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Molecular studies of Canavan Disease

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FLORIDA INTERNATIONAL UNIVERSITY
Miami, Florida

MOLECULAR STUDIES OF CANAVAN DISEASE

A dissertation submitted in partial satisfaction of the requirements for the degree of DOCTOR OF PHILOSOPHY IN BIOLOGY

BY

Guangping, GAO

1994
To: Dean Arthur W. Herriott
     College of Arts and Sciences

This dissertation, written by Guangping, GAO, and entitled, MOLECULAR STUDIES OF CANAVAN DISEASE, having been approved in respect to style and intellectual content, is referred to you for judgement.

We have read this dissertation and recommend that it be approved.

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Dean of Graduate Studies

Florida International University, 1994
I dedicate this dissertation to my wife Qin Su, our daughter Xuan, our son Kevin, my parents, and my father in law. Without their understanding, support, patience, encouragement and most of all love, the completion of this work would not have been possible.
I wish to express my deepest appreciation to my major professor, Dr. Rajinder K. Kaul, and to other committee members: Dr. Reuben Matalon, Dr. Martin L. Tracey, Dr. Gerald Murison, and Dr. Ophelia I. Weeks for their outstanding support, constant encouragement, constructive criticism and guidance.

A special thanks must to go to Dr. Rajinder Kaul who has lead me into the research field of molecular studies of human genetic diseases. This project could not have been accomplished without his patient advice, disciplined training, and enthusiastic help.

I am also deeply grateful to my colleagues at Miami Children’s Hospital: Kuppareddi Balamurugan, Arlene Petrosky, Maria Aloya, Jing Shen, Mei Jin, Isabel Hernandez, Li-Or Davidson and my wife Qin Su for their superior experimental assistance and contributions to some parts of this project.

Finally, I would also like to thank all of those faculty members, students, and staff of both FIU and Miami Children’s Hospital, who gave me help and encouragement in the past three years.
Canavan disease (CD), an autosomal recessive leukodystrophy, is caused by the deficiency of aspartoacylase (ASPA). The human ASPA cDNA spanning 1,435 bp has been isolated and characterized. The single uninterrupted ORF in the cDNA predicted a 313 amino acid long protein. The authenticity of the cDNA has been established by its expression in *E. coli* and Cos1-cells. Human ASPA gene was also cloned and found to span 29 kb of the human genome. Human ASPA is coded by 6 exons intervened by 5 introns. The exon/intron splice junction sites follow the 'gt'/‘ag’ consensus sequence rule. The human ASPA gene was assigned to the 17p13-ter region. Human ASPA coding sequences were demonstrated to be conserved in yeast, chicken, rabbit, cow, dog, mouse, rat and monkey. Sixty-four probands (or 128 chromosomes) with CD were analyzed for mutations in the ASPA gene. Four point mutations have been identified in Canavan alleles. The 693C>A and 914C>A base changes result in non-sense tyr231>ter and missense ala305>glu mutations respectively,
that lead to complete loss of ASPA activity. The 854A>C transversion resulted in a glu285>ala missense mutation, and the mutant ASPA has 2.5% of the activity expressed by the wild type enzyme. The 433-2(A>G) transition at the splice acceptor site in intron 2 would lead to skipping of exon III, accompanied by a frameshift in the final ASPA transcript. Of the 128 unrelated Canavan chromosomes analyzed; 88 were from probands of Ashkenazi Jewish descent and 40 were from non-Jewish probands. The glu285>ala, tyr231>ter and 433-2(A>G) mutations account for 98.8% of the Canavan chromosomes of Ashkenazi Jewish origin. The ala305>glu mutation was found exclusively in non-Jewish probands and constituted 60% of the 40 mutant chromosomes. These results provide the basis for studying epidemiology of CD in at-risk populations; offer a DNA-based pre- and postnatal diagnosis of CD; provide the possibility to create an animal model of CD for understanding its pathophysiology and to develop strategies for possible enzyme and gene therapy.
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CHAPTER I

LITERATURE REVIEW, RESEARCH GOALS, STRATEGIES AND SIGNIFICANCE OF THE STUDY
SECTION I. CANAVAN'S DISEASE

Historical Perspective

Spongy degeneration of the central nervous system (Canavan disease, McKusick 27190) was first described in 1928 by Globus and Strauss. It was the case of a six month old infant (Globus and Strauss, 1928). The baby had died after a brief illness with symptoms including apathy, seizures, and generalized rigidity. The noticeable pathological change was a diffused destruction of myelinated fibers of the central nervous system. Three years later, Canavan (1931) described an affected infant with marked megalencephaly. The authors thought they were dealing with Schilder disease instead of a new category of neural disorder. The nosological entity of spongy degeneration was established by van Bogaert and Bertrand in 1949. van Bogaert and Bertrand described vacuolation in the deep layers of the cortex and adjacent white matter, almost complete lack of myelin in the hemispheric and cerebellar white matter, and hypertrophy of the protoplasmic astrocytes. Banker et al. (1964) noticed that a very high percentage of infants with Canavan disease (CD) had blue eyes and either blond or red hair. Dark hair and brown eyes were characteristic of their unaffected siblings. The reason for decreased pigmentation
in children with spongy degeneration is not understood. A significant number of studies have since been carried out to describe the pathological changes in the brain of patients with CD (Adachi et al., 1972, Adorna et al., 1972, Aleu et al., 1963, Boehme and Marks, 1981, Hagenfeldt et al., 1987, Kamoshita et al., 1968, Kevittingen et al., 1986, Torack et al., 1960, and Wolman, 1958). However, the basic biochemical defect in CD remained unknown. Matalon et al. in 1988, for the first time described the deficiency of aspartoacylase (ASPA; EC 3.5.1.15) as the basic biochemical defect in CD. Aspartoacylase specifically hydrolyzes its substrate N-aspartylaspartic acid (NAA) into aspartate and acetate (Birnbaum et al., 1952, and Birnbaum, 1955). Although NAA was first described by Tallan et al. in 1956, its role in brain metabolism is not known. The pathology seen in CD suggests that aspartoacylase and its hydrolysis of NAA is crucial for the maintenance of normal white matter.

Clinical Features

The clinical features of CD include flaccidity followed by spasticity, severe leukodystrophy, megalencephaly, mental retardation, optic atrophy, and death in early juvenility (Adachi et al., 1973, Banker et al., 1964, Matalon et al., 1993a, and Sacks et al., 1965). The clinical features of CD also include persistent muscle hypotonia (especially the
muscles around the neck), and head lag. The increase in head circumference may not be dramatic and may remain in the upper limit of normal range in early infancy. Children with CD become irritable and suffer sleep disturbance (Matalon et al., 1993a). In most cases, patients with CD are not recognized at early infancy although the signs of delayed development may show as early as 3 months. As the infants grow older, the delay in development becomes more apparent and some developmental milestones such as reaching for objects, rolling over or sitting are not attained (Matalon et al., 1993a). Later in life, seizures may develop and optic atrophy may become another feature of CD. The affected infants may have episodic saccadic eye movements, and can eventually become blind. The optic discs are pale bilaterally (Matalon et al., 1993a). Problems with the gastroesophageal reflex lead to swallowing deterioration during the second or third year of life. In some patients, to overcome the feeding difficulties and improve the weight gain, nasogastric feeding or permanent feeding gastrostomy are necessary (Matalon et al., 1993a). Many patients often run a fever of unknown origin. Head control remains poor during the course of the disease. Most patients with CD die in the first year of life (Banker et al., 1964, van Bogaert and Bertrand, 1949, and Sacks et al., 1965). However, with improved medical and nursing care, more and more of these
children are surviving longer (Matalon et al., 1990 and 1993a).

Adachi et al. (1973) described three clinical variants of CD. 1) Congenital form in which the disease is apparent at birth, or shortly thereafter (Feigin et al., 1968, Wolman, 1958, and Zu Rhein et al., 1960). 2) Infantile form which is the most common and in which symptoms manifest after the first 6 months of life (Adachi et al., 1973, Anderson et al., 1969, Matalon et al., 1993a, and Sacks et al., 1965). 3) Juvenile form in which the disease manifests after the first 5 years of life and has a prolonged course. The life expectancy of patients with CD has a wide variance, though the majority die in their early teens. Matalon et al. (1993a) have reported over 145 patients; there are nine individuals who are over 18 years old and one who survived until the age of 30 years. In the juvenile form of the disease, patients usually survive until late adolescence, show no racial or familial association, and its course is characterized by a progressive cerebellar syndrome with visual and auditory deficits. There are only a few case reports of juvenile CD (Adachi et al., 1973, Goodhue et al., 1979, and Jellinger and Seitelberger, 1969). However, because of the lack of specific biochemical markers in the past, it has been difficult to ascertain whether the three clinical manifestations are separate entities or a spectrum
of clinical severity.

Genetics and Epidemiology

Canavan disease is an autosomal recessive leukodystrophy associated with spongy degeneration in the brain. The disease is particularly prevalent in Jewish people of Ashkenazi origin (Adachi et al., 1972, Adornato et al., 1972, Banker et al., 1964, Buchanan and Davis, 1965, Gambetti et al., 1969, Sacks et al., 1965, Ungar and Goodman, 1983, and van Bogaert and Bertrand, 1949). Many Jewish families with CD have been traced back to founders in Lithuania and northern Poland (Banker et al., 1964, and Ungar and Goodman, 1983). This region overlaps closely with the area in which other diseases with high incidences in Ashkenazi Jews, like Tay-Sachs and dysautonomia, are thought to have arisen (Kaback et al., 1993, and Paw et al., 1990). Banker and Victor (1979) reported 48 unrelated CD patients of whom 28 were Jewish, from Vilna, Lithuania. Patients from other parts of the world with different ethnicities have also been identified (Banker and Vector, 1979, Hogan and Richardson, 1965, Gascon et al, 1990, Mahdi et al., 1986, Matalon et al., 1988, 1989 and 1993a, and Ozand et al., 1990).

The incidence of CD is not well known; it has been thought to be a rare genetic disorder. However, Matalon et
al. (1993a and b) have diagnosed 145 patients since their discovery of aspartoacylase deficiency in CD. Most of these patients are of infantile form and of Ashkenazi Jewish background (Matalon et al., 1993a and b). These studies suggest that CD is more prevalent than previously estimated. The earlier reports of CD rarity may have been due to lack of specific biochemical markers for diagnosis of CD.

Roentgenographic and Sonographic Findings

Computer tomographic (CT) scans reveal diffuse, low-attenuation white matter distributed symmetrically throughout both hemispheres in patients with CD (Andriola, 1982, Patel et al., 1986, and Rushton et al., 1981). Rushton et al. (1981) emphasized the generalized white matter involvement in order to distinguish CD from other leukodystrophies and macrocephaly. Magnetic resonance images (MRI) also unequivocally demonstrate in vivo white matter involvement and grossly delayed (almost absent) myelination in patients with CD (Boltshauser et al., 1991). Matalon et al. (1990) indicated that the white matter involvement is primarily in the cerebral hemispheres. Nuclear magnetic resonance (NMR) spectroscopic analysis of brains in patients with CD revealed diffuse hypointensity of supratentorial white matter on T1-weighted images and hyperintensity on T2-weighted images. The genu of the internal capsule and the
corpus callosum seemed unaffected (Grodd et al., 1991, and McAdams et al., 1990).

The sonographic features of the brain in CD was first described by Patel et al. (1986). The coronal cranial sonography showed multiple anechoic lesions in the cerebrum with increased sonolucency of white matter due to lack of myelin and an increased water content in the affected area.

Histology and Ultrastructural Lesions

Prior to the discovery of N-acetylaspartic aciduria and aspartoacylase deficiency as specific biochemical markers for CD (Matalon et al., 1988), the diagnosis of CD was confirmed only by histological examination of brain biopsy. Under light microscopy, vacuolation is evident in all layers of the cortex, especially as a band of sponginess in subcortical white matter (Adornato et al., 1972). The vacuoles in the white matter are elliptical in shape with their long axes parallel to the nerve fibers (Adornato et al., 1972). The contents of the vacuoles are not stained by routine histological staining. Myelin loss is apparent. The cortical vacuoles are perivascular and perineuronal in location (Adachi et al., 1966 and 1973). There is diffuse increase of microglia in severely spongy areas, and numerous astrocytes show balloon nuclei consistent with Alzheimer's type 2 glia (Kamoshita et al., 1967). Among basal ganglia,
thalamus, and hypothalamus structures, the corpus luysii shows the most severe spongy change. The putamen, pallidum, and thalamus show mild to moderate sponginess. The internal capsule (except for the genu) and optic tracts are almost entirely spared and their myelin appears normal (Kamoshita et al., 1967). Axons in the basal ganglia and thalami are reduced in number, showing focal kinking or fragmentation. Sections of the midbrain show the most pronounced sponginess in the red nuclei and adjacent white matter. The substantia nigra is also spongy, but the neurons remain intact. In the medulla, sponginess is more pronounced in the dorsal than in the ventral region. Pyramidal tracts are entirely spared of sponginess throughout the brain stem. In the cerebellum, the molecular layer appears thin and hypercellular. The Purkinje cell layer is displaced into the molecular layer by the vacuoles, the areas where demyelination has occurred. (Kamoshita et al., 1967).

Ultrastructural findings under the electron microscopy include vacuoles in the cortex interspersed among the normal appearing neuropil (Adachi et al., 1966 and 1973, Adornato et al., 1972, and Gambetti et al., 1969). These vacuoles correspond to swollen cytoplasm of astrocytes containing glial filaments. Invariably present within astrocytes are peculiar elongate mitochondria, measured at between 6 to 15 μm (Adornato et al., 1972). The abnormal mitochondria reveal
a central core containing filaments surrounded by cristae (Gambetti et al., 1969). Myelinic vacuolization results from splitting of myelin lamellae at intraperiod lines between major dense lines (Adachi et al., 1966). Most of the oligodendroglia are normal in morphology in regions of expanded extracellular space and disintegrating myelin sheaths, although vacuolated oligodendroglia are occasionally seen (Adachi et al., 1973, and Adornato et al., 1972). Vacuolated cells resembling macrophages and containing ingested myelin sheaths are evident in the areas of most intense demyelination (Adachi et al., 1973, and Adornato et al., 1972).

**Biochemical Studies**

Since the first report of a patient with Canavan disease in 1928 (Globus and Strauss, 1928), biochemical studies have focused on searching for the possible etiologic factor(s). Wolman (1958) speculated that increased quantities of low-molecular-weight myelin catabolites, derived from abnormal myelin, were responsible for elevated osmotic pressure and consequent edema. Kamoshita et al. (1968) documented that the electrolyte composition of the edema fluid is equivalent to ultrafiltrate of plasma. They concluded that accumulated low-molecular-weight catabolites are characteristic of nonspecific myelin destruction. Spongy
degeneration of the brain was proposed to result from accumulation of water in the astrocytes thereby causing the vacuolization (Torack et al., 1960 and Aleu et al., 1963). Adachi et al. (1972) observed decreased Na-K-Mg-stimulated ATPase activity in astrocytes and blood vessels of a brain biopsy from a patient with spongy degeneration. Inhibition of this enzyme by ouabain, a cardiac glycoside, produces a state of intracellular edema in the CNS that mimics the findings in spongy degeneration (Cornag et al., 1967). Adornato et al. (1972), however, did not find any significant difference in Na-K-Mg-stimulated ATPase activity from patients with CD and normal controls. Boehme and Marks (1981) found that synaptosomal membranes, purified from the corpus striatum of patients with Canavan disease, were deficient in protein kinases. However, the profound degeneration of the striatum may result in an overall reduction in metabolic processes, and protein kinases may be but one of several metabolic enzymes affected.

Kvittingen et al. (1986) described a male child born after an uneventful pregnancy and normal delivery who suffered from abnormal shivering or tremor, mental retardation, leukodystrophy and had excessively high blood, urine, and cerebrospinal fluid (CSF) levels of N-acetylaspartic acid (NAA). There was no evidence showing aspartoacylase deficiency in this patient. Hagenfeldt et al.
(1987) reported another male infant with psychomotor retardation and leukodystrophy. The patient excreted large quantities of NAA in his urine and had deficient aspartoacylase activity in cultured skin fibroblasts. However, the authors did not link the deficiency to CD and indeed thought they were dealing with a new leukodystrophy. The seminal identification of the biochemical defect in CD was made by Matalon et al. (1988). The aspartoacylase deficiency and N-acetylaspartic aciduria were described in three patients, who had earlier been diagnosed to have CD by examination of their brain biopsy. The initial diagnosis of three patients has since been extended to include over 145 patients (Matalon et al., 1988, 1989, and 1993a and b). It was thus established that the deficiency of aspartoacylase leads to CD. The deficiency of aspartoacylase in CD has since been confirmed by several other investigators (Bartalini et al., 1992, Divry et al., 1989, Echenne et al., 1989, Michelakakis et al., 1991, Ozand et al., 1990, Yalaz et al., 1990, and Zlink et al., 1993). The diagnosis of CD now is routinely performed by estimation of NAA in urine from patients with CD and further confirmed by demonstration of aspartoacylase deficiency in cultured skin fibroblast cell lines from such patients.
Section II. N-ACETYL-L-ASPARTIC ACID

Distribution of NAA

N-Acetyl-L-aspartic acid (for review, see Birken and Oldendorf, 1989) is an N-derivative of aspartic acid. The compound was first identified in mammalian and avian brain (Tallan et al., 1956). The concentration of NAA is 1 mg or 5-6 μmol per gram wet brain tissue (Tallan et al., 1956, and 1957). The level of NAA is second only to glutamic acid in the free amino acid pool of the brain and is higher than γ amino butyric acid (Berlinguet and Laliberte, 1966). N-Acetylaspartic acid has been localized exclusively to the central nervous system (Tallan et al., 1956). The highest concentrations of NAA is found in the basal ganglia and cerebral grey matter. The concentration of NAA in the grey matter is twice that found in the white matter, and is lowest in the spinal cord, pons and medulla in bovine (Tallan, 1957) and horse (Curatolo et al., 1965) CNS. The study by Kaul et al. (1991) also demonstrated that NAA concentration in human and bovine brain is three-fold higher in the gray matter compared with white matter. In contrast, NAA is distributed almost uniformly in the rat brain (Marcucci et al, 1966). N-Acetylaspartic acid is found in lesser amounts within peripheral nervous tissues, including the superior cervical ganglion, dorsal root, ventral root,
sciatic nerve, adrenal medulla, and splenic nerve (Nadler and Cooper, 1972, and Ory-Lavollee et al, 1987).

Utilizing immunohistochemical techniques, NAA has been localized in the neurons of rat brain, showing particularly high levels of NAA in many cortical neurons and distinct perikaryal staining which extended into proximal dendrites (Moffett et al., 1991 and 1993). Highest levels of NAA immunoreactivity in cerebral cortex were observed in neurons of layer II (external granular layer) and layer V (internal pyramidal layer). In the retrosplenial cortex, both pyramidal cells and granular cells were strongly stained for NAA. N-Acetylaspartic acid immunoreactivity in the hippocampus was present most notably in pyramidal neurons in all regions (Moffett et al., 1993). The distribution of immunoreactivity did not show primary neurotransmitter characteristics of stained neurons, although neurons with long projections or extensive arbors, such as the locus coeruleus, motoneurons and purkinje cells, stained much more intensively than local circuit neurons.

The concentration of NAA changes during development (Jacobson, 1959, Koller et al., 1984, Miyake and Kakimoto, 1981, Mussini et al., 1967, and Tallan, 1957). In rats, the concentration of NAA at the age of 20 days (120mg%) was six times that at the time of birth (20mg%), and then remained at 110mg% for the whole adult life (Tallan, 1957). This
finding was confirmed by the report from Mussini et al. (1967), in which the concentration of NAA was found to have risen most rapidly between the fifth and twenty-second days of the rat's life. Such rapid postnatal increases in NAA concentration are also observed in every region of both rat brain and guinea pig brain (Miyake and Kakimoto, 1981). A more detailed study by Koller et al. (1984) revealed a three-fold increase of NAA concentration in rat prenatally, and up to a ten-fold increase postnatally. Their data represent a thirty-fold increase in NAA levels of whole rat brain from the fifteenth day of gestation to adulthood. Earlier human studies of the developmental change of NAA levels (Jacobson, 1959) had shown a similar pattern of increase, as described by Koller et al. 1984.

Biosynthesis of NAA

Using uniformly-labeled $^{14}$C-glucose and $1-^{14}$C acetate, both glucose and acetate act as precursors to NAA in vivo. The synthesis of NAA was most rapid in ten-day-old rabbit brains, intermediate in twenty-day-old, and slowest in five-day-old rabbit brains (Jacobson, 1959, and Margolis et al., 1960). Goldstein (1959) first reported that a partially purified preparation from rat brain homogenate had an enzyme activity which was able to catalyze the acetylation of L-aspartic acid by acetyl coenzyme A to produce N-acetyl-L-
aspartic acid. He also suggested that the synthesis of NAA in brain may be represented by the following steps:

\[
\begin{align*}
\text{Acetate + ATP} & \rightleftharpoons \text{Acetyl-AMP + PP} \\
\text{Acetyl-AMP + CoA-SH} & \rightleftharpoons \text{Acetyl-CoA + AMP} \\
\text{Acetyl-CoA + L-aspartate} & \rightarrow \text{NAA + CoA-SH}
\end{align*}
\]  

The enzyme catalyzing these reactions was called aspartic acetylase, and it appears to have an absolute specificity for acetyl coenzyme A and L-aspartic acid. Goldstein (1969) confirmed the particulate location of this enzyme in rat brain, and renamed it acetyl-CoA-L-aspartate N-acetyltransferase. Patel and Clark (1979) further localized the synthesis of NAA to brain mitochondria of both neonatal and adult rats, where the newly produced acetyl CoA combined with aspartate. They proposed that NAA synthesized in mitochondria is transported across the mitochondrial membrane to the cytosol for breakdown, probably by dicarboxylic acid translocase.

Truckenmiller et al. (1985) demonstrated that acetyl-CoA-L-aspartate N-acetyltransferase is a membrane-associated enzyme, and is present only in nervous tissues. The enzyme activity in brain varies as much as 10-fold, with the brain stem and spinal cord exhibiting the highest activity (10-15 pmol/min/mg tissue) and retina the lowest detectable activity (1-1.5 pmol/min/mg). The distribution of acetyl-CoA-L-aspartate N-acetyltransferase in the nervous system
parallels to the distribution of N-acetyl-aspartyl-glutamic acid, a putative excitatory neurotransmitter (Truckenmiller et al., 1985).

Metabolism and Stability of NAA

Several investigators have suggested that NAA is an inert metabolite (Jacobson, 1959, McIntosh and Cooper, 1965, Mukherji and Sloviter, 1973, and Shigematsu et al., 1983). Nadler and Cooper (1972b) indicated that NAA is predominantly a neuronal constituent, with a large metabolically-inactive pool in neurons and a small, but significant, metabolically active pool of NAA is present in oligodendroglia. Using both proton nuclear magnetic resonance and high performance liquid chromatography techniques, Urenjak et al. (1992) suggested that two distinct pools of NAA exist in the CNS. One is present in O-2A progenitors and immature oligodendrocytes during early development and may be contributing to the synthesis of myelin lipids, and another is a neuronal pool of NAA that is not involved in myelination (Urenjak et al., 1992). Berliguet and Laliberte (1966) documented that NAA is mainly synthesized in the brain and is rapidly metabolized in the body.

The acetyl moiety of NAA has been implicated to be involved in the synthesis of fatty acids during neuronal
development in the rat brain (D’Adamo and Yatsu, 1966, and D’Adamo et al., 1968). Deacylation of NAA has been proposed to be a major route for its metabolism (Jacobson, 1959). N-acetylaspartic acid is also suggested to be incorporated into various N-acetyl-aspartyl-di-and tripeptides, which may function as neurotransmitters (Cangro et al., 1987, Lahdesmarki and Timonen, 1982, Moffett et al., 1990, Ory-Lavollee et al., 1987, Reichelt et al., 1969, and Serval et al., 1990).

McIntosh and Cooper (1965) presented evidence for conversion of NAA to a labile product, possibly an azlactone. However, this study could not be reproduced by Nadler and Cooper (1972b).

Biological Role of NAA

Since its discovery by Tallan et al. (1956), the biological role(s) of NAA has been unknown. Early studies suggested that NAA serves essentially as an anion buffer pool to maintain intracellular ionic strength and pH of nervous tissues (Margolis et al., 1960, McIntosh and Cooper, 1965, Mukherji and Sloviter, 1973, and Tallan, 1957). It has been suggested that NAA might play a role in the acetyl transport system for extramitochondrial fatty acid synthesis, especially serving as an acetyl group donor during myelination (D’Adamo et al., 1968, D’Adamo and Yatsu,
1966, and Burri et al., 1991). The incorporation of carbons from pyruvate into lipids might involve a transport mechanism via NAA, which shuttles carbons across the mitochondrial membrane to lipogenic enzymes located in the cytosol (Patel and Clark, 1979 and 1980). Also NAA was found to be the acetyl group donor for the synthesis of acetylcholine in mammalian brain (Buniatian et al., 1965, and Gibson and Shimada, 1979). Shigematsu et al. (1983) presented further evidence that NAA may, indeed, play an important role in myelination. Previous studies had shown that in brain, lignoceric acid (n-tetracosanonic acid) is converted to cerebronic acid (alpha-hydroxylignoceric acid) by the alpha-hydroxylation system. Subsequently, cerebronic acid, as well as lignoceric acid and other fatty acids, are incorporated into ceremide (fatty acid amide of sphingoid base) and then into cerebroside (ceramide-1-beta-galactoside), which is the major lipid component of myelin (Kishimoto et al., 1979, and Nonaka and Kishimoto, 1979). The investigation by Shigematsu et al. (1983) demonstrated that NAA was a component of the heat-stable factor essential for alpha-hydroxylation, beta-oxidation, and the conversion of lignoceric acid to ceramide in rat brain preparations.

Recently, based on their preliminary data, Matalon et al. (1993b) have proposed that NAA in brain may serve as a chemical compartment. They hypothesized that aspartate
released by the action of aspartoacylase may be channelled
to form arginine. Since NAA is very mobile and predominately
unbound, as evidenced by its large, sharp peak in $^1$H-NMR
spectroscopy, it is probably free to inactivate or mobilize
aspartate (for review, see Birken and Oldenforf, 1989).

Since NAA is not present in detectable amounts in lower
species and has been shown to have no effect on the crayfish
stretch receptor (Jacobson, 1959), or on spinal neurons
(Curtis and Watkins, 1960), NAA itself was discounted as a
possible neurotransmitter. Instead, NAA was suggested to act
as a precursor for the synthesis of N-
acetylaspartylglutamate (NAAG), a putative neurotransmitter
with excitatory effects (Cangro et al., 1987). N-
acetylaspartylglutamate is an acidic dipeptide isolated from
the CNS more than 28 years ago (Curatolo et al., 1965) and
has been identified in retinal neurons and their projections
in many vertebrate species (Anderson et al., 1987, Kowalski
et al., 1987, Tieman et al., 1987, 1988 and 1991, and
Williamson et al., 1991). Although the precise physiological
role of this neuropeptide is unclear, Moffett et al. (1990)
proposed that NAAG may act as one of the neurotransmitters
mediating the effects of light in the retinohypothalamic
system. Several reports have raised the possibility that
NAAG may be enzymatically synthesized from its potential
precursor, NAA (Cangro et al., 1987, Lahdesmaki and Timonen,
1982, and Reichelt and Kvämm, 1973). Truckenmiller et al. (1985) suggested that the activity of N-aspartyl-acetyltransferase, the biosynthetic enzyme for NAA, may have a regional distribution similar to that of N-acetylaspartylglutamate. This supports the possible correlation between NAA and N-acetylaspartylglutamate in the CNS. But the data from others documented a lack of such correlation (Koller et al., 1984, and Ory-Lavollee et al., 1987). McIntosh and Cooper (1964) hinted that there was some relationship between NAA and another neurotransmitter, serotonin.

Moreover, some researchers presented studies to demonstrate the involvement of NAA in protein synthesis. Gaetjens and Barany (1966) found NAA at the amino terminus of G-actin. It is not clear whether this is related to the possible function of NAA in the CNS. Reichelt et al. (1973) identified NAA-containing peptides which were synthesized in homogenates of mouse brain cortex through a histamine-dependent mechanism. Clarke et al. (1975) demonstrated that sodium-[1-14C]acetate was enzymatically incorporated into the tRNA fraction from which was isolated N-[1-14C] NAA. The authors suggested that N-acylation of aspartate occurred on aspartyl-t-RNA and NAA-t-RNA might be an initiator of protein synthesis because N-blocked amino acids could only be incorporated into a protein or a polypeptide chain at the
N-terminus.

Finally, NAA has also been shown to be involved in other pathways of brain metabolism. For example, when NAA was incubated with minced rat brain slices at room temperature, both adenyl and guanyl cyclases were activated (Burgal et al., 1982). Further studies by Burgal et al. (1984) documented that, in adult rat, NAA exerted a stronger stimulation on cAMP production than glutamate and aspartate.

NAA and Brain Pathology

Since the biological role(s) of NAA in normal brain is unknown, the role of NAA metabolism in the pathology of CD is highly speculative. N-Acetylaspartic acid is primarily located in the neurons. (Moffett et al., 1992 and 1993, and Urenjak et al., 1992). It has been used as a neuronal marker to assess the physiological status of neurons in brain (Arnold et al., 1990). Gideon et al. (1992) documented that NAA was significantly reduced in the brain after stroke. They also found that such reduction appeared between 6 and 24 hrs after the stroke incident and was mostly irreversible. Meyerhoff et al. (1993) has suggested that reduction in NAA in cognitively impaired HIV-seropositive individuals as an indicator of neuronal damage in early stages of HIV infection. Recently, Dunlop et al. (1992) observed a marked reduction of NAA concentration in certain
brain areas in patients with Huntington’s disease. They proposed to use NAA as a determinant of the course of Huntington’s disease.

Marcucci et al. (1984) observed that significant decrease in brain NAA content in male dystrophic mice might reflect developmental retardation rather than a direct relationship to the dystrophic condition. Using in vivo and in vitro nuclear magnetic resonance spectroscopy, Bell et al. (1991) detected a reduction in NAA levels in the brain of a mouse scrapie model. Such reduction in NAA also was observed in the brain of aggressive mice (Marcucci et al., 1968, and Marcucci and Giacalone, 1969).

The discovery of deficiency of aspartoacylase and N-acetylaspartic aciduria in CD for the first time suggest a specific role for aspartoacylase and its hydrolysis of NAA in normal brain.

Detection and Quantitation of NAA

The method for NAA analysis, described by Tallan et al. (1956), used ninhydrin reagent to detect NAA in the samples. The samples were subjected to ion-exchange chromatography for fractionation. Alternatively, high voltage paper electrophoresis and visualization by aniline/xylose reagent were developed for detection of NAA and other amino acids in brain homogenates (Miyake et al., 1978). High performance
liquid chromatography has also been used for estimation (Koller et al., 1984).

Miyake et al. (1982) reported the quantitation of NAA, NAAG and beta-citryl-L-glutamic acid in human urine using a procedure consisting of ion-exchange chromatography followed by gas-chromatographic analysis. They found 41.21±10.1 and 62.2±16.3 μmol/g creatine in male and female adult humans. Using gas chromatography-mass spectroscopy of the sialylated compounds, Matalon et al. (1988) observed NAA levels of 10.0±2.1 μmol/mmol creatine in the urine of normal individuals.

Recently, the quantification of NAA by gas chromatography-mass spectrometry is achieved by isotope dilution, using 15N-[2H₃]NAA (Kelley and Stamas, 1992, and Matalon et al., 1992). This method can be used with solvent extraction techniques commonly employed in urinary organic acid analysis (Grodd et al., 1990, and Matalon et al., 1992). The mean concentration of NAA in urine from normal control individuals was 12.7±7.8 mmol/mol creatine (Kelley and Stamas, 1992). This method is sufficiently sensitive for estimation of NAA in cerebrospinal fluid. Jakobs et al. (1991) used this method to measure NAA in cerebrospinal fluid, blood, urine and amniotic fluid for accurate postnatal diagnosis of Canavan disease. Control mean values and ranges obtained in the study were, Urine: 19.5 and 6.6-
35.4 μmol/mmol creatine; Plasma: 0.44 and 0.17-0.81 μmol/L; CSF: 1.51 and 0.25-2.83 μmol/L; and Amniotic fluid: 1.27 and 0.30-2.55 μmol/L. The concentration of NAA in a patient with CD is elevated 80-fold in urine and 20-fold in plasma compared to the control means.

To quantify NAA in the CNS in vivo, localized proton nuclear magnetic resonance spectroscopy has been employed (Behar et al., 1983, Bell et al., 1991, Bottomley et al., 1985, Gyngell et al., 1991, Matalon et al., 1991 and Vink et al., 1988). The method has the distinct advantage of being non-invasive and is used for quantitation of NAA in vivo.

SECTION III. ASPARTOACYLASE

Biochemical Characteristics

Aminohydrolases or aminoacylases catalyze the hydrolysis of N-acylated amino acids to free amino acid and acetate. There are two aminoacylases, aminoacylase I and aminoacylase II that differ in their specificity towards amino acid residues (Birnbaum, 1955, Birnbaum et al., 1952, and Goldstein, 1976). Aminoacylase I hydrolyzes all N-acylated amino acids except NAA and the acyl derivative of proline (Goldstein, 1976). The presence of aminoacylase I activity was demonstrated in all the species examined,
including pigeon, mouse, rat, hamster, guinea-pig, rabbit and monkey (Goldstein, 1976). Aminoacylase I, purified from pig kidney, is a homodimer, zinc-binding metalloenzyme with a native molecular mass of 86 kDa and an isoelectric point of pH 5.0 (Kordel and Schneider, 1976). Recently, human aminoacylase I cDNA has been cloned and characterized. The gene has been localized to chromosome 3p21.1 (Cook et al., 1993).

Aminoacylase II or aspartoacylase was characterized in pig kidney homogenate by Birnbaum et al. (1952). There were reports that aspartoacylase was active in both intact brain post mortem (Jacobson, 1959) and brain homogenates (Goldstein, 1959). Kameda et al. (1978) described the purification and properties of an acylase from Ehrlich ascites carcinoma cells. The enzyme hydrolyzed only N-dichloroacetyl-L-aspartate and had no activity towards NAA. The enzyme had a pH optimum around 8.5 and was inhibited by heavy metal divalent cations, p-chloromercuribenzoate, and diisopropyl fluorophosphate.

The discovery of aspartoacylase deficiency as the basic biochemical defect in CD attracted interest to explore its role in normal brain biology. Kaul et al. (1991a) purified bovine brain aspartoacylase more than 73,000-fold to a specific activity of 19,754 mU/mg protein. The native enzyme was a monomer with a molecular weight of 58 kDa. The
pI of this protein was 5.65, as determined by isoelectric focusing gel. The activity of purified aspartoacylase was enhanced 20-40% by low concentration of various divalent cations, such as Zn$^{2+}$ (0.001mM), Mn$^{2+}$ (0.01mM), Mg$^{2+}$ (1.0mM), and Ca$^{2+}$ (1.0mM). Higher concentration of these divalent cations inhibited the ASPA activity (Kaul et al., 1991a). Metal chelators such as EGTA and EDTA up to 5mM did not affect the activity of purified aspartoacylase, whereas 2.0 mM EGTA inhibited 90% of aspartoacylase activity in crude tissue homogenates. The change in the sensitivity of purified enzyme to metal chelators suggests that the purified enzyme is not a metalloenzyme and does not have an absolute requirement for metal ions. The authors postulated that under in vivo conditions, ASPA may not be present in free form. Such structurally bound enzyme may be maintained in an active state by metal ions. During the purification process, the aspartoacylase may have undergone a conformational change so that it did not require metal ions obligatorily for manifestation of its enzyme activity (Kaul et al., 1991a). Sulfhydryl agents such as DTT and BME stabilize the enzyme activity. DTT at concentrations up to 0.5mM slightly increased the enzyme activity. However, increasing concentration of DTT exhibited inhibitory effect on the enzyme and DTT at 5.0 mM concentration resulted in an irreversible and complete loss of activity. The effect of
DTT on the aspartoacylase activity suggested the presence of intramolecular disulfide bond in aspartoacylase (Kaul et al., 1991a).

Bovine aspartoacylase followed the classical Michaelis reaction kinetics of a monoreactant enzyme, with a $K_m$ of 0.85mM and a $V_{max}$ of 43,000 nmol/min/mg of protein. Several N-derivatives of D- and L-aspartic acid including N-carbobenzoxy (N-CBZ)-D-aspartic acid, N-tert-butoxycarbonyl (N-t-BOC)-D-aspartic acid-beta-benzyl ester, and glycyl-L-aspartate inhibited the activity of bovine aspartoacylase. These studies suggested that the carbon backbone of the substrate was primarily involved in its interaction with the active site of the enzyme. It was also concluded that a blocked amino acid group is essential for the catalytic activity of aspartoacylase (Kaul et al., 1991a).

**Tissue Distribution and Localization of Aspartoacylase**

Aspartoacylase has been found in mammalian brain and other peripheral tissues (D'Adamo et al., 1973, Kaul et al., 1991a and 1991b). Goldstein (1976) demonstrated that the enzyme was distributed throughout the rat brain, with higher activities in areas rich in white matter. There is an inverse relationship between the aspartoacylase activity and NAA concentration in homogenates from various parts of brain (Goldstein, 1976, and Kaul et al., 1991a and 1991b).
aspartoacylase activity in white matter of the bovine brain was three times higher than that found in grey matter, whereas the NAA concentration was three-fold higher in the gray matter compared with white matter (Kaul et al., 1991a and 1991b).

Aspartoacylase has also been purified from several other sources, including bovine kidney and human brain, kidney, and cultured skin fibroblasts (Kaul et al., 1991a and 1991b). The aspartoacylase activity was highest in kidney followed by brain and cultured skin fibroblasts, lung, adrenal and liver. Aspartoacylase has been suggested to serve a salvage role in organs other than brain, where NAA hydrolysis seems to be essential for normal brain function (Kaul et al., 1991a and 1991b).

Biochemical studies have demonstrated that aspartoacylase is localized to the white matter (Kaul et al., 1991a and 1991b). The antiserum raised against purified bovine brain aspartoacylase was used for immunohistochemical localization of the enzyme in mammalian brain. Immunostaining was observed positive only in white matter along the myeline tracks (Kaul et al., 1991a). These antibodies were tested in Western blot analyses of crude bovine kidney extract. A single immunoreactive band was observed with a pI around 5.7 (Matalon et al., 1993a). The pI observed was similar to that determined from purification
studies of aspartoacylase (Kaul et al., 1991a).

**Conservation in Evolution**

Both biochemical and immunohistochemical studies have suggested that aspartoacylase is highly conserved during evolution. Human and bovine aspartoacylase share similar biophysical properties (Kaul et al., 1991a). The antiserum raised against purified bovine brain aspartoacylase cross-reacted with aspartoacylase from human brain and bovine kidney and inhibited the aspartoacylase activity from these sources (Kaul et al., 1991a). Studies carried out by Johnson et al. (1989) showed that antiserum to bovine aspartoacylase cross-reacted with the brain enzyme from rats, mice, monkeys and humans, and that the distribution of aspartoacylase in various mammalian brains follows a pattern similar to that seen in bovine brain.

**Section IV. SPONGY DEGENERATION OF BRAIN IN OTHER ANIMALS**

The establishment of animal models for a human disease is crucial for studying its pathogenesis and developing therapeutical strategies. Spongy degeneration of the central nervous system has been reported in dogs, mice, and foxes. The spongy degeneration observed in these animals resembles
the pathology observed in Canavan disease in humans. However, due to the lack of biochemical data in these animals, it is not certain if they indeed are models for CD. The various animals where spongy degeneration has been reported are described below.

Dog

Spongy degeneration of brain in dogs vary with regard to their clinical symptoms. Richard and Kakulas (1978) described spongiform leukoencephalopathy associated with congenital myoclonia syndrome in the dog, characterized with uncontrolled muscular contractures apparent at birth. Mason et al. (1979) reported a Samoyed puppy with hindlimb tremor at 12 days of age, which in 5 days progressed to a generalized tremor. Zachary and O'Brien (1985) reported spongy degeneration of the CNS in two canine littermates. These animals showed the symptoms of extensor rigidity, tremor, ataxia, and opisthotonos. Microscopic examination of brain revealed vacuoles, hypertrophied fibrous astrocytes, myelin loss, and prominent blood vessels. No significant myelinosis, inflammation, or axonal degeneration were observed. The pathological changes observed under electron microscope included intramyelinic vacuoles with separation of lamellae at intraperiod lines and larger spaces formed by coalescence of ruptured vacuoles. The characteristics of
hypertrophied astrocytes include abundant glial filaments, edematous cytosol, membrane-bound crystalline inclusions, dilated cytocavitary system, and abnormal mitochondria (Zachary and O'Brien, 1985). The authors concluded that the clinical, histological, and ultrastructural findings of spongy degeneration of CNS in dogs (Zachary and O'Brien, 1985) were similar to those reported for the juvenile form of Canavan’s disease in children (Adachi et al., 1973, and Matalon et al., 1993a).

Mice

Spontaneous spongy degeneration of the mouse brain was described by Azzam et al. (1984). This naturally-occurring disorder with an autosomal recessive pattern of inheritance was found in the Charles River strain of Swiss-Webster mice. The affected animals showed enlargement of the cranium, failure to thrive and tremor of the hindlimbs when held by the tail in a suspended position. Microscopically, a spongy degeneration of the white matter of the entire neuraxis was apparent. Ultrastructural studies specified the abnormality to the cell body and processes of astrocytes which were distended and enlarged with dispersion of cytoplasmic organelles.
Foxes

Recently, Hagen and Bjerkas (1990) have described spongy degeneration of white matter in the CNS of Norwegian-bred silver foxes (Vulpes vulpes) based on case histories of 21 clinically affected animals. Clinical signs of the disorder in foxes were characterized by caudal limb ataxia which appeared between 2.5 and 4 months of age and progressed over the next 4-8 weeks. Thereafter, the affected animal showed moderate to marked improvement, in contrast with the disorder in humans and other mammalian species. Pathological lesions were restricted to the white matter of brain and spinal cord. A symmetrical spongy change with vacuoles of varying sizes and significant myelin deficiency were observed in the lesion areas. Like the situation in the human disease, an astrocytic hypertrophy was associated with the spongy change. Ultrastructural findings include intramyelin vacuoles resulting from splitting of the myelin lamellae at the intraperiod line, expanded extracellular spaces, watery astrocytic processes, and hypertrophic astrocytic processes in lesioned areas. Again, Hagen and Bjerkas believed that the spongy degeneration of white matter in foxes had clinical and pathological features in common with the juvenile form of Canavan disease in children and spongy degeneration found in dogs (Adachi et al., 1973, Matalon et al., 1993a, and Zachary and O’Brien, 1985).
SECTION V. RESEARCH STRATEGIES FOR IDENTIFICATION OF DISEASE GENES

The biochemical deficiency of aspartoacylase as the basic defect in Canavan disease was established by Matalon et al. (1988). Aspartoacylase has further been purified to homogeneity and its properties and localization were studied (Kaul et al., 1991a and 1991b). In order to understand the molecular basis of Canavan disease, and to develop therapeutical strategies for the disease, the cloning and characterization of the human aspartoacylase gene were undertaken as the main goals of this research project.

Historically, two different approaches have been undertaken for the isolation of genes involved in genetic disorders. These two approaches include: (a) functional cloning and (b) positional cloning. The two strategies have alternatively been called "forward genetics" and "reverse genetics" (Collins, 1992, and Orkin, 1986).

Functional Cloning Or Forward Genetics

Functional cloning of a gene requires knowledge of the protein product of the disease gene. The success of this approach depends on the availability of the amino acid sequence, and/or antibody to the protein. Alternatively, the function of the gene product, such as its role as
receptor/ligand, or a selectable marker, needs to be understood so that it can be used for the isolation of the putative gene clones. The cDNA libraries can be screened with probes that include antibodies to protein and degenerate oligonucleotide probes based upon the known amino acid sequence. Such probes can also be used successfully for isolation of homologous gene products across species. The isolation of cDNA clones has been revolutionized by the availability of techniques based upon reverse transcription and polymerase chain reaction (RT-PCR). The mRNA of the gene of interest is amplified by RT-PCR using oligonucleotides derived form the protein sequence. Cloning of disease genes for hemoglobinopathies (Orkin, 1987) and hypercholesterolemia (Brown and Goldstein, 1986) are excellent examples of the functional cloning process. In these genetic disorders, the proteins adversely affected by mutations associated with the diseases were first identified by traditional cellular and biochemical approaches. Then reagents directed specifically to these proteins, such as antibodies or nucleotide probes based on amino acid sequences, provided the means for isolation of molecular clones (cDNA and genomic clones) encoding these gene products. In turn, these molecular probes provided the tools for dissection of the inherited disorder for understanding the molecular basis of these diseases.
Positional Cloning Or Reverse Genetics

Many inherited disorders lack adequate knowledge of the biochemical basis of the disease. The identification of the disease gene product may be hampered by its low abundance, lack of suitable functional or selection assays, failure to recognize the specific cell type where the product is produced, inconclusive biochemical evidence, complex interactions between proteins, and lack of animal models that faithfully mimic the human disease. In such instances, "reverse genetics" or positional cloning strategies are the method of choice for isolating a disease gene. The search starts from the knowledge of its genetic/physical location in the genome and usually with little useful information regarding its function (Collins, 1992, Ballabio 1993, and Orkin, 1986). In the past seven years about 20 disease genes have been identified using the positional cloning strategy (Ballabio, 1993). It needs to be pointed out that the disease genes isolated by positional cloning were usually associated with relatively common diseases so that a large number of pedigrees were available for genetic mapping of the disease loci. The isolation of cystic fibrosis transmembrane conductance regulatory (CFTR) gene for cystic fibrosis is the classical example for positional cloning of a disease gene (Riordan et al., 1989 and Zielenski et al., 1991). The deletion or translocation observed in certain
disorders greatly facilitate localization and eventual isolation of these disease genes. The disease genes isolated by positional cloning need to be demonstrated to be associated with the disease. The linkage disequilibrium of a locus with a particular genetic disorder is carried out using DNA markers that recognize restriction fragment length polymorphisms (Botstein et al., 1980), chromosomal deletion and translocation. These studies lead to assignment of the disease locus to a precise chromosomal position (Collins, 1992, and Orkin, 1986). Positional cloning of the gene associated with fragile X syndrome (FMR1 gene) is a good example of cloning by this strategy (Fu et al., 1992, and Verkerk et al., 1991).

**Positional Candidate Cloning**

Unfortunately, most genetic disease genes do not fall into the categories discussed above, where either function is known or cytogenetic data is informative or large pedigrees are available. For these diseases in which there are no identified biochemical markers, insufficient families for chromosomal mapping and a precise genetic localization, and no known patients with identifiable chromosomal rearrangements, positional candidate approach is the choice of cloning strategy (Collins, 1992). This approach is not focused on isolating new genes but relies on the
availability of information from previously isolated genes. For instance, the human glycerol kinase gene was recently isolated by mapping a randomly isolated expressed sequence tag (EST) which is homologous to bacterial GK gene to the human Xp21 region (Sargent et al., 1993). In this case, the combination of information on function and position led to the gene's identification.

**Strategy for Isolation of Human Aspartoacylase Gene**

The enzyme deficiency in Canavan disease is known (Matalon et al., 1988). Bovine aspartoacylase has been purified to homogeneity and its partial amino acid sequence has been determined (Kaul et al., 1991a, and 1991b). It is therefore logical to use "forward genetics" or functional cloning approach for cloning the human aspartoacylase gene.
SECTION VI. RESEARCH GOALS OF THE PROJECT AND OUTLINE OF EXPERIMENTAL APPROACHES

The objectives of this project were to: isolate human aspartoacylase cDNA and genomic clones, determine their nucleotide sequences, identify exon/intron boundaries, establish the gene structure, and carry out chromosomal localization of human ASPA gene. The prospectus was to express the human ASPA cDNA in both prokaryotes and eukaryotic cells in culture, and identify the mutations that lead to deficiency of ASPA in patients with CD.

The following experimental approaches were undertaken to accomplish these goals:

1) Isolation of bovine aspartoacylase cDNA clones by RT-PCR using degenerate nucleotides based upon known amino acid sequence, and screening of bovine cDNA phage libraries using partial cDNA clones as probe;

2) Isolation and characterization of human aspartoacylase cDNA clone based on sequence information from bovine ASPA cDNA, using the strategies similar to that described in the first step;

3) Isolation of human and bovine genomic clones for aspartoacylase gene, determination of exon/intron boundaries and establishment of gene structure of aspartoacylase;

4) Chromosomal localization of ASPA locus using somatic
cell hybrid cell lines and fluorescent in situ hybridization procedure;

5) Expression of the human ASPA cDNA clone in both prokaryotic and eukaryotic systems to confirm the legitimacy of the isolated cDNA clone;

6) identification of mutations in the ASPA gene of patients with Canavan disease using single strand confirmation polymorphism (SSCP), PCR and determination of nucleotide sequence;

7) Determination of the frequency of mutations in Canavan alleles from Ashkenazi Jewish and non-Jewish ethnic background; and

8) in vitro mutagenesis of human ASPA cDNA to explore the effect of Canavan mutations on the aspartoacylase activity.
SECTION VII. SIGNIFICANCE OF THE STUDY

cDNA Clones

Isolation and characterization of human ASPA gene should be a significant step forward in our understanding of molecular basis of CD. The isolation of human ASPA cDNA clone will predict the amino acid sequence of ASPA. This information will be useful for predicting the secondary structure of the human aspartoacylase protein, carrying out the study of structure-function relationships of human ASPA by in vitro mutagenesis of human ASPA cDNA, and identifying catalytic domain and/or activity center for the ASPA enzyme. It will be now possible to identify the transcription start site of ASPA gene, using cDNA as a template, by the primer extension method (Weaver and Weissman, 1979). The availability of cDNA will make it possible to study the expression of ASPA in vitro. One can identify the expression of ASPA in different cell types and organs at the transcript level. Identification of cell types in CNS, that express aspartoacylase, is essential for understanding its role in normal brain biology and deciphering the mechanism of pathophysiology in CD. Isolated cDNA clone will identify the initiation AUG codon, presence of any pre- and/or propepetides, and polyadenylation signals for processing of primary transcripts. These studies will provide the
information regarding the biosynthesis, and post-transcriptional and post translational regulation of the human ASPA gene. Characterization of cDNA can also suggest the presence of any alternative spliced transcripts (Lecomte et al., 1987) and/or alternative usage of any multiple promotors of ASPA gene (Rajagopalan et al., 1993). The human cDNA clone will be useful to study the evolution of the ASPA gene across species. Comparative study of aspartoacylase gene from bovine, human and other mammalian sources will provide insight into the evolution of the aspartoacylase gene. The human ASPA sequence will be useful for the identification of mutations in coding sequences and those that result in exon-skipping.

Genomic Clones

Characterization of genomic sequences will be useful for identification of transcription start site by S1-nuclease protection assay (Berk and Sharp, 1977). Genomic clones are also essential for characterization of promoter and other cis-regulatory elements critical to the regulation of ASPA expression. The organization of exons and introns in ASPA gene will be deduced from the genomic clones. The genomic sequences are also important in identification of mutations in the non-coding sequences of the ASPA gene.
Localization of ASPA gene

With the availability of genomic clones spanning the whole ASPA gene, it will be possible to map the ASPA to a specific region of a specific chromosome in the human genome. Chromosome localization of the ASPA gene will provide an additional marker for human gene mapping. It is also possible to study the ASPA and those genes physically linked to it act as a functionally, developmentally and differentially regulated-gene cassette.

Diagnosis and Epidemiology of CD

The identification of mutations in patients with CD will provide a molecular basis for our understanding of CD. The mutation data will be useful to correlate the patient genotype and biochemical property of the ASPA enzyme in patients to their clinical phenotype. Mutation analysis may identify essential functional and regulatory motifs in the ASPA gene and its products. The identification of mutations in patients with CD will offer a DNA-based pre- and postnatal diagnosis of Canavan disease in informative families. Such reliable diagnosis should lead to prevention of the occurrence of CD in at-risk families. Outcome of the mutation study should make it possible to perform carrier screening and molecular epidemiology study of CD in the at-risk populations.
Pathophysiology and Therapy

The human ASPA cDNA will be used to isolate mouse cDNA and genomic clones which will be essential for creating a mouse model for Canavan disease. Such a model is crucial for understanding the molecular basis of the disease. The outcome of this study will provide the possibility to explore alternate strategies for enzyme and gene therapy for CD.
CHAPTER II

Cloning of the human aspartoacylase cDNA and a common missense mutation in Canavan disease

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SUMMARY

Canavan disease, an autosomal recessive leukodystrophy, is caused by deficiency of aspartoacylase and accumulation of N-acetylaspartic acid in brain. Human aspartoacylase (ASPA) cDNA spanning 1,435 bp has been cloned. The isolated cDNA expresses aspartoacylase activity in E. coli. A base change, a854>c, has been found in 85% of the 34 Canavan alleles tested so far. This base change results in a missense glu285>ala mutation that is predicted to be part of the catalytic domain of aspartoacylase. The data suggest that the catalytic center of aspartoacylase involves a triad of ser, his and glu residues.
Canavan disease (CD) or spongy degeneration of brain is an autosomal recessive leukodystrophy associated with mental retardation, megalencephaly, hypotonia, and death, usually in the first decade of life. Brain histology in CD is characterized by spongy degeneration of white matter with astrocytic swelling and elongated mitochondria (Adachi et al., 1972, Adornato et al., 1972, Canavan, 1931, Globus and Strauss, 1928, and van Bogaert and Bertrand, 1949). Canavan disease is more prevalent in Jewish people of Ashkenazi origin (Adachi et al., 1972, Adornato et al., 1972, and van Bogaert and Bertrand, 1949). Matalon et al. in 1988 described aspartoacylase deficiency as the basic biochemical defect in CD (Matalon et al., 1988). Aspartoacylase specifically hydrolyses N-acetyl-L-aspartic acid (NAA) to aspartate and acetate (Birnbaum, 1955, and Birnbaum et al., 1952). The deficiency of aspartoacylase in CD leads to excessive excretion of NAA in urine; and its accumulation in brain (Grodd et al., 1990, and Matalon et al., 1988, 1989, and 1993b). Since the initial report, we have diagnosed 145 patients with CD at our center alone, suggesting that CD is more prevalent than previously thought (Grodd et al., 1990, and Matalon et al., 1993a and 1993b). Aspartoacylase deficiency in CD has also been reported by other
investigators (Michelakakis et al., 1991, and Ozand et al., 1990). The diagnosis of CD is now routinely made by quantitation of NAA in urine and estimation of aspartoacylase activity in cultured skin fibroblast (Matalon et al., 1993a).

Aspartoacylase activity has been found in a variety of mammalian tissues, including kidney, brain white matter, adrenal glands, lung, liver and cultured skin fibroblasts. Brain grey matter and blood constituents do not have any detectable aspartoacylase activity (Kaul et al., unpublished studies). The level of aspartoacylase activity found in direct chorionic villi cells or in cultured chorionic villi cells and amniocytes is about 2 orders of magnitude lower than that found in normal cultured skin fibroblasts. Due to the low aspartoacylase activity in chorionic villi cells and amniocytes, the prenatal diagnosis of CD using aspartoacylase assay is not satisfactory (Matalon et al., 1992).

Aspartoacylase has been purified and characterized from bovine brain and from other bovine and human sources (Kaul et al., 1991a). Stereospecificity of aspartoacylase towards L-analog of NAA has been well documented. The D-analog of NAA acts as a weak inhibitor of NAA hydrolysis by aspartoacylase. Studies have suggested that the carbon backbone of NAA is involved in interaction with the
substrate binding site of aspartoacylase; and that the substitutions at α and β carboxyl groups of aspartate moiety do not have any effect on hydrolysis of NAA by aspartoacylase (Kaul et al., 1991a). Biochemical and immunochemical studies suggest that aspartoacylase has been conserved during evolution. Aspartoacylase in brain has been localized to white matter associated with myelin tracks (Kaul et al., 1991a). How aspartoacylase and the hydrolysis of NAA are involved in keeping white matter intact, is not clear. It is also not understood how the deficiency of aspartoacylase leads to the pathogenesis seen in CD.

This report describes the isolation and expression of human ASPA cDNA. A base change in the human ASPA encoded aspartoacylase has been found in 85% of 34 Canavan alleles. The resulting mis-sense mutation has been localized in the predicted catalytic domain of aspartoacylase.
MATERIALS AND METHODS

**Materials, reagents and bacterial strains.** The materials and reagents used in the study were: Immobilon PVDF transfer membrane (Millipore, Bedford, MA); restriction enzymes (IBI, New Haven, CT; New England Biolabs, Beverly, MA, Promega, Madison, WI and Boehringer Mannheim, Indianapolis, IN); Gene Amp RNA PCR kit and AmpliTaq PCR kit for amplification of DNA (Perkin Elmer Cetus, Norwalk, CT); Random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN); α-[\(^{32}\)P]-dNTP'S, 3000 Ci/mMole (NEN/Dupont, Wilmington, DE); RNAzolB kit for preparation of cytoplasmic RNA from cultured cells (Biotecx, Houston, TX); Biodyne Nylon membranes for Southern and Northern blots (Pall Biosupport, East Hills, NY); Nitrocellulose membranes for screening libraries (Schleicher & Schuell, Keene, NH); and Taq Dye primer and Taq dye-terminator cycle sequencing kits for fluorescent labeled automated DNA sequencing (Applied Biosystems, Foster City, CA). λUni-Zap (host strain XL1 Blue) human lung cDNA library and pBS(+) and pBluescript SK phagemid vectors were from Stratagen (La Jolla, CA); λgt11 (host strain Y1090) human kidney and bovine lung cDNA libraries; λEMBL-3A Sp6/T7 (host strain LE 392) bovine genomic library; and poly(A)\(^{+}\) RNA were from Clontech (Palo Alto, CA).
Amino acid sequence of bovine aspartoacylase peptides.
Amino terminal sequence of bovine brain aspartoacylase could not be obtained due to the autolysis of purified protein during storage. Purified aspartoacylase was digested with cyanogen bromide (Kaul et al., 1983) and peptides were fractionated on a 16X100 mm Mono Q column (FPLC system, Pharmacia LKB). Peptides were eluted with a 0-30% linear gradient of 1 M sodium chloride in buffer A (25 mM Tris.Cl pH 7.2 and 0.1% sodium azide), at a flow rate of 2 ml/min. The amino acid sequence of four peptides, determined at the protein sequencing facility (Yale University, New Haven, CT), were CN8.1: LENSTEIQRT GLEVKPFLTNPRAVKK; CN8.2: KPLIPXDPVFLTDGKTISLGDPQTXYPFXNEXAYY; CN30: XKVDYPRNESGEISAIHPKLQDQ; and CN41: XXXALDFIXNFEXKE.

Oligonucleotides, reverse transcription, PCR amplification, DNA probes. Oligonucleotides were synthesized by phosphoramidite chemistry on 380B DNA Synthesizer (Applied Biosystems, Foster city, CA). First strand cDNA was synthesized by reverse transcription of 20 ng of Poly(A)^+ RNA or 4 μg of cytoplasmic RNA with either oligo(dt)_n or a gene specific primer under standard conditions suggested by the manufacturer. DNA amplification (Saiki et al., 1985) was carried out in DNA Thermal cycler Model 9600 (Perkin-Elmer Cetus, Norwalk, CT). Specific DNA sequences were
amplified in 100 µl volumes with 20 ng of cloned or 500 ng of genomic DNA template according to the standard conditions suggested by the manufacturer. The PCR conditions were: 1 cycle of denaturation at 94°C for 3 min, annealing for 30 sec at temperatures (Tm-4°C) that were primer dependant and extension at 72°C for 1 min; followed by 27 cycles of denaturation at 94°C for 30 sec, annealing for 30 sec and extension at 72°C for 30 sec. The last step was extension at 72°C for 7 min. The tubes were chilled to 4°C until further analysis. For SSCP (Orita et al., 1989) and restriction digestion analysis of mutant alleles, PCR was carried out similarly except that 1 µCi of α-[32P] dCTP was included in the PCR reaction mixture.

Small DNA fragments of less than 200 bp were labeled with 32P by PCR amplification of the desired DNA sequences using α-[32P] dNTP’s. DNA fragments bigger than 200 bp were labeled by Random primer method (Feinberg and Vogelstein, 1984) following the conditions suggested by the manufacturer. The specific activity of probes were 3 - 5 x 10⁸ cpm/µg DNA.

Isolation of cDNA clone, and determination and analysis of the nucleotide sequence. The first strand bovine cDNA was synthesized by reverse transcription (RT) of bovine kidney poly(A)+ RNA with oligo(dt)₂₀. The bovine aspartoacylase
specific coding sequences were amplified by polymerase chain reaction (PCR) using first strand cDNA as template and CN30 peptide based primers CD5 (AAA/GGTIGAC/TTAC/T CCIIGIAA;) and CD8 (TGA/GTCC/TTGIAIC/TTTIGGA/G TG). The cDNA fragment thus amplified was 69 bp long; the size expected from CN30 peptide. The ORF of this fragment predicted the amino acid sequence of CN30 peptide. The 69 bp fragment was used as a probe to screen λgt11 bovine lung cDNA library. One cDNA clone, λABL2, was isolated. The insert in λABL2 had a single 839 bp long ORF. The amino acid sequence predicted by the ORF of λABL2 insert contained CN8.1, CN8.2, CN30, and CN41 peptide sequences described earlier. However, the cDNA clone was truncated at the 5' and 3' termini. The sequences downstream of the 3' termini of λABL2 insert were cloned by RT-PCR amplification of bovine kidney poly(A)+ RNA using oligo(dt)20 and bovine aspartoacylase cDNA specific primer CD48-1 (CCGTGTACCCAGTGTT). A unique 770 bp fragment was amplified that overlapped with the 3' sequence of λABL2 insert. The insert from λABL2 was used to screen bovine liver genomic library cloned in λEMBL3A vector according to the standard conditions (Kaul et al., 1986, and Sambrook et al., 1989). Limited nucleotide sequence of genomic clones identified the missing 5' coding and non-coding sequences of bovine aspartoacylase cDNA.

Bovine cDNA (data not presented), was next used for
isolation of human ASPA cDNA clone. Human cDNA libraries were screened and the isolated clones analyzed by the methods described earlier or according to the standard protocols (Kaul et al., 1986, and Sambrook et al., 1989). The human lung aspartoacetylase cDNA was rescued from λUni-Zap human lung cDNA clone by co-transfection with helper phage R408 into XL-1 Blue strain of E. coli according to the protocol suggested by the manufacturer. The rescued recombinant pHLASP was transfected into E. coli (XLI Blue strain). Large scale recombinant phagemid DNA were purified on cesium chloride gradients (Sambrook et al., 1989).

The nucleotide sequence of double stranded plasmid and amplified DNA fragments was determined by the dideoxy chain termination method (Sanger et al., 1977). M13 universal/reverse and T3/T7 primers, or di-deoxy NTP’s, tagged with fluorescent dyes, were used for sequencing both strands of DNA fragments on automated 373A DNA sequencer (Applied Biosystems, Foster city, CA). Fluorescent DNA sequencing was carried out with DNA sequencing kits and used according to the protocols suggested by the manufacturer. Sequences were analyzed with Lasergene software package for DNA analysis from DNASTar (Madison, WI).

Analysis of RNA. Cytoplasmic RNA was prepared from cultured cell lines (Chomczynski and Sacchi, 1987) using RNAZOL B kit
for isolation of RNA (Biotecx, Houston, TX). Two to three μg of poly(A)+ RNA and 4.5 μg of RNA markers (Promega) were denatured with formamide. The denatured RNA was fractionated on 1% agarose gel in formaldehyde containing buffer. The RNA was transferred overnight onto nylon membrane. The blots were baked in a vacuum oven at 80°C for 90 min, hybridized and washed under stringent conditions and autoradiographed (Sambrook et al., 1989).

Expression of aspartoacylase by human ASPA cDNA clone pHLASP. The E. coli (XLI Blue strain) transformed with recombinant pHLASP or wild type pBluescript SK' phagemid were grown overnight in 15 ml of Luria broth containing glucose (0.1%) and ampicillin (50 μg/ml) in absence or presence of 20 mM IPTG. The bacteria were harvested, treated with 500 μL of 0.05 mg/ml lysozyme in 25 mM Tris.Cl pH 8.0, 10 mM EDTA and 50 mM glucose for 10 min at ambient temperature. The treated bacteria were diluted with ice cold 1.5 ml of 50mM Tris.Cl pH 8.0, 0.01% β-mercaptoethanol and 0.01% sodium azide and sonicated by three 10 sec bursts in ice cold conditions. Protein concentration in bacterial sonicates was determined (Lowry et al., 1951). Aspartoacylase assays were carried out in duplicate with 450 μg protein of bacterial sonicate under standard incubation conditions described elsewhere (Kaul et al., 1991a).
Controls lacking substrate or enzyme during the incubation were run simultaneously to account for background absorption in each assay. One mU of enzyme activity is 1 nanomole of aspartate released/min of incubation time.

**Mutation analysis.** Cytoplasmic RNA (4 µg) from cultured fibroblast of normal controls, probands and their family members was reverse transcribed with HKRT1 (AACCCTACTCTTAAGGAC). Aspartoacylase specific coding sequences were amplified by PCR with HASP9 (CTTCTGAATTGCAGAAATCA) and HASPC7 (GTAAGACACCGTGAAGATG) primers. The full length coding sequence thus amplified was used as template to amplify 200 to 300 bp overlapping cDNA fragments and nucleotide sequence was determined. For a854>c point mutation analysis, 312 bp fragment was amplified with HASP14F (F-CCGGGATGAAAATGGAGAA) and HASPC7R (R-ACCCTGTGAAGATGTAAAGC) primers. The prefix F and R in these oligos stand for M13 universal and reverse primer tags, that allowed determination of nucleotide sequence using fluorescent tagged M13 primers.

For SSCP and restriction digestion analysis, the 237 bp cDNA fragment with a854>c mutation was amplified in presence of α-[³²P] dCTP using HASPG5 (AGGATCAAGACTGGAAACC) and HASPC7 (GTAAGACACCGTGTAAGATG) primers. The analysis for SSCP in the 237 bp amplified cDNA fragments was carried out
in 40 cm long, 5% polyacrylamide gels in 1x TBE and 5% glycerol (Orita et al., 1989). Electrophoresis was carried out at 7.5 Watts at ambient temperature for 16 - 18 h, and the gel was autoradiographed. The a854>c mutation was also analyzed by restriction digestion of 237 bp cDNA fragment with Eag I or Not I. Following electrophoresis of the digest on native 5% polyacrylamide gels in 1XTAE, the gel was autoradiographed.
RESULTS

Isolation and characterization of human ASPA cDNA

Human aspartoacylase specific coding sequences were amplified by RT-PCR of human kidney poly(A)$^+$ RNA using oligo (dt)$_{16}$ for first strand cDNA synthesis followed by PCR using bovine cDNA, (data not shown), based primers CD56I (GGG/ATAIACTGTT/CTGG/ATCICCIC) and CD59I (CCIA/CGIGCIGTIAAA/GAAA/GTG). A specific 676 bp cDNA fragment was amplified and found to have 90% identity to the corresponding region of the bovine ASPA cDNA. The 676 bp partial human cDNA was used as probe to screen human lung λUni-Zap and human kidney λgt11 cDNA libraries. This resulted in the isolation of several overlapping clones. Three of the clones with largest insert of 1.45 kbp, also had identical terminal sequences. One of these, λgt11 HK5-1 was isolated from human kidney library and 2 clones λUni-Zap HL 1 and 2 were isolated from the human lung library. The recombinant pHASP was excised from λUni-Zap HL1 clone. The nucleotide and the predicted amino acid sequence of pHASP is shown in Fig. 1.

The human ASPA cDNA is 1,435 bp with 158 bp 5’ and 316 bp 3’ untranslated sequence. The isolated cDNA has 18 bases long poly(A) tail. A polyadenylation signal ‘aataaa’ is found 48 bases upstream from the poly(A) addition site.
Another consensus polyadenylation signal sequence 'tataaa' is present 23 bases upstream from the poly(A) addition site. The position of 'tataaa' is within the suggested location of 10 - 30 bases upstream from the poly(A) addition site (Birnstiel et al., 1985, and Proudfoot and Brownlee, 1976). Human and bovine ASPA encoded transcripts and proteins do not match any known sequences in the databases.

The open reading frame (ORF) in human ASPA cDNA predicted 313 amino acid long protein that is 92% identical to bovine ASPA encoded protein (see Fig. 2). The molecular weight is estimated to be 36 kd. The predicted protein sequence has potentially one 'N' glycosylation and 7 phosphorylation sites. The amino acid sequence motifs GGTHGNE, DCTV and VNEAAYY identified in both proteins, are similar to the consensus sequences GXXHG/AXE/D, DXXF/V and VXEXXXX involved in the catalysis by esterases (Cygler et al., 1993).

**Tissue distribution and size of aspartoacylase transcripts**

Human poly(A)^+ RNA, 2 μg each from various tissues, were analyzed by Northern blot analysis (Fig. 3). The poly(A)^+ RNA from human liver gave a single band of 1.44 kb, whereas that from other human tissues tested gave two bands of 1.44 and 5.4 kb. The 1.44 kb faster moving band was similar in size to the 1.435 kbp human ASPA cDNA. It appears that
human brain expresses relatively higher proportion of larger transcript, as compared to relative amounts of the two transcripts in each of the tissues analyzed. The intensity of signals for both bands was highest in human skeletal muscles followed by kidney and brain.

**Human lung cDNA expresses aspartoacylase activity**

The orientation of human ASPA cDNA (pHLASP) cloned in pBluescript SK' phagemid vector is shown in Fig. 4. The cDNA was cloned as EcoRI and XhoI insert in pBluescript SK' phagemid in 5' → 3' direction. The transcription of human ASPA cDNA, driven by LACZ promoter, was studied in absence and presence of LACZ inducer isopropylthio-β-D- galactoside (IPTG) in E. coli (XL1 Blue strain). The mean aspartoacylase activity expressed by pHLASP construct was 0.111, (range 0.044 - 0.175), mU/mg protein in absence of IPTG. The activity increased 4-fold to 0.436, (range 0.223 - 0.622), mU/mg protein when cultures were grown in presence of 20 mM IPTG. The level of activity expressed by pHLASP was more than 2 orders of magnitude higher than the mean residual activity of 0.019, (range 0.000 - 0.043), mU/mg protein observed with wild type pBluescript SK' in presence of IPTG. These results demonstrate that human ASPA cDNA codes for aspartoacylase.
Mis-sense mutation in human ASPA cDNA from patients with CD

The ASPA encoded transcripts were analyzed from family members of 17 unrelated pedigrees of Ashkenazi Jewish background. Representative nucleotide sequence data for mutation analysis is shown in Fig. 5. A base change a854>c was identified in patients with CD. Both parents of a854>c homozygous patients were carriers for this mutation. Normal controls and non-carrier sibling of patients with CD did not have the a854>c base change in their ASPA encoded transcript. The a854>c base change would alter the E285 codon in aspartoacylase and result in E285>A mis-sense mutation.

The a854>c base change creates recognition sequences for Eag I and Not I restriction endonuclease in the mutant allele. The mutation was analyzed in an amplified 237 bp cDNA fragment for single strand conformation polymorphism (SSCP) and by digestion with restriction endonuclease Eag I. Typical mutation analysis profile from three representative pedigrees are shown in Fig. 6. Individuals homozygous, heterozygous or non-carriers for a854>c mutation could be differentiated by SSCP analysis (see Fig. 6, Panel A). Following digestion of 237 bp fragment with Eag I (Fig. 6, Panel B), probands homozygous for a854>c point mutation produced two restriction fragments of 125 and 112 bp; obligate carriers and heterozygotes had undigested 237 bp
fragment and two restriction fragments of 125 and 112 bp; while normal controls and non-carriers for a854>c point mutation had only 237 bp fragment. The a854>c base mutation was observed in 85% (29 out of 34) of the Canavan alleles and is inherited as a Mendelian recessive trait. Of the 17 probands, 12 were found to be homozygous and 5 were heterozygous for this mutation. In the 5 compound heterozygote patients with CD, the mutation on second Canavan allele is under investigation. The a854>c base change was not observed in any of 84 normal individuals analysed so far.
Discussion

The discovery of aspartoacylase deficiency and N-acetylaspartic aciduria has for the first time offered a definite diagnosis for CD without the need for brain biopsy (for review, see Matalon et al., 1993). The spongy degeneration of white matter in CD strongly suggests that hydrolysis of NAA by aspartoacylase plays a significant role in the maintenance of intact white matter.

Aspartoacylase activity is present in most of the tissues tested, (Kaul et al., unpublished studies). Northern blot analysis has confirmed the expression of human ASPA in the tissues tested so far. Since the pathology in CD is observed only in brain, it is suggested that aspartoacylase in tissues other than brain acts as scavanger of NAA from body fluids. The relative abundance of 2 human ASPA transcripts is apparently regulated in a tissue dependent manner. The choice of polyadenylation site, and/or alternative splicing of pre-mRNA, as reason for the 2 transcripts is plausible. The polyadenylation signal or/and alternative splicing may provide an additional mode of regulating gene expression (Lemeur et al., 1984).

Hydrolysis of NAA by aspartoacylase is highly specific. N-Acetyl derivatives of amino acids other than aspartic acid are not hydrolysed by aspartoacylase. In contrast, NAA is

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not hydrolysed by aminoacylase I, an enzyme that hydrolyses
N-acetyl derivatives of all other amino acid, including N-
acetyl-L-glutamic acid (Birnbaum, 1955, and Birnbaum et al.,
1952). Human ASPA encoded transcript and protein apparently
do not share any homology with the human aminoacylase I cDNA
(Miller et al., 1990). Human and bovine aspartoacylase have
sequences that have homology to the catalytic domain
sequence motifs reported in esterases and other related
hydrolytic enzymes (Cygler et al., 1993). The invariable
amino acid residues H and E involved in catalysis by
esterases are present in the consensus sequence motifs
GGTHGNE and VNEAAYY in aspartoacylase. The inhibition of
aspartoacylase activity by diisopropyl fluorophosphate,
(Kaul, unpublished data), further suggests that a S amino
acid residue is involved at the active site. It is
therefore proposed that aspartoacylase has an esterase like
activity and that its catalytic domain is predicted to
involve a triad of S, H, and E amino acid residues. The
E285 amino acid residue in wild type aspartoacylase is
indeed part of the VXEXXXY sequence motif involved in
catalysis by esterases, and is conserved in bovine
aspartoacylase. The substitution of the amino acid residue
E with A residue should lead to the inactivation of
aspartoacylase; as is observed in CD in patients with E285>A
mutation.
The mutation data is based on a sample of 17 unrelated pedigrees of Ashkenazi Jewish descent. Since a854>c base change in ASPA has not been observed in any of the 168 normal chromosomes analysed, it suggests that this base change is indeed a mutation causing CD. The apparently predominant nature of a854>c point-mutation, detected in 85% of the Canavan alleles, would suggest a founder effect of this mutation in the Jewish population. Such a predominant mutation should facilitate the study of epidemiology of CD in the population at risk. Mutation analysis should also lead to reliable prenatal diagnosis in informative families.

Canavan disease is the only known genetic disorder which is caused by a defect in the metabolism of a small metabolite, NAA, synthesized exclusively in the brain (Goldstein, 1959 and 1969); in a cell specific manner (Moffett et al., 1991, and 1993, and Urenjak et al., 1992). Since the initial discovery of NAA in brain (Tallan et al., 1956), its biological role has remained unknown (Cangro et al., 1987, Jacobson, 1957, McIntosh and Cooper, 1965, Orlov-Lavollee et al., 1987, and Shigematsu et al., 1983). The stable level of NAA in brain has made it a useful marker in ¹H NMR spectroscopy of brain (for review, see Birken and Oldendorf, 1989). Significantly reduced levels of NAA have been reported in non-CD focal or generalized demyelinating disorders (Grood et al., 1991), Huntington disease (Dunlop
et al., 1992), HIV-seropositive individuals (Meyerhoff et al., 1993), acute stroke (Gideon et al., 1992), myodystrophic mice (Marcucci et al., 1984) and mouse model of scrapie (Bell et al., 1991). While such decrease in NAA level has been proposed as a measure of neuronal loss, it is not specific for any particular pathology. The accumulation of NAA, and dystrophy of white matter, in brain of patients with CD is highly specific and the elevated NAA level has been demonstrated both by biochemical and as well as 1H NMR spectroscopy of brain (Matalon et al., 1989 and 1993b, Kaul et al., 1991, and Grood et al., 1991).

Aspartoacylase is present in a variety of tissues. However, the enzyme seems to have a unique role in maintaining a homeostatic balance of NAA level, particularly in the white matter of brain. The disturbance of this equilibrium, as seen in CD, somehow leads to spongy degeneration of the white matter. The pathology seen in CD is apparently co-localized to the regions of brain that express aspartoacylase activity (Kaul et al., 1991a). The grey matter, despite several fold increased levels of NAA (Kaul et al., 1991a), is spared of any significant pathological changes in CD. It would thus suggest that any critical role of NAA in brain function and biology is manifested mainly through the action of aspartoacylase. The studies conducted so far will provide an opportunity to ask
some of these very basic questions. Further studies should eventually provide some answers towards our understanding of the role of NAA hydrolysis by aspartoacylase and lead to better management of patients with CD.
CHAPTER III

Canavan Disease: Genomic Organization and Localization of Human \textit{ASPA} to 17p13-ter; Conservation of the \textit{ASPA} Gene during Evolution

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SUMMARY

Canavan disease or spongy degeneration of brain, is a severe leukodystrophy caused by the deficiency of aspartoacylase (ASPA). Recently, a missense mutation was identified in human ASPA coding sequence from patients with Canavan disease. Human ASPA gene has been cloned and found to span 29 kb of genome. Human aspartoacylase is coded by 6 exons intervened by 5 introns. The exons vary from 94 (exon III) to 514 (exon VI) bases. The exon/intron splice junction sites follow the 'gt'/'ag' consensus sequence rule. Southern blot analysis of genomic DNA from human/mouse somatic-cell hybrid cell lines localized ASPA to human chromosome 17. The human ASPA locus was further mapped between 17pl3-ter region by fluorescence in situ hybridization. Bovine aspa gene has also been cloned and its exon/intron organization is identical to the human gene. The 500 base sequence upstream of initiator 'ATG' codon in human and bovine genes are 77 % identical. Human ASPA coding sequences cross hybridize with the genomic DNA from yeast, chicken, rabbit, cow, dog, mouse, rat and monkey. The specificity of cross-species hybridization of coding sequences suggests that aspartoacylase has been conserved during evolution. It should now be possible to identify mutations in the non-coding genomic sequences that lead to Canavan disease and study regulation of ASPA.
INTRODUCTION

Canavan disease (CD) is an autosomal recessive leukodystrophy caused by the deficiency of aspartoacylase (ASPA; Matalon et al., 1988). Aspartoacylase (aminoacylase II) specifically hydrolyses N-acetylaspartic acid (NAA) to aspartate and acetate (Birnbaum et al., 1952). The deficiency of aspartoacylase leads to the accumulation of NAA in brain and several order of magnitude increase in the excretion of NAA in urine of patients with CD (Matalon et al., 1988 and 1989, Grodd et al., 1990). The clinical symptoms of CD include megalencephaly, hypotonia, mental retardation, and early death (VanBogaert and Bertrand, 1949). The occurrence of CD is more prevalent among people of Ashkenazi Jewish descent (Adachi et al., 1972 and 1973; Adornato et al., 1972; Banker and Victor, 1979; VanBogaert and Bertrand, 1949). We have so far diagnosed more than 145 patients with CD and majority of these patients indeed are of Jewish descent (Matalon et al., 1993a). The large number of patients diagnosed following identification of aspartoacylase deficiency (Matalon et al., 1988) suggests that the incidence of CD may have been underestimated due to the lack of a biochemical marker for the disease.

Aspartoacylase has been purified and characterized from
mammalian sources and is found to be co-localized to the regions of the brain that are also involved in the pathogenesis of CD (Kaul et al., 1991a). Recently, we have cloned and expressed human \textit{ASPA} cDNA and identified a missense \text{glu285>ala} mutation in 85\% of the 34 Jewish Canavan alleles from 17 independent pedigrees (Kaul et al., 1993). The \text{glu285} residue has been predicted to be part of esterase like catalytic domain in aspartoacylase, that involves a triad of histidine, serine and glutamic acid residues (Kaul et al., 1993). Two other single base changes, resulting in a nonsense \text{tyr231>ter} and a missense \text{ala305>glu} mutations have been identified. These three point mutations result in loss of aspartoacylase activity in \textit{in vitro} expression of mutant CDNA clones (Kaul et al., unpublished data). The availability of cDNA and identification of mutations in the coding sequences of \textit{ASPA} will make it possible to determine the epidemiology of CD in risk population. It will also be possible to carry out prenatal diagnosis in informative families. The prenatal diagnosis is not reliable due to very low to undetectable levels of aspartoacylase activity in chorionic villi samples and amniocytes (Matalon et al., 1992 ). Estimation of NAA in amniotic fluid has been reported to be a reliable marker for predicting pregnancy outcome (Bennett et al., 1993; Kelly, 1993).
Our studies so far have demonstrated that aspartoacylase has been conserved in bovine, mouse and human species during evolution (Kaul et al., 1991a and 1993). Here we report the exon/intron organization of human ASPA and its localization to human chromosome 17p13-ter region. The identity of human and bovine ASPA gene organization and the conservation of the coding sequence during evolution in several eukaryotic species has also been established.
Materials and Methods

Isolation and characterization of genomic clones. Genomic phage libraries prepared from human and bovine lymphocyte genomic DNA and cloned in λEMBL3 vector (Stratagene, La Jolla, CA), were screened with species specific $^{32}$P-labeled CDNA according to the standard protocols (Sambrook et al., 1989). The CDNA probes were labeled with $^{32}$P by random primer method (Feinberg and Vogelstein, 1984). After secondary and tertiary screening, the isolated phage clones were plaque purified. Large scale phage preparations were made by suspension culture and the phage DNAs were prepared according to the protocols described earlier (Kaul et al., 1993). The phage DNAs were digested with restriction endonucleases and the insert fragments subcloned into pBS(+) vector (Stratagene, La Jolla, CA). Southern blot analysis of the phage DNAs was carried out and the exon/intron boundaries were identified by limited sequence analysis of the subcloned fragments. The nucleotide sequence was determined by di-deoxy chain termination method (Sanger et al., 1977) using 373A Automated DNA Sequencer (Applied Biosystems, Foster city, CA), and described earlier (Kaul et al., 1993).

Southern blot hybridization. High molecular weight DNA was
prepared from cultured cell lines or lymphocytes by proteinase K digestion method of Blin and Stafford (1976) described earlier (Jagadeeswaran et al., 1984). Human/mouse somatic-cell hybrid DNA (Mapping Panel #2; NIGMS, MD) was digested with EcoR I and analysed by Southern blot hybridization (Southern, 1975) with $^{32}$P-labeled human ASPA cDNA. The blots were hybridized in 6 X SSC, 0.5 % SDS, 5 % Denhardt's solution and 100 μg/ml of sheared and denatured Salmon sperm DNA at 68°C according to the method described earlier (Jagadeeswaran et al., 1984). The blots were washed twice under high stringency conditions with 0.1 X SSC and 0.5 % SDS at 68°C for 1 hour each. High molecular weight genomic DNA prepared from human lymphocytes or cultured bovine fibroblast cell line (AG08131A, Coriell Institute, Camden, NJ), and the DNA prepared from genomic phage clones, were digested with several restriction endonucleases. The restricted DNA was analysed by Southern blot hybridization to species specific cDNA probe at 68°C. The blots were washed under high stringency washing conditions described above. Zoo-blot with EcoR I digested genomic DNA from eukaryotic species yeast, chicken, rabbit, cow, dog, mouse, rat, monkey and human (Clonetech, Palo Alto, CA) was hybridized to the $^{32}$P labeled human ASPA cDNA at 60°C. The Zoo-blot was washed twice at 60°C for 20 min each with 2 X SSC and 0.5 % SDS. The blots were exposed for appropriate
time to X-Ray film with intensifying screens at -80°C for autoradiography.

Fluorescence in situ hybridization (FISH) mapping. The human ASPA genomic subclones, representing about 25 kbp of human genome, were labeled with biotin-14-dATP by the nick translation method (Rigby et al., 1977). The labeling reaction was carried out using large fragment labeling kit according to the conditions recommended by the manufacturer (Oncor, MD). The fragment size after the labeling reaction was in the range of 300 - 600 bp. A total of 220 ng of labeled ASPA specific DNA along with sheared 440 µg of herring testis and 110 µg of human placental DNA was precipitated and dissolved in 20 µl of Hybrisol VII (50 % formamide and 10 % dextran sulfate; Oncor). The ASPA specific probe was denatured by heating at 70°C for 10 min followed by preannealing at 37°C for 30 min. Biotin labeled chromosome 17 specific α- satellite (D17Z1) probe (2.5 ng of α-satellite specific DNA, Oncor), diluted to 10 µl with Hybrisol VII, was denatured and preannealed simultaneously for use as chromosome marker. Metaphase spreads of cultured normal human leukocytes were prepared. The chromosomes were denatured by heating at 70°C for 2 min in 70 % formamide and 2 X SSC (pH 7.0) and dehydrated through a series of 70, 80 and 95 % ethanol at -20°C. The
metaphase spreads were hybridized with a mixture of ASPA specific and chromosome 17 α-satellite probe at 37°C for 16 to 18 hour. Following hybridization, the slides were washed in 50 % formamide and 2 X SSC at 43°C for 15 min and then with 2 X SSC at 37°C for 8 min. The hybridization signal was detected by incubation at 37°C for 5 min with FITC-avidin. The hybridization signal was amplified by treatment with anti-avidin antibodies followed by a repeat reaction with FITC-avidin. The chromosomes were counterstained with propidium iodide solution containing antifade (1,4-diazabicyclo-(2.2.2)-octane) and observed under Olympus BX50F fluorescent microscope (Olympus Optical Co. Ltd, Japan) and photographed.
RESULTS

Conservation of ASPA coding sequences in eukaryotes. Since bovine and human ASPA coding sequences are 92% identical, and the coding sequences in mouse aspa transcript share 86% identity (Kaul et al., 1993 and unpublished data), we investigated the conservation of human ASPA coding sequences in other eukaryotic species. Southern blot analysis of EcoRI digested genomic DNA from yeast, chicken, rabbit, cow, dog, mouse, rat, monkey and human was carried out with $^{32}$P-labeled human cDNA as probe. The results are shown in Fig. 7. Under the selected hybridization and washing conditions, the specific restriction fragments hybridizing to the human cDNA were observed in all the genomic DNAs tested. Two EcoRI restricted band of 19.5 and 9.8 kbp were observed with human genomic DNA; as compared to the bands seen with similarly restricted genomic DNA from monkey (20 and 12 kbp), rat (19, 5.4, 2.8 and 0.6 kbp), mouse (20 and 6.8 kbp), dog (12, 4.2, 1.4 and 0.5 kbp), cow (9, 6.4 and 4 kbp), rabbit (5, 4, 2.5 and 0.7 kbp), chicken (7.5 kbp) and yeast (4.4 and 2.2 kbp). The intensity of signal seen in various species may be a reflection of the conservation of ASPA coding sequences during evolution.

Genomic organization of human ASPA. Human lymphocyte
genomic phage library cloned in λEMBL3 was screened with $^{32}\text{P}$-labeled human cDNA. Six different overlapping phage clones were isolated. Three of these phage clones, λEMBL3 1-2, 2-1 and 4-3 with insert of 16.5, 19, and 19.5 kbp respectively, were characterized in detail. Southern blot analysis of phage DNA from these clones were compared to the restriction fragment hybridization pattern observed after similar analysis of the normal human genomic DNA. Two EcoRI fragments of 19.5 and 9.8 kbp were observed in the normal human genomic DNA (see Fig. 1). These EcoRI restriction fragments could be reconstructed from the overlapping genomic phage clones. The validity of these phage clones was further established by identification of 7.8, 5.2, 4.8, 3.6 and 0.7 kbp XbaI restricted, hybridization bands in human genomic DNA and the insert DNA from genomic phage clones (data not shown). A restriction map of human ASPA gene showing EcoRI, BamHI, PstI and XbaI restriction sites is shown in Fig. 8. The inserts in the isolated phage clones together represent about 39 kbp of the human genome. The human ASPA spans about 29 kbp DNA. There is about 8 kbp of 5' upstream and 2 kbp of 3' downstream region represented in these clones.

The inserts from human ASPA genomic phage clones were subcloned as BamHI, XbaI or EcoRI restricted fragments.
Limited nucleotide sequence analysis of these subclones was carried out to determine the sequence around the exon/intron boundaries. The results of these analyses are given in Table 1. The human ASPA transcript is coded by 6 exons intervened by 5 introns. The exons vary in size from 94 bases in exon III to 514 bases in exon VI. The introns were from 1.6 (intron 2) to 9.6 kb (intron 3) in length (see Fig. 2). The exon/intron boundaries follow the consensus 'gt' (splice donor) and 'ag' (splice acceptor) rule. The consensus sequences at splice donor site are: G(100%) T(100%) A(100%) A(100%) and (G(50%)T(20%)C(20%)). The splice acceptor sites have a long pyrimidine track terminating in the nucleotide sequence (T/C(80%)G(20%)) A(100%) and G(100%).

**Bovine Aspa exon/intron organization is identical to human gene.** The bovine Aspa cDNA was used as probe to screen bovine lymphocyte genomic phage library cloned in λEMBL3A. Five different overlapping bovine genomic phage clones were isolated. Two of these clones, λEMBL3A 5-1 and 5-3 with insert sizes of 18.5 and 16.5 kbp, respectively were analysed in detail. Southern blot analysis of DNA, prepared from bovine genomic phage clones and cultured bovine fibroblast cell line AG08131A, was carried out with 32P-labeled bovine cDNA as probe. One 9 and 4 kbp and two non-overlapping 6.4 kbp EcoR I restricted fragments were
observed in the phage clones (data not shown). The EcoR I restricted bands observed in the genomic phage clones were identical to the band sizes seen after Southern blot analysis of bovine genomic DNA (see Fig. 7). The bovine Aspa spans about 30 kbp of bovine genomic DNA. The exon/intron organization of bovine gene revealed 6 exons intervened by 5 introns. The exons I to V of bovine and human gene are identical in size and location. Exon VI in bovine Aspa is 835 bases long. The 5’ boundary of exon VI in bovine and human gene is identical. The increased size of exon VI in bovine gene compared to the human ASPA exon VI is due to longer 3’ untranslated region. The nucleotide sequence around exon/intron boundaries of bovine gene is shown in Table 1. The nucleotide sequences at splice junctions in bovine Aspa follow the consensus sequence rule.

The nucleotide sequences upstream of the initiator "ATG" codon were compared between human and bovine species. The data are shown in Fig. 9. There was about 76 % identity in the nucleotide sequence in the 700 bases immediately upstream of the "ATG" codon. The homology dropped beyond this region. The upstream sequences harbor regulatory regions of a gene. Characterization of upstream sequences will be important for identification of transcription initiation site and characterization of transcriptional
regulatory sequences of \textit{ASPA} gene.

**Chromosomal localization of human \textit{ASPA} to 17p13-ter.**

Human/mouse somatic-cell hybrid mapping panel #2 genomic DNA was digested with restriction endonuclease EcoR I. Southern blot analysis of EcoR I restricted genomic DNA was carried out with $^{32}$P-labeled human cDNA as probe. Under the stringent hybridization and washing conditions used, two bands of 19.5 and 9.8 kbp corresponding to the human \textit{ASPA} (see Fig. 7), were observed in control human genomic DNA and in genomic DNA from hybrid cell line GM10498 (data not shown). The human/mouse somatic hybrid cell line GM10498 specifically contains human chromosome 17.

Human \textit{ASPA} gene and chromosome 17 $\alpha$-satellite (D17Z1) sequences, labeled with biotin, were used for chromosomal mapping of human \textit{ASPA} by FISH technique. The data showing chromosomal assignment of human \textit{ASPA} is shown in Fig. 10. Specific hybridization signals were observed on the short arm of chromosome 17 near the telomeric region. The human \textit{ASPA} maps between 17p13-ter region. The identity of chromosome 17 in FISH was established by co-hybridization with chromosome 17 specific $\alpha$-satellite probe (see Fig. 10) or by painting the chromosome 17 with digoxigenin-labeled chromosome 17 paint probe (data not shown).
DISCUSSION

Aspartoacylase (aminoacylase II) specifically hydrolyses NAA to aspartate and acetate (Birnbaum et al., 1952). The ASPA gene is expressed in a wide variety of tissues (Kaul et al., 1991a and 1993), whereas NAA is synthesized only in the central nervous system in a cell specific manner (Goldstein, 1959 and 1969; Moffett et al., 1991 and 1993; Urenjak et al., 1992). The biological role of NAA in brain metabolism has remained unknown (Cangro et al., 1987; Jacobson, 1957; Kaul et al., 1991b; McIntosh and Cooper, 1965; Ory-Lavolle et al., 1987; Shigematsu et al., 1983) since its discovery by Tallan et al. (1956). The derangement of NAA metabolism in CD due to the deficiency of aspartoacylase leads to spongy degeneration of the brain (Matalon et al., 1993b). This finding has for the first time suggested that the hydrolysis of NAA by aspartoacylase is involved in the maintenance of normal white matter. How NAA metabolism by ASPA is involved in the maintenance of normal white matter is not understood at present. The increase in NAA concentration in brain of patients with CD is highly specific (Grodd et al., 1991; Matalon et al., 1989 and 1993a; Kaul et al., 1991a). This is in contrast to the decrease in the level of NAA reported in several unrelated pathological conditions like focal or generalized
demyelinating disorders (Grood et al., 1991), Huntington’s disease (Dunlop et al., 1992), acute stroke (Gideon et al., 1992), mouse model of scrapie (Bell et al., 1991) or HIV seropositive individuals (Meyerhoff et al., 1993). It has been suggested that the drop in NAA concentration is an indicator of neuronal loss (Grood et al., 1991). The pathology in CD is restricted to the white matter; the grey matter is largely spared of any pathology.

ASPA coding sequences are about 90% identical among human, bovine and murine species (Kaul et al., 1993 and unpublished data). The biochemical and immunological studies earlier had also suggested a conserved nature of aspartoacylase in these species (Kaul et al., 1991a). The conservation of sequences has now been shown to extend to the 5’ upstream region in the human and bovine genes. The identity of exon/intron organization between human and bovine ASPA genes, as reported here, is in agreement with generally observed similar organization of conserved genes. The evolutionary conservation of coding sequences of this gene in eukaryotes is now further supported by the specificity of cross hybridization of human ASPA cDNA to genomic DNA from yeast, chicken, rabbit, cow, dog, mouse, rat and monkey. Conserved nature of coding sequences in diverse species would suggest a significant biological role.
for aspartoacylase. However, it remains to be determined how aspartoacylase and its hydrolysis of NAA manifests such biological role.

The three point mutations, tyr231>ter, glu285>ala and ala305>glu, identified in patients with CD affect the coding sequences of aspartoacylase (Kaul et al., 1993 and unpublished data). The mutated amino acid codons in human ASPA are conserved in bovine (Kaul et al., 1993) and murine (Kaul et al., unpublished results) transcripts. Characterization of genomic sequences will facilitate identification of any mutation(s) at the splice site or other sequences that may lead to deficiency of ASPA and thus cause CD. Naturally occurring mutations serve as a genetic repository for identification of necessary functional and regulatory structures in a gene. Availability of genomic sequences will also be useful for characterization of transcription start site and for identification of promoter and other cis-acting regulatory elements involved in the expression of ASPA. We have earlier reported a tissue dependent relative expression of 4.8 and 1.4 kb ASPA transcripts in various human tissues (Kaul et al., 1993). We can now study the reason for differential level of the two transcripts in these tissues.

Human ASPA maps to 17p13-ter region. This region of
human chromosome 17 has genes for Miller-Dieker lissencephaly gene \textit{LIS I} (Reiner et al., 1993) and tumor suppressor gene \textit{P53} (Holstein et al., 1991), whereas neuropathic disorders Charcot-Marie Tooth 1A and Dejerine-Sottas syndrome gene \textit{PMP22} (Roa et al., 1993) maps closely to 17p11.2-p12 region. All these genes are involved in the developmental process, with some of them like \textit{LIS I} and \textit{PMP22} being specifically involved in the growth and development of the central nervous system. It would be interesting to see how closely these, and possibly yet unidentified genes in this region, are physically linked and that if there is a higher order regulation of these genes in the development and differentiation of the nervous system in general and myelin formation in particular. Chromosomal assignment of \textit{ASPA} is an addition to the known markers for chromosome 17 and will be useful in the human gene mapping.

Isolation and characterization of human \textit{ASPA} is a step towards our understanding of the role of aspartoacylase and the hydrolysis of NAA in maintaining normal white matter. In order to understand the pathology in CD, we are in the process of creating a mouse model for CD. Mice with spongy degeneration of brain have been described, though none of these animals are living at present (Azzam et al., 1984). These studies should eventually lead to better management
and new modes of therapy for Canavan disease.
CHAPTER IV

Canavan Disease: Mutations among Jewish and Non-Jewish Patients

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SUMMARY

Canavan disease is an autosomal recessive leukodystrophy caused by a deficiency of aspartoacylase (ASPA). Sixty-four probands were analyzed for mutations in the ASPA gene. Three point mutations, 693C>A, 854A>C, and 914C>A were identified in the coding sequence. The 693C>A and 914C>A base changes, resulting in non-sense tyr231>ter and missense ala305>glu mutations respectively, lead to complete loss of ASPA activity in in vitro expression studies. The 854A>C transversion converted glu to ala in codon 285. The glu285>ala mutant ASPA has 2.5 % of the activity expressed by the wild type enzyme. A fourth mutation, 433-2(A>G) transition, identified at splice acceptor site in intron 2 would lead to skipping of exon III and produce aberrant ASPA transcript. Of the 128 unrelated Canavan chromosomes analyzed; 88 were from proband of Ashkenazi Jewish descent. The glu285>ala mutation was predominant (82.9 %) in this population, followed by tyr231>ter (14.8 %) and 433-2(A>G) (1.2 %) mutations. The three mutations account for 98.8 % of the Canavan chromosomes of Ashkenazi Jewish origin. The ala305>glu mutation was found exclusively in non-Jewish probands of European descent and constituted 60 % of the 40 mutant chromosomes. Predominant occurrence of certain mutations
among Ashkenazi Jewish and non-Jewish patients with Canavan disease would suggest a founding father effect in propagation of these mutant chromosomes.
INTRODUCTION

The deficiency of aspartoacylase (ASPA) leads to spongy degeneration of brain, Canavan disease (CD) (Matalon et al., 1988). The clinical features of this progressive, autosomal recessive leukodystrophy include, mental retardation, megalencephaly and hypotonia. Morphological changes in brain are marked by spongy appearance of the white matter with swollen astrocytes and elongated mitochondria (Adachi et al. 1972; Adornato et al. 1972; Canavan 1931; Globus and Strauss 1928; VanBogaert and Bertrand 1949). Depending upon the age of onset, CD has been classified into congenital, infantile or juvenile forms (Adachi et al. 1973). Children with CD can survive to various ages, though the majority of them die by their early teens (Banker et al. 1964; Banker and Victor, 1979; Van Bogaert and Bertrand 1949 and 1967). Canavan disease is prevalent among people of Ashkenazi Jewish extraction, although patients from other diverse ethnic backgrounds have also been diagnosed (Banker and Victor, 1979; Matalon et al. 1988, 1989 and 1993; Ozand et al. 1990). The incidence of CD may have been underestimated due to the lack of a biochemical marker for diagnosis of this disorder until recently (Matalon et al. 1988, 1989 and 1993).

Aspartoacylase specifically hydrolyses its substrate N-
acetyl aspartic acid (NAA) to aspartate and acetate (Birnbaum et al. 1952; Birnbaum, 1955). N-Acetylaspartic acid was first described in 1956 (Tallan et al. 1956). The synthesis of NAA is localized to certain cell types in brain (Goldstein, 1959 and 1969; Moffett et al. 1991 and 1993; Urenjak et al. 1992). The abundance of NAA in normal brain is second only to glutamate (Miyake et al. 1980). Prior to the discovery of aspartoacylase deficiency in CD (Matalon et al. 1988), the role of NAA in brain was virtually unknown (Congro et al. 1987; Jacobson, 1957; McIntosh and Cooper, 1965; Ory-Lavolle et al. 1987; Shigematsu et al. 1983). The deficiency of ASPA leads to accumulation of NAA in brain (Grood et al. 1990; Kaul et al. 1991b; Matalon et al. 1988 and 1989). These findings suggest that the metabolism of NAA is essential for integrity of the white matter in brain. Biochemically, patients with CD are diagnosed by estimation of excessive amounts of NAA excreted in their urine (Matalon et al. 1988). The diagnosis is confirmed by the deficiency of ASPA activity in cultured skin fibroblasts. There is no demonstrable ASPA activity in blood constituents from normal individuals. The urine from normal individuals and obligate carriers contains less than 10 nmoles NAA per mmole creatinine (Matalon et al. 1993). Carriers of Canavan alleles can only be detected by determination of enzyme activity in their cultured skin
fibroblasts (Matalon et al. 1988, 1989 and 1993). Prenatal diagnosis of CD has been a difficult problem. Direct chorionic villi samples (CVS) and the cultured CVS and amniocytes from normal control pregnancies have very low to undetectable ASPA activity. Determination of ASPA activity for predicting the outcome of pregnancies at risk for CD has proven to be unreliable (Matalon et al. 1992). Estimation of NAA in amniotic fluid has been reported to be reliable in predicting the outcome of risk pregnancy (Bennett et al. 1993; Jakobs et al. 1991; Kelley, 1993; Matalon et al. 1992). However, more studies are needed to establish the reliability of this method.

Aspartoacylase has been purified and characterized from mammalian sources (Kaul et al. 1991a). Human ASPA cDNA has recently been cloned and expressed in E.coli (Kaul et al., 1993). The coding sequence of human ASPA is split into 6 exons intervened by 5 introns in the genome and the gene spans 30 kb of DNA. Human ASPA has been mapped to 17p13-ter region (Kaul et al. 1994). The primary structure of human and bovine ASPA share 92 % identity and the gene organization is also identical between the two species. Such a high level of conservation was suggested earlier by biochemical and immunological studies of ASPA from various mammalian sources (Kaul et al., 1991a). Evolutionary conservation of the coding sequences of ASPA is also
suggested by the specificity of cross hybridization of human cDNA to genomic DNA from yeast, chicken, rabbit, dog, cow, mouse, rat and monkey (Kaul et al. 1994).

We have earlier reported a 854A>C base change in the ASPA gene. The base change was present in 85 % of 17 probands of Ashkenazi Jewish ancestry (Kaul et al. 1993). We now report identification and characterization of four point mutations in ASPA gene from 128 Canavan chromosomes. Three of the mutations account for 98.8 % of the 88 Canavan chromosomes of Ashkenazi Jewish origin. A fourth mutation, identified exclusively in non-jewish patients with CD, accounted for 60 % of the 40 independent chromosomes.
MATERIALS AND METHODS

Patients with CD

Sixty-four patients with CD were analyzed for mutations in the ASPA gene. Forty-four of these probands were of Ashkenazi Jewish extraction, while the other 20 probands were non-Jewish with European ancestry. Whenever possible, the mutations observed in the proband were confirmed in their parents. The frequency of mutations among Ashkenazi Jewish and non-Jewish Canavan chromosomes were determined. In families with more than one affected child, only one proband was included for such analysis.

Mutation analysis

Genomic DNA was prepared from cultured skin fibroblast cell lines or lymphocytes according to the methods described earlier (Kaul et al. 1994). In certain instances, "Guthrie" blood spots were used for mutations analysis. Genomic DNA (500 ng), or "Guthrie" blood spots were used for PCR amplification of ASPA specific coding and exon/intron boundary sequences, as described earlier (Kaul et al. 1993). The mutations were characterized by: a) determination of the nucleotide sequence using dideoxy chain termination chemistry (Sanger et al. 1977); b) analyses of SSCP (Orita et al. 1989); and c) restriction endonuclease digestion, as
described earlier (Kaul et al., 1993). Mutations, that did not result in gain or loss of a restriction endonuclease site, were analyzed by PCR directed site specific mutagenesis (PDSM). A primer with mismatch at a unique position in its sequence was synthesized. Following PCR amplification, the mismatch in the PCR primer created a unique recognition sequence for a restriction endonuclease, either in mutant or WT allele. The mutant and WT alleles could thus be differentiated by restriction digestion of the PCR amplified products.

**DNA constructs**

The transient expression vector pEUK-C1 (Clontech, Palo Alto, CA) was modified to introduce Sac I, Bcl I and Not I restriction sites and destroy the BamH I site at the multiple cloning site. The modified vector was called pGEUK-C1. The wild type (WT) ASPA cDNA was amplified between -108 to +1242 (Kaul et al. 1993) by PCR, using the sense XHASP18 (5’cctagtctagacccctttgggtaaagtctca 3’) and anti-sense NHASPC14 (5’ataagaatgcggcgctatattctgaataacaggctg 3’) strand primers. The primers XHASP18 and NHASPC14 respectively had Xba I and Not I linkers shown in lower case. The amplified cDNA fragment was digested with Xba I and Not I restriction endonuclease and cloned in pGEUK-C1 vector downstream of SV40 late promoter and VP1 intron. The
mutant ASPA cDNA with 693C>A or 914C>A base change were amplified by RT-PCR of cytoplasmic RNA isolated from patient fibroblast cell lines homozygous for either of these two mutations (Kaul et al., 1993). The amplified mutant cDNAs were cloned as described for WT cDNA. Genomic DNA, from a patient homozygous for 854A>C mutation, was amplified with G5 and C7 primers as described earlier (Kaul et al., 1993). The amplified fragment with 854A>C mutation was cleaved at unique BamH I and Hind III restriction sites and cloned in pBS(+) vector. The BamH I/Hind III fragment in the WT cDNA clone pBSHLASP(+) (Kaul et al., 1993) was replaced with the corresponding 854A>C mutant cDNA fragment. The 854A>C mutant cDNA insert was amplified using XHASP18 and NHASPC14 primers, restricted with Xba I and cloned in pGEUK-C1 vector at Xba I/Sma I restriction sites.

The WT and mutant ASPA expression constructs, pSV-ASPA(WT), pSV-ASPA(693A>C), pSV-ASPA(854A>C), and pSV-ASPA(914C>A), were isolated and characterized by restriction endonuclease digestion and by determination of their nucleotide sequence. The plasmid DNAs were isolated and purified on two successive CsCl2 gradients for transfection studies (Sambrook et al. 1989).

**Cell culture, DNA transfection and expression of ASPA**

Human ASPA cDNA expression constructs were expressed in
COS1-cells, (a gift from Dr. Jacob Grimberg, ImClone Systems, NY), grown in RPMI-1640 media supplemented with 20 % fetal bovine serum and 4 % CO₂. Lipofectin mediated DNA transfections (Loeffler and Behr, 1993), were carried out according to the conditions recommended by the manufacturer (Gibco-BRL, MD). The transfection conditions were optimized with pSV-β-galactosidase plasmid (Promega, Madison, WI) with SV40 promoter driven β-galactosidase as the reporter gene. The β-galactosidase activity was determined in situ according to the method of Lim and Chae (1989). It was established that 150 µg of lipofectin, 1.2 X 10⁶ COS1-cell per 100 mm petri dish, and the transfection time of 7 hours was optimal to achieve cell viability of 60-70 % and transfection efficiencies of about 70 percent. Following transfection with expression constructs, the COS-cell sonicates were analyzed for aspartoacylase activity according to the method described earlier (Kaul et al., 1991). Control transfections with pGEUK-C1 vector DNA or mock transfections without any DNA, were carried out and analyzed simultaneously. The expression data presented are means of at least 4 independent experiments.
RESULTS

Splice acceptor site 433 -2(A>G) transition in intron 2

The exon III coding and splice junction sequences were amplified using 3A/3B primers (see Table 2). The nucleotide sequence of the 201 bp PCR amplified product was determined in both strands. The nucleotide sequence data of the sense strand is shown in Fig. 11. An A>G transition was observed at splice acceptor site (433 -2 nucleotide position), in intron 2 of one chromosome. Both A and G base specific signal was observed at this position. The patient is therefore heterozygote for the base change at this position.

The 433 -2(A>G) splice acceptor site mutation was also demonstrated by restriction endonuclease analysis. A sense primer (IVS2SA) with a T>C mismatch (see Table 2) was synthesized. The IVS2SA and 3B primers were used for PCR amplification of 179 bp fragment from the mutant and WT alleles. The results are shown in Fig. 12. The A>G transition in the mutant allele created a Hpa II restriction site by PDSM and is thus cleaved by Hpa II enzyme into 156 and 23 bp fragments. The amplified product from WT allele is not cleaved by Hpa II enzyme. Restriction analysis confirmed that the patient is heterozygous for a 433-2(A>G) splice site mutation.
Identification of 693C>A base change in exon V of ASPA gene

Exon V specific coding and its boundary sequences were amplified with 5A/5B primers (see Table 2). The 235 bp product was analyzed for SSCP to indicate base change in the Canavan alleles. Representative SSCP pattern in exons V of ASPA gene alleles is shown in Fig. 13. The PCR amplified product from mutant and WT alleles had distinct SSCP profiles. Proband homozygous or heterozygous for this base change could be identified by SSCP banding pattern. The nucleotide sequence of the exon V specific amplified fragment from such patients was determined. Representative nucleotide sequence of the sense strand are shown in Fig. 14. A single base change of C>A was observed at position 693 in a patient homozygous for this mutation. The 693C>A base change should result in a nonsense tyr231>ter mutation in the ASPA transcript encoded protein.

The 693C>A base change creates Mse I restriction endonuclease recognition sequence in the mutant alleles. The 693C>A mutation was therefore characterized by digestion of exon V specific amplified fragments with Mse I enzyme. The results of Mse I restriction digestion are shown in Fig. 15. Mse I restriction digestion of the mutant alleles resulted in 104, 73, and 58 bp bands. The WT alleles yielded 177 and 58 bp fragments. Patients homozygous or heterozygous for 693C>A mutation could be identified by
Characterization of 914C>A point mutation in exon VI of ASPA gene

ASPA gene specific exon VI sequences and their exon/intron boundaries were amplified using 6A/RT1 primers (see Table 2). The 347 bp amplified products were analyzed for SSCP in Canavan alleles. Representative SSCP pattern in exon VI is shown in Fig. 13. Two distinct SSCP profiles were observed; and probands homozygous or heterozygous for either of the two profiles were identified. One of the SSCP pattern was due to the 854A>C base change described earlier (Kaul et al., 1993). The base change responsible for the second SSCP patterns (distinct from that due to 854A>C mutation), was characterized by determination of nucleotide sequence. The nucleotide sequence data of the sense strand are shown in Fig. 16. A C>A base change at position 914 was observed in a proband homozygous for this mutation. The 914C>A base change would result in a missense ala305>glu mutation.

The 914C>A base change did not result in a gain or loss of restriction site. A primer C22, with an A>T mismatch, was synthesized (see Table 2) and used with G5 primer for PCR amplification of 194 bp exon VI specific genomic sequences. The results of such analyses are shown in Fig. 17. The PCR amplification of WT allele created Nsi I
specific recognition sequence so that restriction digestion yield 173 and 21 bp fragments. The amplified product from 914C>A mutant alleles do not create a Nsi I restriction site. Heterozygotes for 914C>A base change could be differentiated from homozygous patients and the normal control individuals.

The C22/G5 amplification product can also be used for detection of 854A>C mutations. The 194 bp amplified fragment from mutant alleles yield 112 and 82 bp bands, whereas the WT allele is not cleaved by Eag I restriction endonuclease (see Fig. 17).

Expression of ASPA cDNA in COS1-cells

The schematics of the human ASPA cDNA expression construct pSV-ASPA(WT) are shown in Fig. 18. The cDNA was cloned at Xba I/Not I restriction sites in pGEUK-C1 vector downstream of the SV40 late promoter and VP1 intron. The SV40 polyadenylation signal was present downstream of the ASPA cDNA insert. The relative location of three point mutations in the coding sequence are also shown in Fig. 18.

COS1-cells were transfected with pSV-ASPA(WT) construct at 1.25, 2.5, 5, 10 and 20 μg DNA concentration and the ASPA activity was measured 36 hours post-transfection. Maximal activity of 0.7 mU was observed upon transfection with 5 μg of the WT expression construct. This was compared to the
background activity of 0 to 0.02 mU observed in mock transfected cells or in cells transfected with vector DNA. Transfection with 10 or 20 μg DNA concentration resulted in 40% lower ASPA activity. The activity expressed by 5 μg of pSV-ASPA(WT) construct DNA was measured at 72 hours and 1 and 2 weeks following transfection. The aspartoacylase activity expressed by pSV-ASPA(WT) construct was 2.3 mU at 72 hours and 2.4 mU at 1 week and 1.8 mU at 2 weeks following transfection.

Effect of 693C>A, 854A>C and 914C>A base change on ASPA activity

Mutant ASPA cDNA constructs pSV-ASPA(693C>A), pSV-ASPA(854A>C) and pSV-ASPA(914C>A), with 693C>A, 854A>C and 914C>A point-mutation respectively in their coding sequence (see Fig. 18), were expressed in COS1-cells. The expression of ASPA activity by mutant cDNA constructs was compared to the activity expressed simultaneously by the WT construct. The pSV-ASPA(693C>A) and pSV-ASPA(914C>A) mutant cDNA constructs did not express any measurable ASPA activity after transfection in COS1-cells. These data establish that the 693C>A or 914C>A point mutations result in complete loss of ASPA activity.

The ASPA activity expressed by pSV-ASPA(854A>C) construct at 1 week post-transfection was 0.06 mU, compared
to 2.4 mU activity expressed by the pSV-ASPA\(^{\text{WT}}\) construct. The residual ASPA activity expressed by the 854A>C mutant cDNA was thus 2.5 % of the activity expressed by the WT construct. Kinetic analysis of glu285>ala mutant ASPA expressed by pSV-ASPA\(^{\text{854A>C}}\) construct was carried out and compared to that of the WT enzyme expressed by the pSV-ASPA\(^{\text{WT}}\) construct. The apparent \(K_m\) of the WT human ASPA was determined to be \(3.6 \times 10^{-4}\) M. The glu285>ala mutant enzyme did not reach the apparent maximum velocity (\(V_{\text{max}}\)) even at substrate concentrations 3-fold higher than in a standard assay system. The kinetic parameters thus could not be determined accurately.

**Relative frequency of 4 Canavan mutations**

We have analyzed 128 independent Canavan chromosomes for four point mutations. Of these, 88 Canavan chromosomes were from probands of Ashkenazi Jewish extraction. The other 40 chromosomes were from non-Jewish probands of European descent. The frequency of mutations among the two population groups are given in Table 3. Only 433-2(A>G), tyr231>ter and glu285>ala mutations were observed among chromosomes of Ashkenazi Jewish ancestry and together they accounted for 98.8 % of the 88 Canavan chromosomes. The glu285>ala missense mutation was predominant in 82.9 % or 73 chromosomes, followed by nonsense tyr231>ter in 14.8 % or 13
chromosomes and 433-2(A>G) splice site mutation in 1.1 % or 1 chromosome in this population. None of the chromosomes from Ashkenazi Jewish probands with CD carried ala305>glu missense mutation. Among the 40 non-jewish Canavan chromosomes analyzed, ala305>glu mutation was identified in 60 % or 24 chromosomes, while glu285>ala mutation was found in 2.5 % or 1 chromosome. The tyr231>ter nonsense and 433-2(A>G) splice acceptor site mutations were ruled out in all 40 chromosomes from non-jewish probands of european descent.
DISCUSSION

Canavan disease is a progressive, autosomal recessive leukodystrophy marked by severe neurological impairment. A rapid regression between the ages of 3 to 8 months occurs in the majority of these patients. Canavan disease is caused by the deficiency of aspartoacylase; and the consequent lack of hydrolysis of its substrate NAA leads to absence or dissociation of myelin by large number of vacuoles giving it a spongy appearance (Matalon et al. 1988, 1989 and 1993; VanBogaert and Bertrand, 1949).

Excretion of NAA in urine of patients with CD is specific; and has not been observed in other non-Canavan white matter diseases (Matalon et al. 1989). Cultured skin fibroblasts are needed for confirmation of ASPA deficiency in patients with CD; and determination of carrier status of individuals at risk. Lack of aspartoacylase activity in blood constituents has made testing for carriers a time consuming process. Canavan disease is prevalent among Ashkenazi Jewish people, indeed, the majority of cases diagnosed at our center are from this background (Matalon et al. 1993). However, it has not been possible to carry out epidemiology of CD in the risk population, since cultured cells are required for determination of ASPA activity. The isolation and characterization of the human aspartoacylase
gene (Kaul et al. 1993 and 1994) has made it possible to identify mutations that lead to deficiency of aspartoacylase. Deficiency of ASPA activity due to nonsense tyr231>ter or missense glu285>ala or ala305>glu mutation establish that the three coding sequence mutations indeed lead to CD. The 433-2(A>G) transition in the splice acceptor site in intron 2 would lead to skipping of exon III. The skipping of 94 base exon III (Kaul et al., 1994) will also change the reading frame in the final transcripts. Such exon skipping accompanied by frameshift would result in deficiency of aspartoacylase.

The glu285 residue has been predicted to form a triad of ser, his and glu residues at the catalytic center of ASPA (Kaul et al. 1993). Kinetic analysis suggests that the glu285>ala mutation significantly compromises the maximal reaction velocity (Vmax) of the mutant ASPA. This would be expected of mutations at the catalytic center of the enzyme. The presence of residual activity in glu285>ala mutant ASPA would suggest a relatively less severe brain pathology in patients homozygous for this mutation; in contrast to the complete loss of ASPA activity observed with nonsense tyr231>ter and missense ala305>glu mutations. Such studies for correlation of patient genotype and the residual ASPA activity to their clinical phenotype are currently in progress. Complete loss of activity due to tyr231>ter
nonsense mutation should be due to premature termination of the ASPA polypeptide chain during translation of the mutant mRNA. The reason for complete loss of activity in ala305>glu mutant ASPA is not understood at present.

The predominant nature of glu285>ala mutation (82.9%) among Ashkenazi Jewish patients with CD has now been strengthened by inclusion of larger sample in the current study. Only one chromosome from a non-Jewish patient carried this mutation. The ala305>glu mutation, on the other hand, is found exclusively among non-Jewish patients of European descent. The ala305>glu mutation was not represented among Ashkenazi Jewish probands. The relative frequency of CD mutations among Ashkenazi Jewish and non-Jewish patients follow a pattern reminiscent of the Tay-Sachs mutations in the two population groups (Paw et al. 1990). Both CD and Tay-Sachs mutations in Ashkenazi Jewish people are believed to have originated around Vilnus in the current day Lithuania (Banker and Victor, 1979; Unger and Goodman 1983). The predominant nature of certain mutations would suggest a founding father phenomenon. It would be interesting to explore the genetic background on which Canavan and Tay-Sachs mutations might have originally occurred among Ashkenazi Jewish people.

Characterization of Canavan mutations will make it possible to have a reliable DNA based method for prenatal
diagnosis in informative families. The three mutations identified in Jewish patients account for 98.8% of the mutant alleles. This should make it feasible to carry out the epidemiology of CD in the risk population. Furthermore, carrier testing can be offered as a routine preventive measure similar to Tay-Sachs screening (Kaback et al. 1993). Genotyping of risk population for Canavan mutations should provide a chance at preventing the occurrence of this progressive autosomal recessive leukodystrophy.
CONCLUSIONS

1. Canavan disease is an autosomal recessive leukodystrophy, caused by the deficiency of ASPA. Full length cDNA clones of human and bovine ASPA have been isolated and characterized. The human and bovine ASPA cDNA clone are 1435 bp and 1766 bp, respectively. The two cDNA clones are 92% identical at the primary structure level. The difference in the size of cDNA clones between human and bovine species is due to the length of their 3’UTR, as determined by the location of polyadenylation signal. The authenticity of the cDNA clone has been established by its expression in E. coli and COS1-cells.

2. The single uninterrupted ORF in human and bovine cDNA clones predicted protein with 313 amino acid residues and an estimated molecular weight of 36 kd. Both proteins have potentially one N-glycosylation and five phosphorylation sites. The predicted amino acid sequence of the protein from two species are 92% identical.

3. Northern blot analysis of poly(A)+ RNA from several human tissues revealed a single band of 1.44 kb in
liver sample and an additional 5.4 kb band in all other tissues including heart, brain, placenta, lung, skeletal muscle and kidney. The 1.44 kb band in Northern blot agrees well with the size of the isolated 1.435 kb cDNA. The human brain expresses a relatively higher proportion of the larger transcripts, as compared to the relative amounts of the two transcripts expressed in each tissue. The expression of both transcripts are highest in human skeletal muscle, followed by kidney and brain.

4. Genomic clones for both human and bovine ASPA genes were isolated and the exon-intron organization has been established. The ASPA gene consists of 6 exons intervened by 5 introns. The exon/intron splice junction sites follow the 'gt'/‘ag’ consensus sequence rule. The human ASPA gene has been mapped to 17p13-ter region, using somatic cell hybrids and fluorescent in situ hybridization techniques.

5. The exon/intron organization of bovine ASPA gene is identical to the human gene. These data suggests that the ASPA coding sequences are highly conserved during evolution. This is supported by the fact that Human ASPA coding sequences cross hybridize with the genomic
DNA from yeast, chicken, rabbit, cow, dog, mouse, rat, and monkey.

6. Four point mutations, 433-2(A>G), 693C>A, 854A>C and 914C>A, have been identified in ASPA alleles from patients with CD. The 854A>C and 914C>A base changes lead to glu285>ala and ala305>glu missense mutations. The 693C>A base change results in non-sense tyr231>ter mutation. The fourth mutation, 433 -2(A>G) transition, occurs at splice acceptor site in intron 2.

7. In vitro expression of mutant cDNA with 693C>A or 914C>A base change lead to complete loss of ASPA activity. The ASPA expressed by 854A>C mutant cDNA shows 2.5% of residual activity compared to that expressed by wild type ASPA cDNA construct. The tyr231>ter non-sense mutation should result in premature termination of the polypeptide chain. The mutation at splice acceptor site should lead to skipping of exon III, accompanied by a frameshift in the final transcript.

8. One hundred and twenty-eight Canavan chromosomes from 64 unrelated probands have been analyzed for mutations in the ASPA gene. Eighty-eight of these Canavan
chromosomes were from 44 probands of Ashkenazi Jewish descent; while 40 chromosomes were from 20 probands of non-Jewish European extraction. Among chromosomes from Ashkenazi Jewish people, the glu285>ala mutation was identified in 73 chromosomes (82.9%). The tyr231>ter non-sense mutation was detected in 13 chromosomes (14.8%) and the 433-2(A>G) splice acceptor site mutation was observed in 1 chromosome (1.1%). The three mutations account for 98.8% of the Canavan chromosomes of Ashkenazi Jewish origin. The ala305>glu mutation was not detected among Jewish chromosomes. However, ala305>glu mutation was found exclusively in 60% of the 40 non-Jewish chromosomes of European descent, whereas glu285>ala mutation was identified in 1 chromosome (2.5%). These findings suggest a founding father effect of these mutations in propagation of mutant chromosomes.

9. The findings from this study provide the basis for study of epidemiology of CD in the risk population. The prenatal diagnosis of CD by DNA-based technology is also possible in informative families. This should offer the availability of a reliable method of prenatal diagnosis in such families, so that the occurrence of CD in risk families can be prevented. It is now also
feasible to create an animal model of CD for understanding the pathophysiology of the disease at the molecular level. It should also be possible, then, to explore enzyme and gene therapy for CD.
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APPENDIX I

FIGURES AND LEGENDS
TTGTAACAGA AAATTAAAAT ATACTCCACT CAAGGAATT CTGTACTTTG CCCTTTTGGT -99
AAAGTCTCAT TTACATTTCT AAACCTTTCT TAAGAAATAC GAATTTCCCT TGATCTCTCT -39
1 -1MTSCH1
TCTGAATTGC AGAAATCAGA TAAAAACTAC TTGGTGAA ATG ACT TCT TGT CAC ATT 18

7 A E E H I Q K V A I F G G T H G
GCT GAA GAA CAT ATACAA AAG GTT GCT ATC TTT GGA GGA ACC CAT GGG 66
23 N E L T G V F L V K H W L E N G
AAT GAG CTA ACC GGA GTA TTT CTG GTT AAG CAT TGG CTA GAG AAT GCC 114
39 A E I Q R T G L E V K P F I T N
GCT GAG ATT CAG AGA ACA GGG CTG GAG GTA AAA CCA TTT ATT ACT AAC 162
55 P R A V K K C T R Y I D C D L N
CCC AGA GTG AAG TGT ACC AGA TAT ATT GAC TGT GAC CTG AAT 210
71 R I F D L E N L G K K M S E D L
CGC ATT TTT GAC CTT GAA AAT CTT GGC AAA AAA ATG TCA GAA GAT TTG 258
87 P Y E V R R A Q E I N H L F G P
CCA TAT GAA GTG AGA AGG GCTCAA GAA ATA AAT CAT TTA TTT GTT CCA 306
103 K D S E D S Y D I I F D L H N' T
AAA GAC AGT GAA GAT TCC TAT GAC ATT ATT TTT GAC CTT CAC AAC ACC 354
119 T S N M G C T L I L E D S R N N
ACC TCT AAC ATG GGG TGC ACT CTT ATT CTT GAG GAT TCC AGG AAT AAC 402
135 F L I Q M F H Y I K T S L A P L
TTT TTA ATT CAG ATG TTT CAT TAC ATT AAG ACT TCT CTG GCT CCA CTA 450
151 P C Y V Y L I E H P S L K Y A T
CCC TGC TAC GTT TAT CTG ATT GAG CAT CCT TCC CTC AAA TAT GCG ACC 498
167 T R S I A K Y P V G I E V G P Q
ACT CGT TCC ATA GCC AAG TAT CTT GTG GGT ATA GAA GTT GGT CCT CAG 546
183 P Q G V L R A D I L D Q M R K M
CCT CAA GGG GTT CTT AGA GCT GAT ATC TTT GAT CAA ATG AGA AAA ATG 594
199 I K H A L D F I H H F N E G K E
ATT AAA CAT GCT CTT GAT TTT ATA CAT CAT TCC AAT GAA GGA AAA GAA 642
215 F P P C A I E V Y K I I E K V D
TTT CCT CCC TGC GCC ATT GAG GTC TAT AAA ATT ATA GAG AAA GTT GAT 690
231 Y P R D E N G E I A A I I H P N
Fig. 1 Nucleotide and predicted amino acid sequence of human ASPA encoded transcript and protein. The cDNA is 1,435 bp and initiator 'atg' marks base 1 of coding sequence. The polyadenylation signal sequences are shaded. Also shown are 18 base poly(A) tail. Amino acid sequence predicted from the human ASPA cDNA is depicted as single letter code and initiator amino acid residue M is residue 1. There are several in frame termination codons present in human ASPA cDNA upstream of the initiator 'atg' codon. The potential 'N' glycosylation site is shown in bold type and marked by an asterisk (*). The numbers on left show amino acid residues while those on right are nucleotide base position.
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Fig. 2  Alignment of human (HLASP) and bovine (BASPCDN) ASPA encoded protein sequence using the AALIGN program (Gap penalty 4, Deletion penalty 12, PAMfile STANDARD.PAM) in Lasergene software package from DNAstar (Madison, WI). The human and bovine aspartoacylase amino acid sequences share 92.3% identity in 313 aa overlap. Consensus amino acid sequence motifs predicted to be involved in catalytic center of aspartoacylase are shown as shaded areas. The potential 'N' glycosylation site is marked by an asterisk (*), and phosphorylation sites are highlighted as shadows.
Fig. 3 Autoradiogram of Northern blot analysis of poly(A)$^+$ RNA isolated from various human tissues. Two $\mu$g of poly(A)$^+$ RNA from each tissue were fractionated on agarose gel and blotted on nylon membrane and blots were hybridized to human cDNA under stringent conditions. The human poly(A)$^+$ RNA are from: 1: heart, 2: brain, 3: placenta 4: lung, 5: liver, 6: skeletal muscle and 7: kidney.
Fig. 4 Schematic representation of pHLASP cloned in pBluescript SK'. The human ASPA cDNA was cloned between EcoRI and Xho I sites in 5' → 3' direction and the transcription was driven by LACZ promoter. The initiator 'atg' and terminator 'tag' codons are shown. Other vector regions are not represented in the schematics. The figure is not drawn to scale.
Fig. 5 Nucleotide sequence of 312 bp cDNA fragment in the region of a854>c mutation from normal controls (WT, Panel A) and a patient with CD (MUT, Panel B). Reverse transcription of cytoplasmic RNA with HKRT1 (AACCCTACTCTTAAGGAC) primer was followed by amplification with HASP14F (F→CCGGGATGAAAATGGAGAA) and HASP7C7R (R→ACCGTGTAAGATGTAAGC) primers. The prefix F and R in these oligos stand for M13 universal and reverse primer tags. Fluorescent di-deoxy sequencing of both strands were carried out with M13 universal and reverse primers. The patient with CD (MUT) was homozygous for a854>c point mutation and the base involved is shown by a down arrow (↓). The mutation would result in E285>A mis-sense mutation at the amino acid level.
Fig. 6 Single strand conformation polymorphism (Panel A) and Eag I restriction endonuclease digestion (Panel B) of 237 bp cDNA fragment amplified by RT-PCR of cytoplasmic RNA from normal controls, and Canavan probands and their family members. After initial reverse transcription of cytoplasmic RNA with HKRT1 (AACCCCTACTCTTAAGGAC) primer the 237 bp cDNA fragment was amplified using HASPG5 (AGGATCAAGACTGGAAACC) and HASPC7 (GTAAGACACCGTGTAAAGA TG) primers. Representative SSCP and restriction digestion analysis of a854>c point-mutation in 3 families is shown and the pedigrees are drawn at the top. One of the normal controls (Lane 4) and the non-carrier sibling of a patient with CD (Lane 1) are also shown.
Fig. 7. Southern blot analysis of genomic DNA from eukaryotic species. Genomic DNA (8 µg) was digested with EcoR I and electrophoresed on 1% agarose gel in 1XTAE. The DNA in the gel was denatured and neutralized, and blotted on nitrocellulose membrane. The blots were baked, hybridized to $^{32}$P-labeled human ASPA cDNA, washed under conditions described in the text and autoradiographed. The specific EcoR I restricted fragments that hybridized to human cDNA were: yeast (4.4 and 2.2 kbp); chicken (7.5 kbp); rabbit (5.4, 2.5 and 0.7 kbp); cow (9, 6.4 and 4 kbp); dog (12.4, 4.2, 1.4 and 0.5 kbp); mouse (20 and 6.8 kbp); rat (19, 5.4, 2.8 and 0.6 kbp); monkey (20 and 6.8 kbp); and human (19.5 and 9.8 kbp). Hind III digest of λ DNA served as size markers.
Fig. 8. Restriction map of human ASPA gene constructed from the isolated genomic phage clones λEMBL3 1-2, 2-1, and 4-3. The restriction fragments hybridizing to cDNA in phage clones were similar to those observed upon analysis of the human genomic DNA. The exon/intron boundaries were mapped by limited sequence analysis of the phage insert subclones that hybridized to the cDNA and using primers derived from the cDNA sequence. The position of exons I to VI in ASPA gene are shown by rectangles (■). The solid rectangles represent the coding sequences and open rectangles show the non-coding sequences in the human cDNA clone. The length of introns in the restriction map are close approximations.
Fig. 9. Comparison of nucleotide sequence of human (HASPS: 680 bases) and bovine (BASP5: 721 bases) genomic sequences upstream of the initiator "ATG" codon in ASPA gene from these two species. The alignment of these sequences was carried out by ALIGN program version 5.90 (K-tuple size = 3, range = 20, gap penalty = 3) in Lasergene software package from DNAstar (Madison, WI). The human and bovine sequences were about 75% identical in the region compared. The homology dropped upstream of this region. The HASP5 sequences in bold type are the transcribed region identified in the cDNA clone. The position of initiator "ATG" codon are marked by down-arrow (↓) and the base preceding it has been numbered as -1. The initiator "ATG" codons in human and bovine sequences are underlined.
Fig. 10. Chromosomal localization of human ASPA gene by fluorescent in situ hybridization. A human metaphase spread showing specific hybridization of human ASPA sequences in p13-ter region on chromosome 17. The chromosome 17 was identified by co-hybridization with D17Z1 (α-satellite) probe. The probes were labeled with biotin-14-dATP by nick translation according to the conditions described in text.
Fig. 11. Nucleotide sequence of exon III specific amplified fragment from a normal control (WT, Upper panel) and a patient (mutant, Lower panel) ASPA gene. Exon III specific sequences were amplified by PCR with primers 3A and 3B. The nucleotide sequence is shown around 433 -2(A→G) mutation. The proband was a heterozygote for A→G transition and the base is therefore identified as N at this position (Lower panel).
Fig. 12. Restriction endonuclease digestion of PCR amplified fragments for identification of 433 -2(A>g) mutations. The PCR amplification was carried out with IVS2SA and 3B primers and 179 bp amplified fragment was digested with Hpa II. The digest was electrophoresed on 6\% native polyacrylamide gels and stained with ethidium bromide. The mutant allele results in 156 and 29 bp (not seen in the gel) fragments. The WT allele is not cleaved by Hpa II enzyme.
Fig. 13. Autoradiography showing SSCP in exon specific PCR amplified fragments. Exon VI sequences (Panels A and B) were amplified with 6A and RT1 primers as 347 bp fragments and electrophoresed on SSCP gel. Lanes 1 and 6: 854A>C heterozygote; Lanes 2, 4 and 7: normal controls; Lane 3: 854A>C homozygote; Lane 5: 914C>A homozygote; Lane 8: 914C>A heterozygote. Exon V (Panel C) sequences were amplified with 5A/5B primers and 235 bp amplified fragment was electrophoresed on SSCP gel. Lane 9: normal control; Lane 10: 693C>A heterozygote; Lane 11: 693C>A homozygote.
**Fig. 14.** Nucleotide sequence of exon V specific amplified fragment from a normal control (WT, Upper panel) and a patient (mutant, Lower panel) ASPA gene. Exon V specific sequences were amplified by PCR with primers 5A and 5B. The nucleotide sequence of sense strand is shown around 693C>A mutation. The proband (Lower panel) was homozygous for 693C>A base change.
Fig. 15. Restriction endonuclease digestion of PCR amplified fragments for identification of 693C>A mutation. Exon V specific sequences were amplified using 5A/5B primers. The 235 bp amplified fragment was digested with Mse I restriction enzyme and electrophoresed on 6% native polyacrylamide gels and stained with ethidium bromide. The mutant alleles yield 104, 73 and 58 bp and WT alleles produce 177 and 58 bp fragments.
Fig. 16. Nucleotide sequence of sense strand from exon VI specific amplified fragment from a normal control (WT, Upper panel) and a patient (mutant, Lower panel) ASPA gene. Exon VI specific sequences were amplified by PCR with primers 6A and RT1. The nucleotide sequence shown is around 914C>A base change. The proband (Lower panel) was homozygous for 914C>A mutation.
Fig. 17. Panel A, see next page for legend.
Heterozygous

Homozygous

Control

Control, uncut

pBR322, Haelll
Fig. 17. Restriction endonuclease digestion of PCR amplified fragments for identification of 854A>C and 914C>A mutation. Exon VI specific sequences were amplified using G5/C22 primers. The 194 bp fragment was digested with Eag I (Panel A) or Nsi I restriction enzyme (Panel B) for identification of glu285>ala or ala305>glu mutations respectively. The digests were electrophoresed on 6% native polyacrylamide gels and stained with ethidium bromide. The mutant allele with glu285>ala mutation yield 112 and 82 bp fragments upon digestion with Eag I restriction endonuclease, while the glu285 WT alleles is not cleaved. Following PCR amplification, the T>A mismatch in C22 primer creates Nsi I recognition sequence in ala305 WT alleles. Restriction digestion of amplified WT allele results in 173 and 21 bp (not seen in the gel) bands. Ala305>glu mutant alleles do not create Nsi I recognition sequence following PCR amplification.
Fig. 18. Schematic representation of pSV-ASPA(WT) expression construct. The WT cDNA insert (base positions -108 to +1248) was cloned in pGEUK-C1 vector down stream of SV40 late promoter and VP1 intron. The SV40 polyadenylation signal was present at 3' of the cDNA insert. The relative position of 693C>A, 854A>C or 914C>A mutations, present in pSV-ASPA(693C>A), pSV-ASPA(854A>C) or pSV-ASPA(914C>A) expression constructs respectively, are also indicated. The figure is not drawn to scale.
APPENDIX II

TABLES
TABLE 1

Exon sizes and DNA sequence at exon/intron boundaries of ASPA

<table>
<thead>
<tr>
<th>Exon bp</th>
<th>5’ Splice donor</th>
<th>Intron</th>
<th>3’ Splice acceptor</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>CTTGG gtaaga</td>
<td>1</td>
<td>ttataacag</td>
<td>CAAAAAA Human</td>
</tr>
<tr>
<td></td>
<td>CTTGG gtaaga</td>
<td></td>
<td>taataacag</td>
<td>CAAAAAA Bovine</td>
</tr>
<tr>
<td>II</td>
<td>ATTAAG gtaatg</td>
<td>2</td>
<td>ttttttcag</td>
<td>ACTTCT Human</td>
</tr>
<tr>
<td></td>
<td>ATTAAG gtaatg</td>
<td></td>
<td>tcttttcag</td>
<td>ACGTCT Bovine</td>
</tr>
<tr>
<td>III</td>
<td>GTGG gtaagt</td>
<td>3</td>
<td>tgtacctag</td>
<td>GTATAGAA Human</td>
</tr>
<tr>
<td></td>
<td>GTGG gtaagt</td>
<td></td>
<td>tgtacctag</td>
<td>GTATAGAA Bovine</td>
</tr>
<tr>
<td>IV</td>
<td>GAAG gtaagt</td>
<td>4</td>
<td>ttgtcatag</td>
<td>GAAAAGAA Human</td>
</tr>
<tr>
<td></td>
<td>GAAG gtaagt</td>
<td></td>
<td>ttgccccag</td>
<td>GAAAAGAA Bovine</td>
</tr>
<tr>
<td>V</td>
<td>CTGCAG gtaaca</td>
<td>5</td>
<td>tcctgagag</td>
<td>GATCAA Human</td>
</tr>
<tr>
<td></td>
<td>CTGCAG gtaatg</td>
<td></td>
<td>tcctgaaag</td>
<td>GATCAA Bovine</td>
</tr>
<tr>
<td>VI</td>
<td>514</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>835</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
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Consensus sequence:  gtaag/yu/t  tnn/y ana/yyag
### Table 2

Oligonucleotides synthesized\(^1\) for PCR amplification and detection of mutations in Canavan alleles.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide Sequence</th>
<th>Size</th>
<th>S/AS(^2)</th>
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<tbody>
<tr>
<td>3A</td>
<td>5' AACATACGGTTTTTACCTAAG 3'</td>
<td>21-mer</td>
<td>S</td>
</tr>
<tr>
<td>3B</td>
<td>5' TCTCTGAGTTTCAGCTAGG 3'</td>
<td>19-mer</td>
<td>AS</td>
</tr>
<tr>
<td>IVS2SA-2</td>
<td>5'GAAAGACGTTTTTGTATTTTCTC 3'</td>
<td>22-mer</td>
<td>S</td>
</tr>
<tr>
<td>5A</td>
<td>5' CCAGAGATGGTTTTTAGTTGC 3'</td>
<td>20-mer</td>
<td>S</td>
</tr>
<tr>
<td>5B</td>
<td>5' TGCTGTATGAGCTATAACTTT 3'</td>
<td>21-mer</td>
<td>AS</td>
</tr>
<tr>
<td>6A</td>
<td>5' GTCTAGAGTCGACATAAAATT 3'</td>
<td>21-mer</td>
<td>S</td>
</tr>
<tr>
<td>RT1</td>
<td>5' AACCCTACTCTAAAGGAGC 3'</td>
<td>19-mer</td>
<td>AS</td>
</tr>
<tr>
<td>G5</td>
<td>5' CCAGAGATGTTTTTAGTTGC 3'</td>
<td>20-mer</td>
<td>S</td>
</tr>
<tr>
<td>C22</td>
<td>5' TAAACAGCAGCGAATACTTTAT 3'</td>
<td>22-mer</td>
<td>AS</td>
</tr>
</tbody>
</table>

1. Primers were synthesized on 380B DNA-synthesizer (Applied Biosystems, Foster City, CA) using phosphoramidite chemistry.
* The base with an asterisk is the mismatch introduced in the primer sequence for PDSM.
Table 3

Relative frequency of Canavan mutations among Ashkenazi Jewish and non-Jewish proband.

<table>
<thead>
<tr>
<th>Position</th>
<th>Mutation</th>
<th>Type</th>
<th>Relative Frequency</th>
<th>Ashkenazi Jewish</th>
<th>Non-Jewish</th>
<th>European</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ashkenazi Jewish</td>
<td></td>
<td></td>
<td>European</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>%</td>
<td>N</td>
<td>%</td>
<td>N</td>
</tr>
<tr>
<td>433 -2</td>
<td>a&gt;g</td>
<td>Intron 2</td>
<td>1.2</td>
<td>1</td>
<td>-</td>
<td>None</td>
</tr>
<tr>
<td>693c&gt;a</td>
<td>Y231&gt;X</td>
<td>Nonsense</td>
<td>14.6</td>
<td>12</td>
<td>-</td>
<td>None</td>
</tr>
<tr>
<td>854a&gt;c</td>
<td>E285&gt;A</td>
<td>Missense</td>
<td>80.5</td>
<td>66</td>
<td>2.8</td>
<td>1</td>
</tr>
<tr>
<td>914c&gt;a</td>
<td>A305&gt;E</td>
<td>Missense</td>
<td>-</td>
<td>None</td>
<td>44.4</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>96.3</td>
<td>79</td>
<td>47.3</td>
<td>17</td>
</tr>
</tbody>
</table>

*: Non-Jewish patients studied were caucasians of European ancestry.
1: 82 chromosomes from Ashkenazi Jewish proband were analyzed.
2: 36 chromosomes from non-Jewish proband were analyzed.
VITAE

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PRESENTATIONS:


AWARD:

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The Genetics Society of America, U.S.A.

The American society of Human Genetics. U.S.A.