-off experiments were attempted to determine if changes seen in the steady-state levels of *Adh-1* and *Ahd-2* mRNA in the liver with ethanol feeding were due to differences in transcription of the genes or differential stability of the messages (section 3.5.7). Nuclei were isolated from the livers of control and ethanol-fed animals, and the new RNA transcribed *in vitro* was labelled with ^{32}P-UTP. It was possible to obtain an adequate number of nuclei from the livers of animals fed the control diet and from those fed the ethanol diet. Differences in the amount of newly transcribed RNA were obtained. When an equal number of nuclei were added, the c.p.m.s of the labelled RNA isolated from ethanol-fed mice was approximately one-third that of the RNA isolated from

control mice. Others studies have also shown that the overall RNA transcription rate and total RNA content is significantly reduced in several tissues with ethanol feeding in rats (Preedy and Peters, 1990 and Marway et al., 1990). The low level of labelled RNA resulted in autoradiograms with little cDNA specific hybridization apparent above the background (section 3.5.7).

Earlier results (section 3.2) indicated a genotype-dependent change in the steady-state levels of *Adh-1* and *Ahd-2* mRNA in the livers of ethanol-fed animals. Changes in the steady state levels may be a result of changes in gene transcription or changes in mRNA stability. Differential transcription may occur due to changes in the concentration of DNA-binding proteins. Sites for three such possible binding proteins have been identified in the BALB/c *Ahd-2* promoter (section 3.5.7). One of these proteins, C/EBP, may also bind to the *Adh-1* promoter (Stewart et al., 1990). Ethanol consumption is known to alter protein metabolism (Lieber, 1982). Theoretically, DNA binding proteins in the livers of the three inbred strains of mice could be affected, in a genotype-dependent manner, by ethanol consumption. This could then alter gene transcription in the livers of these mice. Analysis of the steady-state levels of *Adh-1* and *Ahd-2* mRNA on day 14 of the ethanol diet does not detect possible changes in gene transcription during the course of the experiment (i.e. days 1 to 13). Differential transcription of the two genes, in combination with differential stability of the mRNAs, may result in the genotype-dependent changes seen in the final steady-state levels.

4.6 Molecular Analysis of a Functional Domain from AHD-2.

Antabuse (disulfiram) is a drug given to alcoholics to reinforce abstinence. Antabuse irreversibly inhibits approximately 90% of all cytosolic ALDH ($ALDH_1$) activity and may partially inhibit $ALDH_2$ activity (Goedde et al., 1983 and Helander et al., 1988). Antabuse treatment therefore results in a build-up of acetaldehyde following ethanol consumption. Antabuse is believed to bind to $ALDH_1$ near the active site (gly-gln-cys-cys-ile-ala; Hsu et al. 1985). Steric hinderance likely limits the binding of Antabuse to $ALDH_2$, which has a slightly different amino acid sequence at this site (gly-gln-cys-cys-cys-ala; Hsu et al., 1985 and Goedde and Agarwal., 1990).

Sequencing of the BALB/c *Ahd-2* cDNA revealed a difference of one amino acid in the putative Antabuse binding region from human $ALDH_1$ (val in BALB/c and ile in human $ALDH_1$; Figure 13). This represents an amino acid substitution very similar in size and charge (Lehninger, 1975). To explore the possibility that differences in this region of the *Ahd-2* polypeptide may affect ethanol sensitivity among different strains of mice, a 369 nucleotide fragment surrounding this region was RT-PCR amplified and sequenced. Analysis of RT-PCR fragments from two C57BL/6J mice, two BALB/c mice, a 129/ReJ mouse, a C57BL/6J x BALB/c recombinant inbred #2 and recombinant inbred #13 mouse are given in Figure 21. In the 180 readable nucleotides, only one alteration at position 906 (C in 129/ReJ and RI #2 and #13, T in the others) was detected. The C-T alteration, however, does not change the amino acid (cys) at this site. Thus differences in ethanol sensitivity among the strains cannot be explained by

changes in the sequence surrounding the putative Antabuse binding region and this region may not contribute to differences observed in ethanol sensitivity among mouse strains..

The sequencing reactions for this 369 nucleotide RT-PCR fragment consisted of two sets of bands; major bands, corresponding to the BALB/c *Ahd-2* sequence, and minor bands. The additional sequences present must represent genes which are similar to *Ahd-2* and are actively transcribed in the livers of these mice, as all sequences were obtained by RT-PCR amplification of RNA (section 3.6.1).

Sequencing of an individual recombinant clone from an RT-PCR amplification from a C57BL/6J mouse revealed the presence of a sequence similar, but not identical to *Ahd-2*. The results of this sequence, referred to as '*Ahd-2* related', are given in Figure 23. A difference in amino acid 302 in the putative Antabuse binding site between *Ahd-2* (cys) and '*Ahd-2* related' (ile) was found. A number of other differences between '*Ahd-2* related' and the corresponding sequence of BALB/c *Ahd-2* were also noted. The sequence '*Ahd-2* related' is 90.8% identical to the BALB/c *Ahd-2* sequence at the nucleotide level and 91.0% identical at the deduced amino acid level for the 369 nucleotides examined (Table 9).

The gene '*Ahd-2* related' has not yet been identified. Further work isolating the complete cDNA sequence and characterization of the resulting polypeptide is necessary. The available sequence for '*Ahd-2* related' clusters it with the known cytosolic ALDH sequences in dendograms (Figure 26). It does not appear to be a mitochondrial ALDH or the murine equivalent of ALDHχ based upon Euclidean distances. '*Ahd-2* related' may, however, represent the murine equivalent of γ-aminobutyraldehyde dehydrogenase. In humans γ-aminobutyraldehyde dehydrogenase has an

amino acid composition and subunit molecular weight similar to $ALDH_1$ and $ALDH_2$. It interacts with anti-$ALDH_1$ and anti-$ALDH_2$ antibodies, has a K_m value for acetaldehyde of approximately 45 μM, and is relatively insensitive to Antabuse (Kurys et al., 1989). To date it is the only ALDH enzyme which has been found in humans but not yet identified in the mouse (Sladek et al., 1991).

4.7 Summary

Acetaldehyde is the toxic metabolite in the ethanol metabolism pathway and its rate of clearance from the body has been hypothesized to affect ethanol sensitivity. It was the aim of this project, therefore, to characterize the genes involved in alcohol metabolism in the mouse, particularly *Ahd-2*, to assess possible differences at the molecular level which could affect the ability to tolerate ethanol. Mice from three strains (BALB/c, C57BL/6J and 129/ReJ) were fed the Lieber DeCarli liquid diet for a two week period. Control-fed mice had no apparent weight gain/loss or change in consumption over the experimental period. Mice fed the ethanol diet, on the other hand, generally showed decreased consumption over the duration of the experiment. Weight loss also occurred, and appeared to be strain-specific.

Levels of *Adh-1* and *Ahd-2* mRNA in the liver were assessed in ethanol challenged mice and compared to levels in control-fed animals of the same strain. Only the C57BL/6J mice showed increased levels of both mRNA species after ethanol feeding. BALB/c mice had a small increase in *Adh-1* mRNA and no apparent change in *Ahd-2* mRNA. Mice from the strain 129/ReJ, which are particularly sensitive to the effects of ethanol,

had an apparent decrease in both mRNA species. The increased levels of *Ahd-2* mRNA in the C57BL/6J ethanol-fed mice corresponded to previously reported increases in the AHD-2 enzyme activity. Such a regulation may facilitate acetaldehyde clearance and contribute to relative ethanol sensitivity. C57BL/6J mice should, therefore, be better able to cope with the adverse effects of alcohol and would show a greater preference for ethanol than mice which could not metabolize acetaldehyde as effectively.

DNA from 11 mouse strains could be categorized into two groups based on DNA banding patterns. Group 1 consisted of DNA from alcohol preferring mice, with the exception of BALB/c, while group 2 was DNA from alcohol avoiding strains. Further work examined the mouse *Ahd-2* cDNA, including two different functional regions, to examine possible strain-specific changes which could explain the relative ethanol sensitivity of the strains.

The cDNA for *Ahd-2* has 1503 nucleotides coding for a 501 amino acid polypeptide. It has an 84.7% identity to human cytosolic acetaldehyde dehydrogenase cDNA (nucleotide level) and an 87.2% identity to the deduced amino acid sequence. No sequence differences existed among the strains in the 5' untranslated region, and only minor differences were found in the putative promoter and the Antabuse binding regions. The variable response of the three mouse strains to the effect of ethanol can not be explained by the differences observed in the DNA sequences of the two regions. Analysis of the putative promoter region of *Ahd-2* revealed a number of possible enhancer sequences, including LVa, LVc and c/EBP. Binding of proteins to these putative enhancer sequences, their regulation of gene transcription, and the possible effects of ethanol on this regulation

in the different genotypes have yet to be determined. An additional gene, related to *Ahd-2*, was also detected. The sequence of '*Ahd-2* related' is 90.8% identical to the BALB/c *Ahd-2* sequence at the nucleotide level and 91.0% identical at the deduced amino acid level at the 369 nucleotides surrounding the putative Antabuse binding region examined. The function of this previously unreported gene and its role in ethanol metabolism and effect on ethanol sensitivity remain to be examined.

in the different genotypes have yet to be determined. An additional gene, related to *Ahd-2*, was also detected. The sequence of '*Ahd-2* related' is 90.8% identical to the BALB/c *Ahd-2* sequence at the nucleotide level and 91.0% identical at the deduced amino acid level at the 369 nucleotides surrounding the putative Antabuse binding region examined. The function of this previously unreported gene and its role in ethanol metabolism and effect on ethanol sensitivity remain to be examined.

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