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Molecular genetic studies of human and feline *N*-acetylgalactosamine 4-sulfatase (arylsulfatase B)

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Molecular Genetic Studies of Human and Feline *N*-acetylgalactosamine 4-sulfatase (Arylsulfatase B)

by

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Abstract

Molecular Genetic Studies of Human and Feline

***N*-acetylgalactosamine 4-sulfatase (Arylsulfatase B)**

by

Christine Elise Jackson

Advisor: Edward H. Schuchman, Ph.D.

N-acetylgalactosamine 4-sulfatase (Arylsulfatase B; ASB; EC 3.1.6.1) is the lysosomal enzyme responsible for the hydrolysis of 4-sulfate groups from *N*-acetylgalactosamine 4-sulfate moieties present in the glycosaminoglycan, dermatan sulfate. A deficiency of this enzyme in man results in the lysosomal storage disorder, Maroteaux-Lamy disease (Mucopolysaccharidosis Type VI; MPS VI). Notably, a naturally occurring model of MPS VI has been identified in Siamese cats, and the biochemical and pathological properties of the human and feline diseases have been well characterized.

Thus, the molecular genetic studies described in this thesis were undertaken in order to provide insights into the nature of this lysosomal hydrolase, and to facilitate the future use of this prototypic animal model system for the development of enzyme

and/or gene replacement therapy for MPS VI. Human ASB was purified from liver and five tryptic peptides (112 residues) were microsequenced. Degenerative oligonucleotide probes were constructed and used to isolate overlapping partial cDNAs for ASB by the mixed oligonucleotide-primed amplification of cDNA (MOPAC) cloning strategy. The complete human ASB cDNA sequence is 2,802 bp and includes 559 bp of 5′ untranslated sequence, 644 bp of 3′ untranslated sequence, and a 1,599 bp open reading frame encoding 533 amino acids. There are six potential *N*-glycosylation sites. Two full-length cDNAs, differing only in the length of their 5′ untranslated sequences, were constructed from the partial cDNAs, and transiently expressed in COS-1 cells to demonstrate their functional integrity. These cDNAs have also been subcloned into the retroviral vectors, DCTK and pBC140, so that gene transfer studies may be initiated.

Feline ASB cDNAs have also been isolated using a 2.2 kb human ASB cDNA as a probe. The feline cDNA sequence, compiled from the overlapping cDNA clones, is 1,939 bp and includes 3 bp of 5′ untranslated sequence, 331 bp of 3′ untranslated sequence, and 1,605 bp coding for 535 amino acids. There are five potential *N*-glycosylation sites. A full-length feline cDNA was constructed and transiently expressed to demonstrate its functional integrity. This feline cDNA is now available for retroviral studies, if necessary. Additionally, the feline ASB gene was mapped to feline chromosome A1 by PCR analysis of somatic cell hybrid panels.

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Abbreviations

ASA	Arylsulfatase A
ASB	Arylsulfatase B
ASC	Arylsulfatase C
BMTs	Bone marrow transplantations
fASB	Feline Arylsulfatase B
fMPS VI	Feline Mucopolysaccharidosis VI
G6S	Glucosamine 6-sulfatase
GAGs	Glycosaminoglycans
GalNAc4SO_4	*N*-acetylgalactosamine 4-sulfate
hASB	Human Arylsulfatase B
hMPS VI	Human Mucopolysaccharidosis VI
IDS	Iduronate 2-sulfatase
MLD	Metachromatic Leukodystrophy
MOPAC	Mixed oligonucleotide-primed amplification of cDNA
MPS VI	Mucopolysaccharidosis VI
MSD	Multiple Sulfatase Deficiency
NCS	Nitrocatechol Sulfate
PCR	Polymerase Chain Reaction

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Chapter One: Molecular Genetic Studies of Human Arylsulfatase B

Chapter Two: Molecular Genetic Studies of Feline Arylsulfatase B

Background

A. Characterization of Human and Feline Arylsulfatase B

Characterization of Human ASB

Human *N*-acetylgalactosamine 4-sulfatase (Arylsulfatase B, ASB, E.C. 3.1.6.1) is the lysosomal enzyme responsible for the hydrolysis of 4-sulfate groups from *N*-acetylgalactosamine 4-sulfate (GalNAc4SO_4) moieties present primarily in the glycosaminoglycan, dermatan sulfate (87). ASB has been purified from many human tissue sources, however, the reported molecular weight and subunit determinations vary, in part due to the aberrant electrophoretic behavior characteristic of glycoproteins. McGovern *et al.* determined that the liver ASB was a monomer with a M_r of 38 kDa (81). Wasserman's lung ASB was a monomer that had a M_r of 60 kDa (151), and there is a report of an eosinophil ASB which is a homotetramer composed of 17.5 kDa subunits (152). Gibson *et al.* found that ASB from liver, kidney, lung, and urine had a M_r of 57 kDa which is dissociable by reducing agents into subunits of 43 and 13 kDa (39).

Pulse-chase studies have provided the most reliable data. These studies, performed by Steckel *et al.* on fibroblast ASB, identified a 64 kDa precursor which may be processed to two forms. Form I is composed of 47 and 11.5 kDa chains, while form II is composed of 40 and 31 kDa chains. Only the precursor and form I are

enzymatically active. These same investigators determined that in placental ASB, form I ASB subunits were held together by disulfide linkages (125). In a later experiment, investigators from the same laboratory (Peters *et al.*) overexpressed a human ASB cDNA in BHK cells. When the metabolically labelled ASB was immunoprecipitated, only the 64 kDa precursor and the form I (47 kDa and 11.5 kDa) ASB were observed (99). Taylor *et al.* have also studied the biosynthesis and maturation of ASB in fibroblasts. They observed a 66 kDa precursor which was processed to a 57 kDa intermediate. The mature form of ASB consisted of 43 kDa and 8 kDa disulfide linked subunits (135). Both of these groups now believe that the protein previously identified as ASB form II is an occasional contaminant which is unrelated to ASB.

ASB has been mapped to chromosome 5 by several groups (52,24,37,33) and localized to 5q13-5q14 by Litjens *et al.* by *in situ* hybridization with a partial genomic clone (74).

Minor anionic (acidic) forms of ASB have been reported in liver (117,132), fibroblasts (131), kidney, testis, placenta (132), and brain (80,132). In brain, this anionic form designated Bm may be 15-25% of the total ASB activity (132). While Shapira and Nadler found that the two forms of ASB in liver were identical in all their physical and enzymatic properties except for net charge (117), Stevens *et al.* found that the brain Bm was more thermolabile than ASB. Brain and fibroblast Bm migrated as a diffuse band on PAGE, and isoelectric points were found at 6.8, 7.0, and 7.2 for brain Bm, suggesting the presence of a heterogeneous population of minor anionic species (132). Additionally, Stevens and Fluharty have both suggested the possibility

of a membrane-associated form of ASB (132,36) . This claim was mainly based on the observation that not all ASB was recovered easily by usual extraction procedures, i.e., some required a higher concentration of buffer for extraction. For example, Stevens isolated an additional 25% of total ASB activity by using a higher concentration of Tris on the residue obtained from normal brain ASB extraction. He suggested that the brain Bm form may be membrane-associated (132). Stumpf *et al.* have observed that significant ASB activity remains in pellets following procedures sufficient to extract soluble enzymes from lysosomes. This residual ASB is identical to the soluble ASB in heat stability, pH optimum, and response to inhibitors. They too suggested that ASB may exist in a membrane-associated form (133). ASB has a pH optimum close to 5.6 when determined with the commonly used artificial substrate nitrocatechol sulfate (NCS) (117). Assays employing an GalNAc4SO_4-containing trisaccharide substrate, which is more similar to the natural substrate, indicated a pH optimum of about 4.0 (39). In addition, Fluharty *et al.* have found a pH optimum of about 3.5 using UDP-GalNAc4SO_4 and a trisaccharide derived from chondroitin sulfate (36). The isoelectric point of ASB is about 8.0 (81).

NCS is not specific for ASB, and is hydrolyzed by ASA as well (43,118). Since both of these arylsulfatases are soluble, difficulties arise in determination of the true ASB levels when using this substrate (118). Both enzymes are inhibited by sulfite, sulfate, and phosphate ions (19). ASB may be specifically inhibited by chloride ions (122,7), and ASA may be similarly inhibited by silver ions (19). Barium acetate also has been shown to inhibit ASA, but there are many conflicting reports (7,122). The

best means of specifically assaying is to separate ASA and ASB by batch DEAE chromatography (43,118).

Studies on mutant human ASB

Several studies have been performed on the mutant ASB present in MPS VI patients. One MPS VI family has been reported with no residual ASB activity, but since the affected individuals from this family are CRIM +, it is likely that a stable, but nonfunctional, protein is made (14). Fluharty *et al.* studied three MPS VI fibroblast cell lines which had about 10% residual activity. Levels of the anionic (Bm) form of ASB were depressed in these cell lines as well, indicating that ASB and Bm are from the same gene product. The properties of the residual ASB were identical to those for normal enzyme (pH activity for 4-methylumbelliferyl sulfate (4MUS), K_m, heat stability, DEAE separation) (34). Shapira *et al.* have also reported the isolation of a residual ASB from CRIM+ MPS VI fibroblasts (116).

Recently, Brooks *et al.* developed a sensitive immunoquantification assay for ASB. In this assay, they used seven monoclonal antibodies to quantitate ASB in sixteen MPS VI patients. In general, fibroblasts from the most severely affected MPS VI patients had the lowest amounts of protein and the fewest epitopes detected. All patients had $\leq$ 5% normal ASB levels, and $\leq$ 5% catalytic capacity as determined with the ASB-specific trisaccharide substrate, GalNAc4S-GlcA-GalNAcitol4S. Catalytic capacity, or the capacity of the fibroblasts to degrade substrate, was defined as the product of ASB catalytic efficiency (moles per min per mole substrate/K_m) and the

content of ASB in the fibroblasts (17).

Taylor *et al.* have studied the biosynthesis and maturation of ASB in eight MPS VI fibroblast cell lines. Immunoprecipitation of metabolically labelled ASB from all eight lines showed that the incorporation of radioactivity was less than 10% of normal in these cells. Pulse chase experiments indicated that in all eight lines, ASB was processed differently than normal. Some of the MPS VI lines had ASB maturation processes which might indicate that there was a delay in intracellular transport, decreased stability of the enzyme, failure to target to the lysosome or resistance to enzymatic processing (135). Wicker *et al.* have studied an intermediate type MPS VI fibroblast line. By immunoquantification with three ASB-specific monoclonal antibodies, it was determined that the cells produced seventy-fold less ASB than control fibroblasts. However, the K_m and V_{max} of this enzyme towards GalNAc4S-GlcA-GalNAcitol4S were near normal (154). Clearly, there is considerable heterogeneity of mutant ASB in unrelated MPS VI patients.

Notably, a deficiency of ASB, and of six other sulfatases, is seen in the neurodegenerative lysosomal disorder Multiple Sulfatase Deficiency (MSD) (61). This disorder will be discussed further in the Chapter One section entitled "Amino Acid Homology Between the Sulfatases."

Other properties of ASB

The work which will be presented in this thesis is mainly concerned with ASB's function as hydrolase of the glycosaminoglycans (GAGs), dermatan sulfate and

chondroitin sulfate, and the loss of this function which produces the lysosomal storage disorder MPS VI. ASB does, however, have other functions and characteristics which have fueled a number of interesting studies. Farooqui *et al.* have found that ASB can hydrolyze sulfate groups from glucosamine 4,6 disulfate moieties as well as from *N*-acetylgalactosamine 4-sulfate moieties (32). ASB from lung or eosinophils has been shown to inactivate the slow-reacting substance of anaphylaxis, which is a mediator of immediate-type hypersensitivity (151). Human kidney ASB was found to hydrolyze the catecholamines adrenaline-3-sulfate and noradrenaline-3-sulfate *in vitro*, but the kinetic parameters of these reactions are unfavorable, and it is unclear if this reaction is physiologically relevant (25). The minor acidic form of ASB is phosphorylated by a cAMP-dependent protein kinase (3). In normal cells, the acidic ASB is phosphorylated at its serine residues, while in lung cancer tumors or chronic myelogenous leukemia leukocytes, the ASB is phosphorylated at threonine residues as well (140). The overall increase in phosphorylated ASB in these disease states may be useful as a prognostic indicator for therapy. Pronounced increase in arylsulfatase activity has been found in patients with tumors of the stomach, colon, breast, and skin. However, increased arylsulfatase levels were not observed in the sera of these patients (32). Greatly increased arylsulfatase levels have also been noted in the urine of patients with myeloid leukemia, diseases of the bladder, testes, and uterus, and cancer of the prostate. ASB levels are increased in the sera of patients with colorectal cancer (15).

Characterization of normal and mutant feline ASB

Feline ASB (fASB) has been studied in normal and MPS VI Siamese cats (81,83). The normal enzyme isolated from feline liver is a functional homodimer with subunits of 50 kDa (81). It has a pH optimum of about 5.7 when determined with the NCS substrate, has a pI of about 7.8, and is stable over the pH range of 3.5-8.5 (146). There are two types of defective fASB residual enzymes which have been isolated from two different families with feline MPS VI. The first is referred to as the MPS VIa mutant. This mutant protein is a monomer when analyzed by gel filtration, SDS-PAGE, and subunit alkylation. Also, it differed from normal fASB by having a 100-fold increased K_m, and being more thermo-, cryo-, and pH labile (145). Its K_i using dermatan sulfate as an inhibitor of NCS hydrolysis is increased five-fold and its V_{max} is decreased. The pH optimum is about 5.7, similar to normal fASB (83). Activity of the mutant enzyme may be enhanced several fold by treatment with the sulfhydryl reagents DTT or cysteamine. These reagents also restored the normal molecular weight and normal electrophoretic mobility to the MPS VIa mutant enzyme. Vine *et al.* proposed that a point mutation resulted in substitution of a cysteine residue at or near the subunit association site of the fMPS VIa residual enzyme, or that a point mutation caused a conformational change which exposed a previously masked cysteine residue. A disulfide bridge is formed within the subunit, and the subunits fail to dimerize. In the presence of a sulfhydryl reagent, the disulfide bond is reduced and the dimers may form (146).

The second fASB mutant is referred to as the MPS VIb mutant. In contrast to the MPS VIa mutant, its molecular weight was similar to that of the normal feline enzyme. However, the pH optimum differed from that of normal fASB, i.e., the pH curve was biphasic with optima at pH 5.2 and 6.2. The K_m was slightly higher than normal, and it was slightly more thermo- and cryo-stable. No stimulation by DTT was observed. There was a marked decrease in V_{max} towards the artificial substrate NCS and the K_i using dermatan sulfate as inhibitor of NCS hydrolysis showed a 15-fold increase (83).

A heterozygous MPS VI a/b kitten has been bred. Its enzyme showed characteristics intermediate to those of the a/a or b/b homozygotes. Residual ASB activity in a/a, b/b, or a/b cats is about 6% of normal (83).

Thompson and Daniel have studied ASB and ASA in eleven different mammals, including the domestic cat. The feline ASB characterized in their study appeared to be a monomer of 59 kDa by gel filtration. They suggested that in some feline species ASB may exist as a monomer. Interestingly, rodent, dog, whale, and pig ASB were all found to have minor ASB isozymes which were anionic and dimeric (137).

B. The Human Mucopolysaccharidoses and MPS VI (Maroteaux-Lamy Disease)

The Mucopolysaccharidoses are a group of lysosomal storage disorders caused by deficiencies of specific lysosomal enzymes required for the degradation of glycosaminoglycans (GAGs) (87). First described by Hunter in 1917 (54) and Hurler

in 1919 (79), these disorders have a chronic and progressive course and display common characteristics such as coarse facies, dysostosis multiplex, and multisystem involvement. There can be a wide variability in clinical characteristics and disease severity both between MPS disorders and within a specific enzyme deficiency. For example, MPS IH, Hurler syndrome, is characterized by profound mental retardation, corneal clouding, dysostosis multiplex, organomegaly, heart disease, and early death, while MPS IS, Scheie syndrome, is characterized by normal intelligence, stiffness of joints, corneal clouding, and normal life span. Both of these disorders are caused by allelic mutations leading to the deficiency of α-L-iduronidase. MPS IV, the Morquio syndrome, is characterized by corneal clouding and distinctive skeletal abnormalities, which serves to visually distinguish MPS IV patients from other MPS patients. Five of the six MPS classifications display autosomal recessive inheritance, and the sixth, Hunter syndrome, is X-linked recessive (87).

Traditionally, the MPS disorders have been diagnosed by clinical features, by the identification of specific cetylpyridinium chloride-precipitable GAGs which are secreted in large quantities in the urine of these patients, and by radiologic features. Identification of the enzyme deficiencies causative in these disorders has made enzyme assays on leukocytes, plasma, or cultured fibroblasts the preferred means of disease diagnosis. Molecular characterization of the MPS disorders is proceeding at a rapid rate. Full-length cDNAs encoding α-L-iduronidase (MPS I) (115), iduronate 2-sulfatase (MPS II) (156), glucosamine 6-sulfatase (MPS IIID) (106), β-D-galactosidase (MPS IVB) (94), *N*-acetylgalactosamine 4-sulfatase (MPS VI) (114,99), and β-D-

glucuronidase (MPS VII) (93) have been isolated. A full-length genomic clone for α-L-iduronidase has been isolated (115), as have partial genomic clones for *N*-acetylgalactosamine 4-sulfatase (75,99) and iduronate 2-sulfatase (156). MPS causative point mutations have been identified in MPS VI (59,154) and MPS VII (139). Further molecular characterization and identification of mutations should facilitate heterozygote and homozygote detection for the MPS disorders.

Individuals with MPS VI generally present with dysostosis multiplex, hepatosplenomegaly, corneal clouding, and normal intelligence. MPS VI patients display excessive urinary secretion of dermatan sulfate, and are deficient in the lysosomal enzyme ASB. Dermatan sulfate is a component of connective tissue. It is generally found along with other GAGs complexed to a base protein, forming a proteoglycan. Degradation of dermatan sulfate begins with the proteolysis of the proteoglycan to produce single dermatan sulfate chains. It is presumed that an endoglycosidase then clips dermatan sulfate into oligosaccharides, which are degraded in a stepwise fashion in the lysosome. It is here that the blockage in MPS VI occurs. Because the oligosaccharide degradation is generally sequential, it cannot proceed unless the 4-sulfate moieties are cleaved from GalNAc4SO_4. The oligosaccharides are then trapped, and the lysosomes become engorged (54).

Mild, intermediate, and severe forms of MPS VI have been observed. Most MPS VI patients with the severe phenotype die of heart failure in their second or third decade. There is no specific treatment for this disorder. Supportive care is given and complications are treated as they occur (87).

MPS VI was first classified as a separate mucopolysaccharidosis by Maroteaux and Lamy in 1963. They were able to identify the disease by distinct clinical, radiologic, genetic, and biochemical findings. Their fractionation of urinary GAGs and identification of large levels of chondroitin sulfate B (dermatan sulfate) in the urine of their patient was instrumental in the disease identification. They called this disorder polydystrophic dwarfism, and found five similar cases described in the literature, including one case from 1938 which had been classified as Hurler syndrome (79).

In 1970, Spranger *et al.* reviewed the data for nineteen MPS VI patients. They found that some features of the syndrome could often be observed at birth, including a large head and deformed chest with thickened ribs which tapered at the vertebral ends. In later infancy, respiratory infections occurred frequently. Consanguinity was seen in four of thirteen MPS VI families suggesting an autosomal recessive inheritance. Features found to be diagnostic for MPS VI were progressive dysmorphism, or a coarse facial appearance, after four years of age, failure to grow after age four, moderate to severe dysostosis multiplex, corneal opacities, normal intelligence, and an increase in urinary GAGs of which 70% or greater is dermatan sulfate (123). Paterson *et al.* described a mild form of MPS VI, termed MPS VIb, characterized by mild aches and pains, corneal opacities, minimal excretion of urinary GAGs, and absence of metachromatic granules in leukocytes (97). Pilz *et al.* also described a mild form of MPS VI in two brothers who were 38 and 40 years of age. The clinical findings in these cases differed slightly from Paterson's patients', and included metachromatic lymphocyte inclusions (101). Vestermark *et al.* described a case of MPS VI with

severe mental retardation, but since there was a mentally retarded sibling with no MPS VI, it is possible the two disorders were not connected (144).

Other studies performed in the 1970s characterized the biochemical deficiency in MPS VI. In 1972, Barton and Neufeld performed complementation studies using MPS VI fibroblasts. These fibroblasts were able to correct the deficiency in Hurler (MPS IH), Hunter (MPS II), and Sanfilippo (MPS III) syndrome fibroblasts, and in turn had their enzyme deficiency corrected by the "correction factor" from any of these other cells (6). This gave further evidence that a distinct, specific factor was causative in MPS VI. Stumpf *et al.* found ASB deficiencies in lung, kidney, and spleen of three MPS VI patients from two families (133). In 1974, Fluharty *et al.* found that ASB added to media increased degradation of GAGs by MPS VI fibroblasts four-fold. They concluded that ASB deficiency was the primary cause of MPS VI (34). That same year, O'Brien *et al.* were able to conclude that deficiency of *N*-acetylgalactosamine 4-sulfatase (GalNAc4-SO_4) caused at least one form of MPS VI (89). The study of Fluharty *et al.* in 1975, which demonstrated that ASB and GalNAc4-SO_4 were one and the same, tied these results together (35).

Prenatal diagnosis for MPS VI has been performed by ASB assay on cultured amniotic fluid cells (107,142). The incidence of MPS VI is rarer than that for MPS IH; estimates vary from 1 in 36,000 in Israel to 1 in 132,000 in Great Britain (87). It has been suggested that incidence of MPS in general may be as high as 1 in 10,000, or even higher if patients with mild phenotypes remain undetected (54).

C. Feline MPS VI

Characteristics of feline MPS VI; comparison of human and feline MPS VI

The first feline model of mucopolysaccharidosis was characterized by Cowell *et al.* in 1976. A 21 month old female Siamese cat, the product of a mother/son mating, presented with skeletal deformities, dwarfism, and abnormal facial features (21). Further investigations revealed metachromatic inclusion bodies in circulating leukocytes, a 17-fold excess of cetylpyridinium chloride-precipitable GAGs in the urine, normal cranial nerve function, and slight corneal clouding (57). Characterization of the urinary GAGs revealed that they consisted mostly of dermatan sulfate. Sulfate incorporation studies performed on cultured skin fibroblasts also indicated that these cells were defective in GAG degradation (47). Enzyme assays performed on fibroblasts from the affected cat showed increased activity of the lysosomal enzymes arylsulfatse A, β-D-glucuronidase, α-D-mannosidase, β-D-*N*-acetylglucosaminidase, and β-D-galactosidase. Notably, the activity of ASB was about 10% of normal feline levels (57). The parents of the affected cat were phenotypically normal and displayed no excess urinary GAG excretion, indicating an autosomal recessive mode of inheritance (46).

The clinical findings of the feline disease closely paralleled those for human MPS VI (hMPS VI), and since ASB deficiency and excretion of excess urinary dermatan sulfate are diagnostic for hMPS VI, the cat was classified as a naturally occurring feline model for this debilitating lysosomal storage disease (57). At about the

time of this initial study, the investigators were able to locate two additional families with four affected cats apparently suffering from the same disorder (46). This facilitated further characterization of the disease, as well as the establishment of a breeding colony for this valuable animal model. By 1979, the breeding colony was firmly established. A mating between obligate heterozygotes from the two different feline families produced an MPS VI kitten, demonstrating that the same locus was involved in the disease phenotypes (46). Examination of the pedigrees from the three families supported the theory of autosomal recessive inheritance of fMPS VI.

Enzyme studies on normal and mutant fASB have determined that there are at least two different genetic lesions which may give rise to fMPS VI. These studies are discussed above in the section entitled "Characterization of Human and Feline Arylsulfatase B". The heterozygous kitten bred from the two different families presented with a pathology which followed a similar course in the homozygous animals (83).

The similarities between human and feline MPS VI include facial dysmorphia, corneal clouding, epiphyseal dysplasia, peripheral leukocyte inclusions, urinary excretion of excessive amounts of dermatan sulfate, storage of GAGs in membrane-bound cytoplasmic inclusions, bony lesions, and lack of mental deterioration. One difference is the decreased severity of hepatosplenomegaly in the cats. However, membrane-bound inclusions are noted by electron microscopy in feline hepatocytes, Kupffer cells, interlobular connective tissue cells, and splenic smooth muscle cells (47).

Heterozygote detection is essential for the maintenance of the fMPS VI breeding

colony. Since heterozygote levels of ASB expression have a wide range, carrier detection can be extremely difficult. The use of a DEAE separation step helps in this determination by separating ASB and arylsulfatase A (ASA). The same assay is, of course, useful for detection of affected kittens. Additionally, affected homozygotes can be identified within a week after birth by the Berry spot test for excessive urinary GAGs. By 6-8 weeks of age, these kittens show characteristics of MPS VI such as facial dysmorphia, small ears, diffuse corneal clouding, large forepaws, and pectus excavatum. In addition, they are smaller than littermates of the same gender (47).

Bone marrow transplantations in MPS animal models

There have been twenty-one bone marrow transplantations (BMTs) performed on MPS VI cats (49). Gasper *et al.* transplanted the bone marrow of a histocompatible sibling into a totally irradiated two year old male with advanced fMPS VI. By day 232 posttransplantation, this cat's leukocyte ASB activity had increased 30-fold, and the urinary dermatan sulfate was decreased about 15-fold. Corneal clouding was completely cleared, and facial dysmorphia continued to resolve. Subjective changes included improved locomotion, improved demeanor, greater suppleness of coat, and increased head, neck, and mandibular mobility (38). Later, two more BMTs were performed by Wenger *et al.* As an interesting control, a five month-old affected cat was used as the marrow donor for a five month-old normal sibling. The same affected kitten was irradiated one month later and received a BMT from a normal sibling. Improvements in the affected cat seen at 330 days posttransplant included an increase

in liver ASB activity from 5 to 19% of normal, a drop in the level of urinary dermatan sulfate excretion, increased joint mobility, and significant resolution of facial dysmorphia and suppleness of coat. The normal cat transplanted with affected marrow only developed a coarser coat. Although it had displayed almost no leukocyte ASB activity for almost 330 days posttransplantation, urinary dermatan sulfate levels at this time had not increased (153). By 1988, eleven MPS VI cats had received BMTs. Thrall *et al.* have reported the cumulative results of allogenic BMT in these cats. Engraftment was successful in eight of eleven cats. The six surviving cats at the time of this report ranged from 94 to 1,730 days posttransplantation. Overall, the clinical findings indicated that these cats had greater mobility than non-transplanted MPS VI cats, and had only slight corneal clouding. Leukocyte ASB activity was in the normal range in these cats, and urinary GAG excretion was at normal levels (138). Currently, it is believed that corneal clouding is not significantly resolved by BMT in MPS VI cats (Dr. M. Haskins, personal communication).

Other MPS animal models include feline (45) and canine (120) MPS I models, and canine (48) and murine (10) MPS VII models. Six BMTs have been performed on MPS I dogs. At nine months posttransplantation, α-L-iduronidase levels were increased in the dogs' livers, brain, and cerebrospinal fluid, and α-L-iduronidase levels were normal in the recipients' leukocytes. Reduction in GAG accumulation was seen in the dogs' livers and brains. Other clinical improvements noted were decreases in corneal clouding and joint instability, and a decrease of urinary GAGs to a normal level (121). Two of three surviving dogs were further characterized twenty months post BMT. The

results were encouraging. Excess GAGs were almost completely cleared from the hemi-lymphatic, hepatic, renal, ocular, cardiovascular, and nervous systems. In the musculoskeletal system, lesions were less severe, but not completely cured. GAG accumulation was partly cleared from brain tissue, but some vacuolation was still seen in the cytoplasm of neurons. GAG levels in brain tissue were similar to normal control dogs, and there were detectable α-L-iduronidase levels in brain tissue (16).

A BMT has been performed on an MPS VII dog. One year after transplantation, the dog appears much healthier than untreated MPS VII dogs of a similar age. Less bone degeneration is observed, and the transplanted dog is able to run or walk with a near-normal gait. Untreated MPS VII animals cannot walk after six months of age. Additionally, there is a marked improvement in corneal clarity. Serum β-glucuronidase activity of this dog has ranged from 35-87% of normal since one month post BMT. Previously, the dog's serum β-glucuronidase activity had been 15% (50). Murine MPS VII has been corrected by BMT as well by Birkenmeier (11).

D. Bone Marrow Transplantation and Gene Therapy for Lysosomal Storage Diseases

Enzyme replacement and bone marrow transplantation

Enzyme replacement therapy has long been considered a possible treatment for lysosomal storage disorders (26) and, in particular, for the mucopolysaccharidoses (66). Several types of experiments were performed in the 1960s and 1970s which helped to

evaluate the usefulness of enzyme replacement therapy. The first type of studies performed were correction studies in which pure or partially purified enzymes were introduced into fibroblast cell lines deficient in specific enzymatic activities. Studies on fibroblasts from patients with MPS IH, MPS IS, MPS II, MPS IIIB, MPS VI, Fabry disease, and GM2 gangliosidosis showed that the substrate accumulation levels in these disorders could be normalized by uptake of the enzymatic factors deficient in these cell lines (26,86). At about the same time, other experiments were performed which involved enzyme replacement using limited quantities of painstakingly isolated enzyme. Such diverse studies as injection of fungal enzyme into patients with type II or IV glycogenoses and injection of human urinary ASA into a Metachromatic Leukodystrophy patient led to the conclusion that enzyme replacement therapy had possibilities for storage diseases, but required larger amounts of human enzyme to be evaluated completely. Other clinical correction studies performed at around this time included kidney, liver, and spleen transplantations. Despite limited success, these transplantation trials were not seen as useful approaches for enzyme replacement therapy because these tissues do not have donor cells with the capability to repopulate other affected tissues (26). Currently, enzyme replacement trials are being performed for Type I (non-neurologic) Gaucher disease, using a commercially available mannose-terminated glucocerebrosidase ("Ceredase") (9). Previous studies have indicated the effectiveness of enzyme replacement in treatment of Type I Gaucher disease, and the results of the current clinical trial are extremely encouraging (5,96).

Ultimately, the best type of enzyme replacement therapy is permanent enzyme

replacement by somatic gene transfer. One means of accomplishing this goal is to use bone marrow transplantation (BMT) as a means to introduce normal genes into an affected person. Obviously, this approach requires a histocompatible donor. The transplant should, of course, include the pluripotent stem cells, which proliferation and differentiation would ideally result in the repopulation of tissues by new, healthy cells of the monocyte/macrophage lineage. In theory, these cells could also provide secreted lysosomal enzymes to the surrounding cells as well (66).

Over 150 BMTs have been performed in storage disease patients. The successful grafts have generally arrested or improved the disease pathology. One issue which constantly surfaces is the effectiveness of treating those diseases with neurological involvement. The bone marrow-derived microglial cells do reach the brain, but may take six months to one year to do so. BMT results differ due to the variability in transplantation procedures and individual genetic differences within a disease (68).

Seven BMTs have been performed in children with Metachromatic Leukodystrophy (MLD) (68). MLD is a progressive neurodegenerative disease caused by a deficiency of the lysosomal enzyme ASA. The first child received a BMT at age four, and continued to deteriorate neuroradiologically and neurophysiologically, but at a slower rate than is usual for the severe form of this disease. Additionally, no deterioration was seen in the walking or running gait, or by neurologic examination (73). A recent report on this same patient, now five years post BMT, indicated that she was doing well and that cognitive decay had been arrested since one year post BMT

(67). A second MLD BMT was performed on an 11 month-old child, and this case has provided some optimistic data. At three years posttransplantation the patient continued to progress developmentally. Of his two other affected siblings, one had died and the other was totally unresponsive at the same age. In this patient, bone marrow-derived donor cells were shown to have crossed the blood brain barrier, and there was preliminary data suggesting ASA activity in the cerebrospinal fluid (69).

Seven BMTs have been performed on MPS VI patients (68). The first BMT was performed in a 13 year old MPS VI patient. Twenty-four months posttransplantation, improvements were seen. Leukocyte ASB had reached normal levels, and urinary GAG excretion decreased to normal levels. No storage products were seen by EM in any biopsied endothelial cells or in hepatocytes or Kupffer cells. The spleen was reduced to a normal size and the liver decreased in size, there was improved joint mobility and visual acuity, and possibly some improvement in cardiopulmonary function (65). At 40 months posttransplantation, levels of ASB in lymphocytes, granulocytes, and liver continued to increase, e.g., in liver reaching 16% of normal values. Striking improvement in cardiac function was seen by ECG. There have, however, been no improvements in corneal clouding or in bone pathology, perhaps due to the age of the patient (82). These improvements seen after BMT are similar to changes seen in MPS VI cats that have received BMTs. However, resolution of corneal clouding is occasionally seen in the cats. This may be because the cats are generally given BMTs as early as possible in life.

A number of MPS IH, MPS I H/S, and MPS IS patients have received BMTs,

as have ten MPS II, fourteen MPS III, and five MPS IV patients (66,68,92,85). For MPS BMTs overall, consistent improvement of visceral lesions were seen. Also, some bone growth occurred in these patients, and severe facial dysmorphia no longer occurred. Neurological results were variable (68). The cumulative data for the Hurler cases showed stabilization or improvement for these patients. It was implied that since magnetic resonance imaging abnormalities seen in the Hurler syndrome patients had improved post-BMT, that CNS meninges and blood vessels had been altered by the therapy (66).

BMT performed on patients with both neuronopathic and nonneuronopathic Gaucher disease indicated that the donor bone marrow-derived macrophages will repopulate the recipient's tissue slowly. There was no available evidence from these cases to suggest an increase in CNS glucocerebrosidase levels. As the major disease pathology in Gaucher disease is seen in cells of monocyte/macrophage lineage, BMT seems to have potential as a therapy for at least nonneuronopathic type I Gaucher disease (104,31).

The general agreement from all of these cases is that BMT should be performed as soon in life as possible, before irreversible pathologic changes occur. Additionally, occurrence of graft-versus host disease is much less frequent in patients transplanted in their first decade, and the potential for cell growth and differentiation (hematopoiesis) is greater in the young (91). Recently, an *in utero* BMT to a Hurler fetus was reported. Fetal stem cells (from an aborted ectopic pregnancy) were injected through the uterine wall into the chest cavity of this MPS IH fetus. Because the fetus' immune

system was not yet mature, the allogenic graft was not recognized as foreign, and the donor cells were not rejected. This baby is now one year old and displays no signs of Hurler disease except slight corneal clouding. While this treatment would seem to be the best of all possible treatments, one must bear in mind that it was possible because the parents had two other MPS IH children. Therefore, prenatal screening was routinely done on the fetus (53). Also, many more studies need to be performed to evaluate the efficacy of this therapy, and the U.S. government maintains a ban on most fetal tissue research.

Retroviral vectors and gene therapy

A major limitation of BMT is the frequent lack of histocompatible bone marrow donors. The ideal solution to this problem would be to bypass the need for a histocompatible donor and perform somatic cell gene therapy directly on the bone marrow of the patient. Once the deficient gene was introduced and expressed in the bone marrow, the patient would receive an autologous transplant. Introduction of the gene into bone marrow stem cells would permit the establishment of a population of healthy bone marrow-derived cells (including tissue macrophages) in the patient. Of course, this therapy should work very well for blood diseases such as the thalassemias. The rationale for using this approach to gene therapy for lysosomal storage diseases is that since five to twenty percent of newly synthesized lysosomal enzymes are normally secreted from cells (63), this enzyme may be internalized by other tissue cells through the ubiquitous mannose 6-phosphate receptor, and the enzyme may reach all pathologic

tissues.

One very efficient way to introduce a gene into bone marrow (or other cells) is by the use of retroviral vectors. Retroviruses were first described about eighty years ago as infectious cancer causing agents in chickens. They are retrotransposons, or cellular movable genetic elements which use reverse transcriptase for transposition (143). Retroviruses may transduce, or capture, a piece of the host's genome. Thus, they are natural vectors. Because of this transducing property, retroviruses have been instrumental to cancer researchers by helping to identify cellular sequences which have oncogenic properties (143,30).

Retroviruses are single-stranded RNA viruses which replicate through a double-stranded DNA intermediate, which must be integrated into the chromosome of a host cell. The integrated double-stranded copy, known as the provirus, can replicate with the host chromosome, or may make use of the host cell machinery to produce new infectious virus. Viral progeny will bud from the host cell membrane. No lysis is involved, and retroviruses do not generally harm the host cell (136).

Retroviral vectors have several features which make them particularly useful for gene therapy studies. They will infect close to 100% of the target cells, they express very efficiently, they can be taken up by a wide variety of cell types, many of which are resistant to transfection, and they tend to integrate in only one copy per cell, without forming the concatemers which may occur in microinjected DNA (30).

Since introduction of a foreign gene into a retroviral vector requires replacement of most of the retroviral trans functions, complementation systems for retroviral transfer

have been developed. The gene-containing retroviral construct is used to transform a packaging cell line, which contains a helper virus. Early helper virus constructs were intact except for the deletion of the viral packaging signal. The helper virus supplied all the essential viral proteins, and only the retroviral gene construct was packaged, since it had the necessary packaging signal. Once integrated into the host genome, the gene-containing construct would remain stably integrated. Problems arose using this system because the helper virus and the gene-containing construct could recombine and generate an infection-competent helper virus, which could undesirably continue to infect host cells, possibly triggering oncogenesis (136). Development of new packaging cell lines has greatly decreased the rate of recombination (78,84).

Retroviral vectors have been successfully employed in *in vitro* gene transfer experiments, and many *in vivo* gene transfer experiments are currently under way in animal systems. These generally involve autologous BMT in animal models after introduction of a selectable and/or a therapeutic retroviral construct into the bone marrow cultures grown in the laboratory. The *in vitro* experiments are numerous and include such diverse studies as expression of adenosine deaminase (ADA) in human and primate lymphocytes (60), expression of ADA in murine hematopoietic progenitor cells (8,155), and expression of parathyroid hormone in pituitary cells (51). Wolfe *et al.* have used a retroviral vector to introduce and express β-glucuronidase in MPS VII human and canine fibroblasts. The enzyme deficiency was corrected in both of these cell lines. Additionally, retrovirally introduced β-glucuronidase was expressed at high levels in canine MPS VII bone marrow cells, and was found to specifically degrade

GAGs and correct the pathology in canine MPS VII retinal pigment epithelial cells (157). Suchi *et al.* have corrected the enzymatic deficiency in cultured Niemann Pick type A fibroblasts by introduction of retroviral constructs containing the acid sphingomyelinase cDNA (134).

In vivo expression of a retrovirally introduced gene has been demonstrated in mice, dogs, monkeys, cats, and man (155,60,124,76,1). These studies have proven that successful reintroduction of retrovirally infected bone marrow is possible (98). In the feline study, Lothrop *et al.* retrovirally introduced the neomycin resistance (neo^r) gene into feline bone marrow cells which were then used to reconstitute four cats. All four cats expressed the neo^r gene in bone marrow for 30 days, and three cats still expressed at a low level 200 days later. An unexpected complication arose 90 days post BMT when two of the cats developed diabetes mellitus. This occurrence remains unexplained. It does, however, highlight the importance of using animal models initially, when possible, in retroviral gene replacement studies (76).

Currently, two retroviral-mediated gene therapy studies are underway in humans. The first of these studies, gene replacement therapy for adenosine deaminase (ADA) deficiency, or severe combined immunodeficiency, was begun in September 1990. An ADA retroviral construct was introduced *ex vivo* into T cells of a four year old patient, and after G418 selection the cells were introduced back into the patient. Trials were begun on a second patient in January 1991. Currently, both patients are doing well, and are reportedly more resistant to infections than at any other time in their lives. These two patients do, however, receive enzyme replacement as well. The

second human gene therapy trial began in January of 1991. Tumor-infiltrating lymphocytes (TIL) were marked with a retrovirally introduced neo^r gene and autologously transplanted into patients with melanoma. The patients suffered no ill effects due to the presence of the retrovirus, but the results of the study were unclear. In two cases, the TIL, which had been stimulated with interleukin 2, homed to the sites of the cancer and destroyed the tumor. In the other two cases, the tumors were not destroyed. While it is unclear why this treatment works some times and not others, the results were encouraging enough that further studies of this type have been approved (1,2). An NIH study has just begun which involves *ex vivo* treatment of TIL cells with a retroviral vector carrying the tumor necrosis factor gene, followed by subcutaneous implantation of the TIL. A similar study using the interleukin 2 gene will soon begin. Additionally, a trial has been approved at the University of Michigan in which hypercholesterolemia patients will be treated with liver cells into which the LDL receptor has been introduced using retroviral vectors (2).

Chapter One

Molecular Genetic Studies of Human Arylsulfatase B

Abstract

Arylsulfatase B was purified to homogeneity from human liver. Five peptides from tryptic digestion were chosen for microsequencing, and a total of 112 amino acids were determined from these peptides. After traditional screening attempts using antibodies or degenerative oligonucleotide probes were unsuccessful, a small N-terminal portion (55 bp) of the ASB cDNA was isolated by mixed oligonucleotide-primed amplification of cDNA (MOPAC). A longer (66 mer) probe was built based on this PCR sequence, mammalian codon usage, and sequence from arylsulfatases A and C. This probe was used to isolate partial ASB cDNAs (#22, #35, and #38) from a human hepatoma cDNA library. One of these cDNAs, #35, was used as a probe to screen a λgt11 testis cDNA library, from which a clone (#39) was isolated which contained the remainder of the ASB coding sequence. The full length ASB cDNA sequence is 2,802 bp and includes 559 bp of 5′ untranslated sequence, 644 bp of 3′ untranslated sequence including a polyadenylation signal, and 1,599 bp coding for 533 amino acids. There are six potential *N*-glycosylation sites. Significant homology is seen between the ASB protein and arylsulfatases A and C. There is also homology between ASB and two other sulfatases, iduronate 2-sulfatase and glucosamine 6-sulfatase. These five sulfatases (and two others) are deficient in the severe neurodegenerative disease, Multiple Sulfatase Deficiency. It is postulated that a common posttranslational modification occurs in all seven sulfatases. Two full length ASB cDNAs, one with 10 and one with 165 bp of

5′ untranslated sequence, were constructed and expressed transiently in COS-1 cells to prove their functional integrity. Before the construction was done, a coding difference between two partial cDNAs made it necessary to screen twenty normal individuals by PCR to determine if methionine or valine was the prevalent amino acid in the population at ASB amino acid 376. The allele coding for valine was seen in all twenty individuals. The human full length cDNAs were subcloned into the retroviral vectors, DCTK and pBC140, for future gene replacement studies in human and feline MPS VI. The availability of the ASB cDNA sequence has also made the molecular characterization of point mutations in MPS VI possible, and may prove useful in functional studies of ASB, of the other sulfatases, and of Multiple Sulfatase Deficiency.

Experimental Procedures

A. Protein Purification and Microsequencing

ASB was purified about 42,000 fold from human liver by a variation of the method of McGovern *et al.* (81). Each enzyme preparation was performed on 5-8 kg of human liver and all steps were carried out at 4 °C. Liver pieces were suspended in 25 mM Tris-HCl buffer, pH 7.5 containing 0.05% Triton X-100 (1:1.5 ratio) and homogenized for 90 sec. in a Waring blender. Further homogenization was performed in a Brinkmann polytron for 30 sec. in order to disrupt cell membranes. Following centrifugation in a Sorvall centrifuge for 45 min. at 7,000 X g, the supernatant was filtered through cheesecloth to remove lipids and any remaining debris. Salts were then added to a final concentration of 0.5 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$. The total volume at this time was about 5 l. Con-A Sepharose was added to the supernatant at a concentration of 1 ml Con A/mg of protein, and mixed overnight using an overhead stirrer. After passing the Con-A mixture through a scintered glass funnel (the flow-through was saved for assay), the beads were washed well with a buffer of 25 mM Tris-HCl, pH 7.5 containing the above salts, and the bound glycoprotein was eluted with 1 M α-methyl mannopyranoside. This eluate was transferred to dialysis tubing and dialyzed overnight against 3-4 changes of 100 mM Tris-HCl, pH 7.5. Following this, the eluted enzyme sample was concentrated to 400 ml by Amicon filtration using a PM-10 membrane. This concentrate was applied to a DEAE-Cellulose column which had been equilibrated with 100 mM Tris-HCl, pH 7.5. Under these

conditions, ASA will bind to the resin and ASB will not. The column was run at about 1 ml/min. using a peristaltic pump. The eluate was concentrated to 10 ml and dialyzed against 10 mM Tris-Acetate, pH 6.0. The concentrate was run over a Blue Sepharose column which had been equilibrated with 10 mM Tris-Acetate, pH 6.0. ASB was eluted using a linear salt gradient (0 to 0.3 M NaCl in 10 mM Tris-HCl, pH 7.4) at 0.5 ml/min. Fractions were collected and those containing the highest ASB activity were pooled, concentrated, dialyzed, and run on a Sephadex G-200 column which had been equilibrated with 0.5 M NaCl in 10 mM Tris-HCl, pH 7.5. The column was run at 0.1-0.2 ml/min to achieve good separation, and fractions containing the ASB activity were pooled. The final step in the purification was a DEAE-Sepharose column equilibrated with 5 mM Tris-HCl, pH 8.3. Using these conditions, ASB will not bind to this column. Enzyme isolated in the above fashion produced an active protein of approximately 50 kDa, which was apparently homogeneous by SDS-PAGE. In order to ensure purity for subsequent microsequencing, the 50 kDa band was electroeluted. The first of the two enzyme preparations was digested with trypsin and the tryptic peptides were isolated by reverse-phase HPLC. The second preparation was subjected to N-terminal sequencing. N-terminal and tryptic peptide microsequencing were performed by Dr. Ken Williams (Yale University School of Medicine) on an Applied Biosystems automated gas-phase microsequenator.

B. Construction of Synthetic Oligonucleotides

Synthetic oligonucleotide probes were synthesized on an Applied Biosystems

DNA synthesizer using phosphoramidite chemistry. Oligos which were to be used for screening were analyzed by electrophoresis on 14-20% polyacrylamide/7M urea gels. 5′ end-labelling of the oligos was performed using (γ-^{32}P) dATP and polynucleotide kinase as described by Maniatis *et al.* (77).

C. cDNA Library Screening and Analysis of Positive Human ASB cDNAs

For library screenings using oligonucleotide probes, hybridization and wash conditions were determined in pilot experiments. First, the Tm range was estimated by the TDR program (Dr. J. Wetmur). In general, the initial screening conditions were prehybridization and hybridization at 5-7 °C below the low end of the Tm range, followed by washing for 2 hours (with shaking and 3 changes of solution) at 3-5 °C below the low end of the Tm. If the background was too high, prehybridization, hybridization, and wash temperatures would be raised in 2 °C increments. Occasionally, washing time was increased. For oligonucleotide probes, the prehybridization solution was 6X SSPE, 10X Denhardt's, and 0.5% SDS; the hybridization solution was 6X SSPE, 5X Denhardt's, and 0.5% SDS. For these experiments, the wash solution contained 6X SSC and 0.5% SDS. The filters were prehybridized for a minimum of 2 hours, and the hybridization was performed overnight. The oligonucleotide probes were end-labelled with (γ-^{32}P) dATP.

For cDNA probes, the prehybridization solution contained 5X SSC, 0.1% SDS, and 10X Denhardt's, and the hybridization solution contained 5X SSC, 0.1% SDS, and 5X Denhardt's. Washing was performed in 0.1X SSC, 0.1% SDS. Prehybridization,

hybridization, and washing were routinely done at 65 °C, with an additional hour of wash if the background was too high. Probes were labelled using (α-^{32}P) dCTP and an Amersham or New England Biolabs random prime labelling kit according to the manufacturer's instructions.

For the phage library screenings, primaries were plated at a density of 2 X 10^4 pfu/150mm plate. Positive plaques were picked and soaked for a minimum of 4 hours at 4 °C in λdiluent (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 0.1 mM EDTA). After several rounds of purification, DNA was prepared by the mini-preparation method and analyzed by electrophoresis on 1% agarose gels followed by Southern hybridization. The phage mini-preps were performed by a modification of the method described by Maniatis *et al.* (77). Differences in the protocol included a longer, overnight soak with 3 ml SM to elute the phage out of the top agar, and the addition of these steps after PEG precipitation:

1) Resuspend the pellet in 200 μl T:E, pH 8, 2) Spin 2 min. and transfer to a new microfuge tube, 3) Add SDS to 0.25%, and 1/8 vol. of 2 M Tris/0.2M EDTA, pH 8. Leave at 70 °C for 20 min. 4) While still hot add 1/10 vol. of 5 M KAc. Leave on ice 30 min. 5) Spin 5 min. at 4 °C, transfer supernatant to a fresh tube, 6) Add Proteinase K to 50 μg/ml, leave at 42 °C for 1hr., 7) Extract 2X with phenol, 1X with phenol/chloroform, 1X with chloroform, 8) Precipitate DNA and dry in speed vac. Additional RNase A was added during the restriction digest. These preparations produced a good yield of clean DNA.

The Stratagene λZAP library (hepatoma cell) has some unique features which

require special techniques, and may cause special problems. λZAP is a modified λ vector which contains the bluescript SK- plasmid within its λ arms. Using the λZAP system it is possible to "rescue" the plasmid containing insert. This is accomplished with the help of an f1 bacteriophage. The system permits the screening of a λ phage vector library, which is simpler and more efficient than screening of a plasmid vector library. It also permits the large scale preparation of a pure clone in a plasmid vector, which is more efficient than large scale λ preparation. Also, the need to subclone the purified insert for sequencing is eliminated, since single-stranded template may be rescued or double-stranded sequencing may be performed directly on the plasmid.

Rescue of the plasmid from the λZAP vector was performed by the manufacturer's instructions. For this system, incubation of host cells, phage stock, and helper phage together permits rescue of the bluescript plasmid. F1 proteins supplied by the phage in trans recognize and bind to f1 sites in the vector and regulate DNA synthesis. These sites are present in the λ arms directly flanking the bluescript plasmid (and the insert it contains). One strand of DNA is nicked at the f1 site and the plasmid is duplicated. The new single-stranded DNA is circularized by a gene II product from the f1 phage. Thirty to 50 plasmids are packaged per phage head, and the phage is secreted from the *E. coli* host cells. Heating the sample at 70 °C for 20 min. followed by a brief centrifugation step will precipitate the *E. coli*, leaving a phage stock. After these steps, competent XL1-Blue host cells were transformed with the phagemid and plated out on LB plates which contained 50 μg/ml ampicillin. The system worked easily all three times it was tried.

ASB clone 39, which was isolated from a λgt11 testis library (Clontech), was subcloned into the bluescript SK + vector. In order to produce enough pure insert for the subcloning, a 500 ml phage preparation was done according to the method of Maniatis *et al.* (77). After restriction digestion with *Eco* RI, the DNA was run on a 1% agarose gel. The insert was isolated by the method of Dr. M. Suchi (personal communication). The insert band was cut out from the gel and homogenized by passage through a 1 cc syringe from which the needle had been removed. A polypropylene microfuge tube was filled with about 500 μl of this homogenate, and 700 μl of phenol was added to the tube. The tube was vortexed and frozen at - 70 °C. After thawing, the tube was microfuged for 10 min., and the aqueous phase was saved. The organic phase was reextracted with 100 μl T:E, pH 8.0, frozen, thawed, microfuged, and the aqueous layers were combined. This solution was extracted one time each with phenol, phenol/chloroform, and chloroform. The DNA was then ethanol precipitated. Approximately 70% of the insert was recovered using this method. After subcloning into the bluescript SK+ vector, the plasmid was used to transform competent XL1-Blue host cells. Since the bluescript system includes a tetracycline resistant host cell, the ampicillin resistent plasmid, and the lac Z gene, selection is provided by plating on LB plates with 80 μg/ml X gal, 50 μg/ml amp, 12.5 μg/ml tet, and 100 μl of 100 mM IPTG which is spread over the plates 30 min. before the transformants are plated.

When transformation and plating of competent cells was necessary, this was usually done by the TCM method, which proved to be more efficient than any other

method used. In this method, 44 μl of ice-cold TCM (5 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 40 mM $MgCl_2$) is added to 50 μl competent cells and the ligated DNA. The cells are gently mixed, not vortexed. The mixture is left on ice for 15 min., then at 25 °C for 4 min., on ice for 4 min., and again at 25 °C for 4 min. Five hundred μl LB media is then added to each tube of cells, and these are left at 37 °C for one hour before plating.

D. DNA Sequencing

All DNA sequencing was performed by the dideoxynucleotide method using a Sequenase kit according to the manufacturer's instructions, and (α-^{35}S) dATP obtained from Amersham or New England Nuclear. For single-stranded sequencing, 0.5-1 μg of template was used per reaction, and for double-stranded sequencing, 2 μg or more of template was used per reaction. The amount of primer (17-mer) used was 2.76 ng for the single-stranded reactions and 10 ng for the double-stranded reactions. Double-stranded template was prepared from either purified large scale DNA preparations (77) or rapid boiling mini-preps (148). Denaturation of double-stranded template prior to labelling was performed according to Promega. Single-stranded m13mp18 template was prepared either by the New England Biolabs protocol or by the method of Dale *et al.* (22). Single-stranded bluescript templates were prepared by the manufacturer's instructions.

E. MOPAC Polymerase Chain Reaction

PCR was performed by a variation of the method of Lee *et al.* (71). First strand cDNA was made from 5 μg of Clontech human placental poly A+ mRNA using a Boehringer-Mannheim cDNA synthesis kit, following a modification of the manufacturer's directions and the methods described in Maniatis *et al.* (77). The mRNA was hydrolyzed with 0.1M NaOH at 65 °C for 20 min., then neutralized with an equal volume of 1M Tris, pH 7.5. This mixture was phenol/chloroform extracted, and the organic phase was reextracted with 10 mM Tris-HCl, pH 8, 100 mM NaCl, and 1 mM EDTA. The single-stranded reaction cDNA was then ethanol precipitated. For each PCR reaction, about 25% of this cDNA was used.

For the MOPAC procedure, 4 μM of each primer was used (2 μM A and 2 μM B for sense primer). The reactions were in a 100 μl volume (30 mM Tris-Ac, pH 7.9, 60 mM NaAc, 10 mM MgAc, 10 mM DTT, and 1.5 mM each dNTP). MOPAC was performed for 25 cycles. Each cycle included a 2 min. denaturation step at 100 °C, annealing at 28 °C for 30 sec., addition of 10 U Klenow, and extension for 5 min. at 28 °C. Following PCR, reactions were analyzed by PAGE using 1/2 of each reaction on a 6% benchtop gel. Electroblotting was performed in TAE buffer at 4 °C using a BioRad apparatus. Bands to be subcloned were cut out of the 6% polyacrylamide gels and homogenized with a plastic pestle in 700 μl T:E, pH 8. DNA was eluted from the gel pieces by rotation overnight. The microfuge tubes were then spun 2 min. and the supernatant was removed. Supernatants were run through Boehringer-Mannheim G-25 spun columns to remove residual polyacrylamide, and DNA was precipitated with 1/2

volume ammonium acetate, pH 7.5 and an equal volume of isopropanol.

The MOPAC fragment which was subcloned into the *Eco* RI and *Hind* III sites of m13mp18 had been digested with these enzymes prior to PAGE. Ligation, transformations, and platings were done following the protocol from the New England Biolabs M13 cloning manual, except that IBI ligase buffer was used. Filters were pulled off transformant platings and duplicate filters were hybridized with oligo int A and B or 9.

F. Northern Analysis

Guanidine isothiocyanate preparation of total RNA was performed by the method of Davis *et al.* (23). Fibroblast RNA was isolated from 4 T-75 flasks of normal human fibroblasts. Lymphoblast RNA was the gift of Tom Fitzmaurice. For all RNA solutions DEPC treated dH_2O was used. Formaldehyde gel preparation, sample preparation, and Northern transfer were all done by the method of Maniatis *et al.* (77). Gels were run at 45V for at least 15 hours. Ten to 50 μg total RNA was run on gels, along with the Boehringer Mannheim RNA molecular weight marker I. The random primed ASB clone 39 or hASB 1 was used to probe the Northern blots. Prehybridization, hybridization, and wash conditions were as follows: Prehybridization and hybridization, 5X Denhardt's, 5X SSPE, 0.1% SDS, and 100 μl sheared salmon sperm DNA (10mg/ml stock). Washing was done in 6X SSC, 0.1% SDS twice at room temperature with shaking for 15 min., and then 1 hr. at 65 °C.

G. PCR for Human Polymorphism Analysis

The primers used for this PCR were sense primer 38-3R (5′-TTGCTGAAGCAG AAGGG-3′) and antisense primer 3′U3 (5′-TGGAAGGGAACCAGTAAC-3′). PCRs were run for 30 cycles of 94 °C denaturation for 1 min., 50 °C annealing for 2 min., and 72 °C extension for 1 min. Optimal amplification occurred in booster buffer, which consists of: 67 μl 1M Tris pH 8.8, 16.6 μl 1M $(NH_4)_2SO_4$, 3 μl 1M $MgCl_2$, 0.7 μl 14M β-mercaptoethanol, 40 μl 2.5mM dNTPs, 3.4 μl 50 mg/ml BSA, 10 μl Triton X-100, and 689.3 μl dH_2O. The total volume of 870 μl was aliquoted to 87 μl/PCR tube. For this PCR, 1 μg genomic DNA, 0.5 μl Promega *Taq* Polymerase, 1 μM each primer, and dH_2O to 100 μl were added to each tube. Thirty μl of each PCR reaction was run on a 1.5% agarose gel, and gels were transferred to Biotrace nylon membrane by standard Southern using 0.4N NaOH. Blots were hybridized using either oligo Met (5′-GCTTCGACATGTGGAAA-3′) or Val (5′-GCTTCGACGTGTGGAAA-3′). These oligos were end labelled with (γ-^{32}P)dATP. Prehybridization and hybridization for Met were at 47 °C and for Val at 49 °C, and blots were washed at 49 °C and 51 °C, respectively in 5X SSC, 0.5% SDS.

H. Construction of Full-length Human ASB cDNAs

The two full-length hASB cDNAs were constructed from partial cDNAs #35 and #39. This construction is outlined in Figure 12. To make the first full-length cDNA, hASB 1, cDNA clone 35 was sequentially digested with restriction endonucleases *Sma* I and *Aat* II, and the resultant 345 bp band was gel purified. cDNA clone 39 was

sequentially digested with restriction endonucleases *Stu* I and *Aat* II, and this 1.5 kb band was gel purified. The two cDNA pieces were combined in a ligation reaction to allow joining at the *Aat* II overhang to occur; after two hours *Sma* I digested vector pGEM-7 was added to the ligation. A full-length cDNA could be blunt-end ligated into this vector, as enzymes *Stu* I and *Sma* I are blunt-cutting enzymes.

JM 109 competent cells were transformed with the ligation and, after plating, colonies were mini-prepped and plasmid DNA analyzed for correct size insert by digestion with restriction endonucleases *Eco* RI and *Bam* HI, which flank the *Sma* I cloning site in the pGEM-7 polylinker. After Southern analysis with random-primed cDNA #39 as probe, two inserts of the expected size, 1.8 kb, hybridized strongly with cDNA #39. One of these was grown up in a large scale plasmid prep, and was fully sequenced on both strands. No mutations were present.

In order to subclone this cDNA into the expression vector, p91023(B), it was necessary to add an *Eco* RI linker. The hASB in pGEM-7 was linearized with restriction endonuclease *Bam* HI, which cuts in the pGEM-7 polylinker 3′ of the ASB cDNA. The recessed 3′ end was filled in with Klenow by the method of Sambrook *et al.* (110), with the omission of the G-50 spin. The construct was then phosphatased with Promega calf intestine alkaline phosphatase (CIAP) by the method of the manufacturer. *Eco* RI phosphorylated linkers were purchased from New England Biolabs. These were resuspended in 1 ml dH_2O to give a concentration of .001 OD_{260} unit/μl. Linkers were added in at least 50 molar excess as recommended. After transformation of JM 109 competent cells with the ligation, the plated colonies were

mini-prepped and the plasmid DNA was analyzed for addition of linker by digestion with restriction endonuclease *Eco* RI. This completed construct is referred to as hASB 1.

The full-length hASB 2 construct was generated by digestion of hASB 1 with restriction endonucleases *Sac* II, which cuts at -12 of the cDNA, and *Kpn* I, which cleaves the pGEM-7 polylinker 5′ of the ASB cDNA. Initial attempts at *Sac* II digestion were unsuccessful. Since *Sac* II is a methylation sensitive enzyme, GM 2163 competent cells were transformed with the hASB 1 construct. GM 2163 is a *dam-/dcm-* cell line. Plasmid DNA grown in GM 2163 was sequentially digested with *Sac* II and *Kpn* I, and gel purified. The protruding 3′ termini produced by the *Sac* II cut were removed with T4 DNA polymerase by the method of Sambrook *et al.* (110), leaving 10 bp of 5′ untranslated sequence on the cDNA. One hundred ng of the now blunt product was self-ligated. After transformation and plating, positive clones were mini-prepped and the plasmid DNA was analyzed by agarose gel electrophoresis after digestion with *Eco* RI. A positive clone was chosen, grown up in a large scale plasmid prep, and sequenced totally on both strands. No mutations were found.

I. Transient Expression of Human Full-length ASB cDNAs in COS-1 Cells

Eco RI digested hASB 1 and hASB 2 inserts were gel purified. Expression vector p91023(B) (gift of Dr. R. Kaufman, Genetics Inst., Boston, MA) was digested with *Eco* RI and treated with CIAP. Each insert was ligated into this vector, and after transformation and plating, alkaline lysis mini-preps were performed on 10-20 colonies.

Plasmid DNA was sequentially digested with *Bgl* II and *Pst* I to determine the insert orientation in the vector.

Sense constructs were grown up in large scale boiling preps and plasmid DNA was purified as recommended by Sambrook *et al.* (110). All expression studies were done in COS-1 cells (ATCC, Rockville, MD), which were grown in DMEM supplemented with 1% glutamine, 1% penicillin/streptomycin mix, and 10% fetal bovine serum. COS cells were transfected by electroporation using the BioRad gene pulser apparatus by a modification of the manufacturer's instructions. The electroporation buffer was phosphate buffered sucrose (PBSu; 272 mM sucrose, 7 mM $NaPO_4$ pH 7.4, 1 mM $MgCl_2$). Twenty μg of DNA was routinely used for each electroporation. Cells were harvested by trypsinization and pooled, and the cell pellet was washed in 10 ml PBSu and then resuspended in 0.8 ml PBSu/electroporation. To each electroporation cuvette 0.8 ml of cells were added. Experimental DNA, which had been ethanol precipitated, was resuspended in 50 μl PBSu and was added to the cells in the cuvette. Fifty μl of PBSu alone was added to cells in one cuvette as a negative control. Cuvettes were left on ice 5-10 min., and then pulsed in the gene pulser at 350V, 25 μF capacitance. Cuvettes were then left on ice for 10 min., after which cells were gently added to 10 ml media in 100 mm tissue culture dishes. The cells were incubated for 3 days at 37 °C prior to harvesting. For expression studies with colchicine, 100 μl Colcemid (10 μg/ml, Gibco) was added to each 30% confluent 75 ml flask of COS-1 cells. Twenty-four hours after the addition of Colcemid, cells were harvested as usual. An additional PBSu wash was done. For the initial

expression studies on hASB 1, an expression construct of human α-galactosidase A in p91023(B) (gift of Dr. Yiannis Ioannou) was used as a positive control. Cell pellets were resuspended in 0.5 ml-1 ml of saline and lysed by sonication for 4 X 15 sec. Debris was removed by centrifugation at 4 °C for 5 min. at 10K. Protein was determined by fluorescamine (12), and α-galactosidase A activity was assayed with a 4 MU substrate as described in (27). ASB assays were performed by a variation of the method of Roy *et al.* (108) using 200 μl of substrate and up to 200 μl of sample. The ASB substrate is 50 mM NCS in 0.5 M NaAc, and 10 mM BaAc, adjusted to pH 6.0. Reactions were incubated at 37 °C for 1 hour, and stopped by the addition of 2N NaOH to 1 ml. The OD_{515} was read immediately on the samples. ASB activity units are nmoles of NCS hydrolyzed/hour/ml sample.

For expression studies of hASB 2, hASB 1 was the positive control. Cell pellets were resuspended in 0.5-1 ml of 10 mM Tris-HCl, pH 7.5, and protein was determined by standard Bradford assay using the BioRad reagent. As a control for these experiments, ASA levels were determined as well. The ASA substrate is 10 mM NCS in 0.5 M NaAc, 0.5 mM $Na_4P_2O_7$, and 1.7 M NaCl, adjusted to pH 5.2. The samples were incubated and read as for ASB.

J. Retroviral Constructs

hASB 1 and hASB 2 cDNAs were subcloned into the DCTK retroviral vector (gift of Dr. Eli Gilboa, Sloan Kettering, NY). This vector was linearized by digestion with restriction enzyme *Sna* BI, a blunt-end cutting enzyme, and treated with CIAP.

hASB 1 and hASB 2 cDNAs were excised from pGEM-7 plasmids by *Eco* RI digestion, the overhang was filled in with Klenow, and the cDNAs were gel purified. The insert orientation in this vector was determined by digestion with *Bam* HI. Constructs were also digested with *Eco* RI and analyzed by Southern hybridization with random primed hASB 1 insert.

The hASB 2 cDNA was subcloned into the pBC140 vector (gift of Dr. Eli Gilboa). This vector was linearized by digestion with *Xho* I, the overhang was filled in with Klenow, and treated with CIAP. The hASB 2 cDNA was prepared as described above. The insert orientation in the pBC140 vector was determined by digestion with *Bam* HI.

Retroviral sense constructs were grown up by large scale boiling plasmid preps, and were purified over a cesium chloride gradient by the method of Sambrook *et al.* (110).

Results and Discussion

A. Purification and Microsequencing of Human ASB

ASB was purified 42,000 fold from human liver by a variation of the method of McGovern *et al.* (81). Two separate preparations yielded ASB with a specific activity of about 1.7 X 10^6 U/mg protein and 1.8 X 10^6 U/mg protein, respectively. For both preparations, SDS-PAGE revealed a single band of about 50 kDa. Preparation 1 was subjected to tryptic digestion and the peptides were separated by reverse-phase

HPLC. Five peptides (69, 84, 105, 116, and 124) were then chosen for microsequencing (Figure 1). A total of 112 amino acids residues were determined from these peptides (Figure 2). Purified ASB from preparation 2 was subjected to N-terminal microsequencing. The N-terminal sequence was identical to that of peptide 116, indicating that this was the N-terminal tryptic peptide.

Figure 1 HPLC profile of tryptic peptides from human liver ASB (tryptic digestion and HPLC were performed by Dr. Ken Williams, Yale University School of Medicine). The inset is an SDS PAGE of purified ASB. Lane 1, Molecular mass standards; lane 2, 10 μg ASB; lane 3, 20 μg ASB.

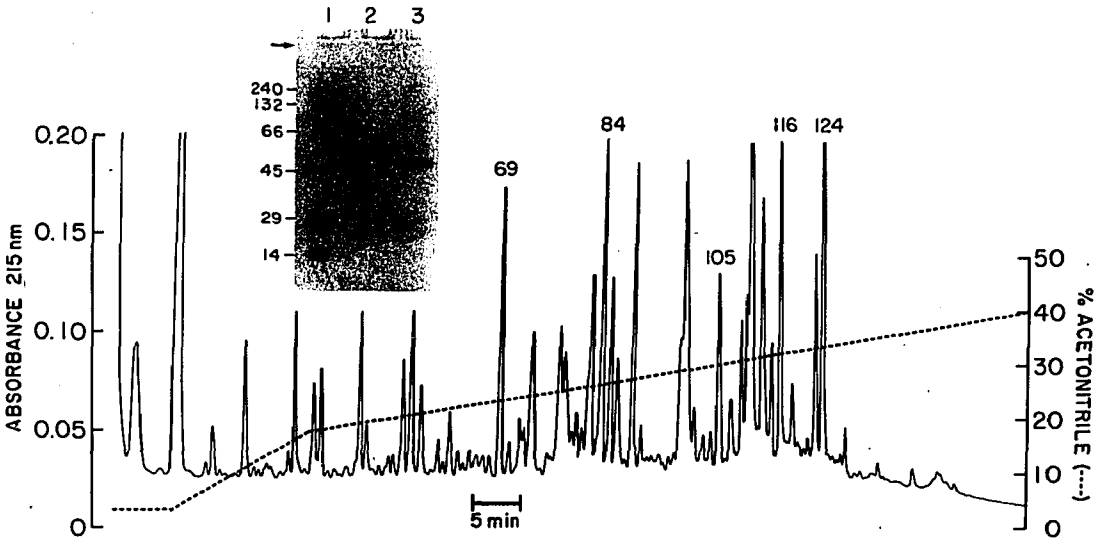

1 2 3
240
132
66
45
29
14
0.20
0.15
0.10
0.05
0
ABSORBANCE 215 nm
69
84
105
116
124
5 min
50
40
30
20
10
0
% ACETONITRILE (----)

Figure 2 Amino acid sequence of tryptic digest peptides from human liver ASB. The N-terminal peptide was independently sequenced twice.

Amino Acid Sequences From Human Liver ASB

Peptide	Sequence
N-Terminal:	
T-116	A-S-R-P-P-H-L-V-F-L-L-A-D-D-L-G-W-N-D-V-G-F-H-G-S- (25)
Internal:	
T-69	-H-S-V-P-V-Y-F-P-A-Q-D-P-R- (13)
T-84	-T-G-L-Q-H-Q-I-I-W-P-C-Q-P-S-C-V-P-L-D-E-K- (21)
T-105	-T-L-W-L-F-D-I-D-R-D-P-E-E-R- (14)
T-124*	-A-I-(A)-L-I-T-N-H-P-P-E-K-P-(L)-F-L-Y-(L)-A-L-Q-S-V-(H)-E-P-L-Q-V-P-E-E-Y-L-K-P-Y-(D)-F- (39)

*Residues in parentheses were uncertain and represent most likely identification

B. Initial cDNA Library Screening

The initial attempts at isolating a hASB cDNA are summarized in Figure 3, and the oligonucleotide probes used for these screenings are depicted in Figure 4. First, a cDNA expression library (Woo λgt11) was screened with two polyclonal antibodies and one monoclonal antibody (gift of Dr. E. Shapira, Tulane U.). These screenings were unsuccessful, since none of the clones positive with two or more antibodies hybridized with oligos 1, 2, 3, or 4 when analyzed by Southern hybridization. As can be seen in Figure 3, screenings using synthetic oligonucleotide mixtures were then exhaustively performed on cDNA libraries from various tissue sources. The earliest screenings were performed using small (17 mer), degenerative probes derived from the amino acid sequence of purified ASB. Later screenings favored the use of duplicate filters and mixtures of two or more longer probes. The final screenings employed long N-terminal probes on duplicate filters. Since the N-terminal peptide had been microsequenced twice from different enzyme preparations, this sequence was considered the most reliable. Therefore, two long (62 or 66-mer) probes were constructed. Oligo 9 was based upon a mammalian codon usage chart as well as the N-terminal peptide sequence. Oligo 10 used the degenerative nucleotide sequence for ASB, but incorporated the nucleotide inosine at selected positions of redundancy to reduce the probe complexity.

Again, these numerous screenings were unsuccessful since none of the N-terminal positive clones hybridized to internal probe mixtures. Thus, despite exhaustive screenings and the isolation of numerous putative positive clones, an

authentic ASB cDNA was not isolated by these conventional methods.

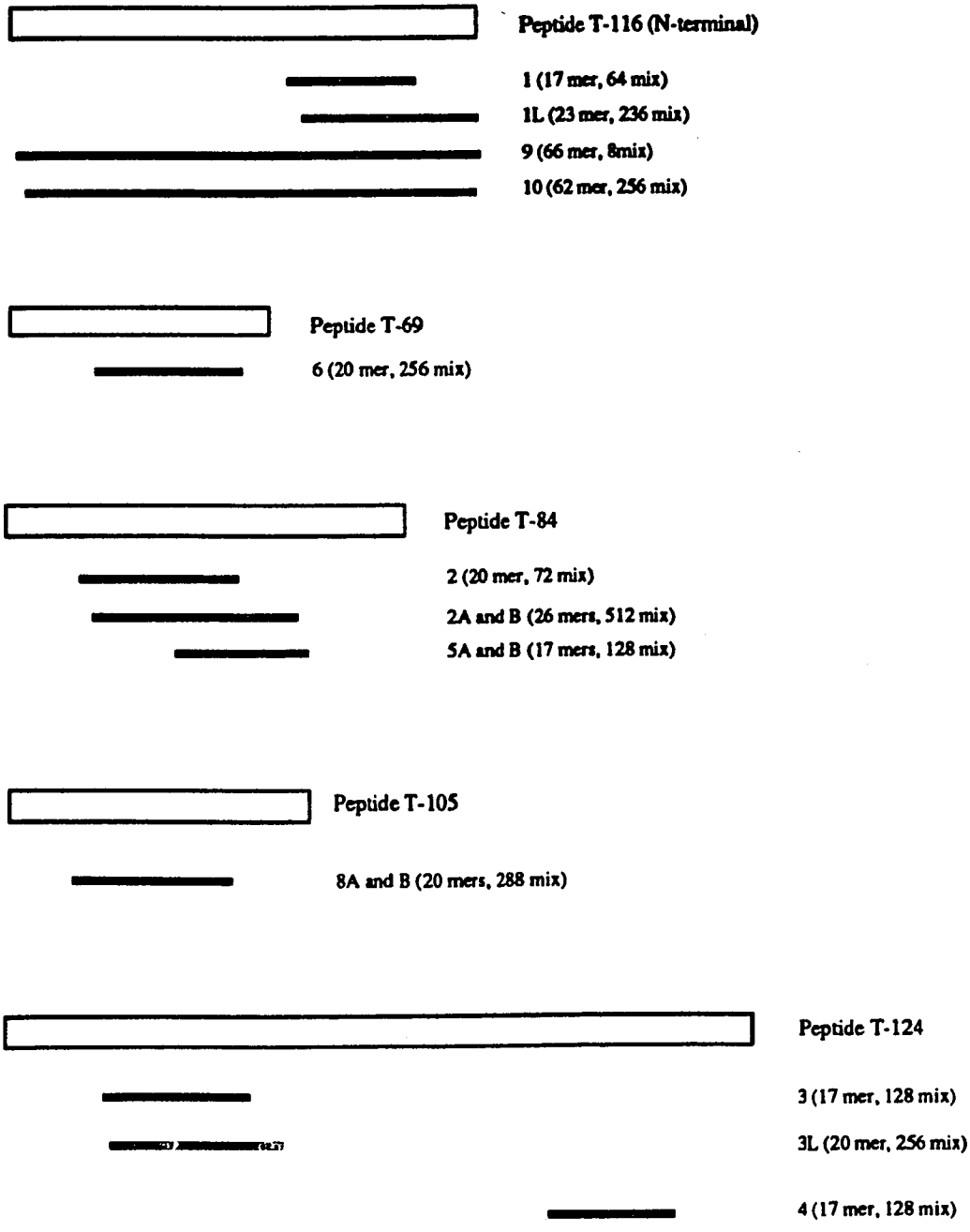
Figure 3 Summary of the initial ASB cDNA library screenings.

Summary of ASB cDNA Library Screenings

Library	Tissue Source	Probe
Woo λgt11	liver	polyclonal antibody I
Woo λgt11	liver	polyclonal antibody II
Woo λgt11	liver	monoclonal antibody
Woo λgt11	liver	oligo 2
Orkin	fetal liver	oligos 1 and 3
Orkin	fetal liver	oligo 2
Orkin	fetal liver	oligo 4
PCD 9	fibroblast	oligos 1L, 2A and B, 3L
PCD 9	fibroblast	oligos 2A and B, 3L, 4, 5A and B, 6, 8A and B, 9
PCD 4	fibroblast	oligos 1L, 9, 10
PCD 4	fibroblast	oligos 2, 3, 5A and B, 6, 8A and B
Clontech λgt11	testis	oligos 1L, 9 10
Clontech λgt11	placenta	oligos 1L, 9, 10

Figure 4 ASB synthetic oligonucleotide probes. The probes are shown beneath the peptide of origin.

ASB Synthetic Oligonucleotides



Oligonucleotide mixtures labelled A and B were synthesized in two parts to reduce primer degeneracy.

C. Generation of a Partial cDNA Encoding Human ASB by MOPAC

Due to the lack of success of the cDNA library screenings, efforts were redirected to use of the mixed oligonucleotide-primed amplification of cDNA (MOPAC) technique to amplify a portion of the hASB cDNA. Initial attempts were made to amplify a piece of the hASB cDNA using a degenerative sense primer from the N-terminal peptide and one of four antisense primers from each of the internal ASB tryptic peptides. Two different templates were used, either DNA from a large scale prep of the total PCD fibroblast library, or first-strand cDNA generated from poly A+ liver mRNA. PCR was performed according to the method of Lee (see Experimental Procedures) with the Klenow fragment of DNA Polymerase I, or by following the Perkin Elmer Cetus protocol using *Taq* polymerase. These attempts at amplification of the hASB cDNA were unsuccessful. It is possible that the PCD library did not contain any hASB clones long enough for the sense primer to hybridize to. Also, it is difficult to optimize conditions for PCR when the size of the fragment to be amplified is unknown. With PCR, it may be necessary to perform many different trials with varying conditions in order to optimize amplification.

After these initial trials at amplification of the hASB cDNA, it was decided to attempt amplification of a small piece of the cDNA using primers within the N-terminal peptide of hASB. Because of the large number of leucine residues in this peptide, high probe degeneracy was a concern. Therefore, inosine residues were incorporated in selected positions of redundancy to reduce this degeneracy. The 25 nucleotide primers made for this experiment can be seen in Figure 5a. The sense primer was synthesized

Figure 5 **A.** A portion of the N-terminal peptide of ASB and the sense and antisense primers built for MOPAC. **B.** The internal primer used to hybridize an electroblot of the gel. The probe hybridized very strongly to the band at 78 bp, but not to the band at 55 bp. **C.** Polyacrylamide gel analysis of the MOPAC products. Lane 1, molecular weight markers; lane 2, MOPAC products. A major band is visible at 55 bp, and a minor band of the expected size, 78 bp. Lane 3, an electroblot of this gel, hybridized with primer Int A and B.

A. ASB MOPAC Primers

Oligonucleotide 1 →

HINDIII	P	P	H	L	V	F	L	L	A	D	D	L	G	W	N	D	V	G	F	H	G	*ECORI*
CAAGCTTG	CCI	CCI	CAC	CTI	GTI	TT										CTA	CAI	CCI	AAG	GTG	CC	CCTTAAGG
			T	T												G			A	A		

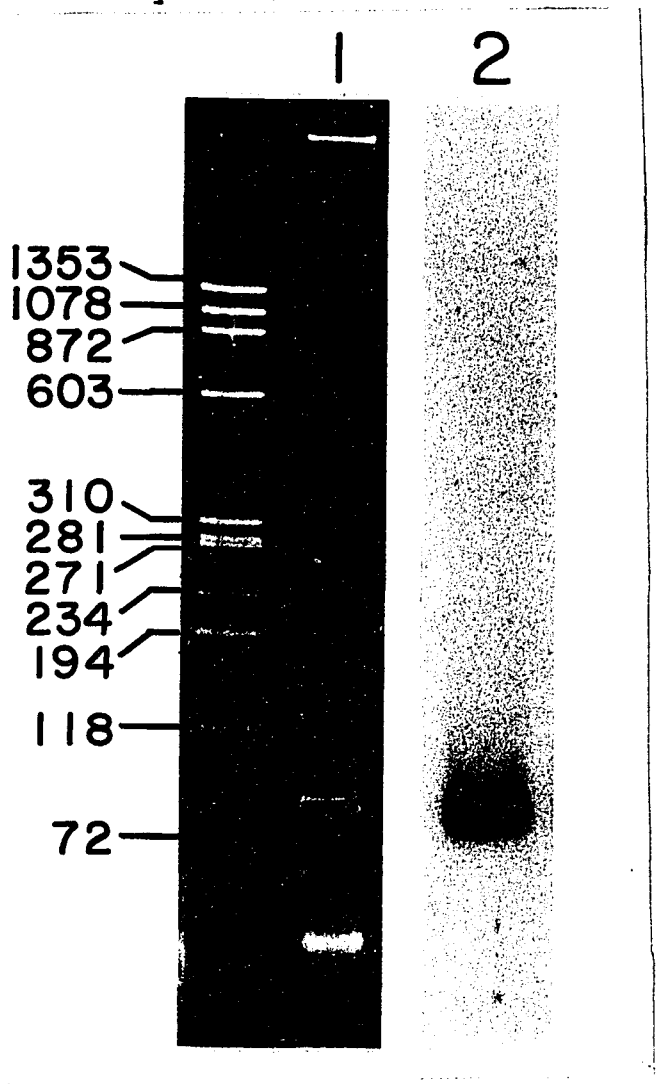
← Oligonucleotide 2

B. ASB Internal N-terminal Primer

A	D	D	L	G	W	N
GCI	GAT	GAT	CTI	GGI	TGG	AA
	C	C				
GCI	GAT	GAT	TTA	GGI	TGG	AA
	C	C	G			

Int A and B, 20 mer; 12 mix

C. Polyacrylamide Gel Analysis of MOPAC Products



in two parts to reduce degeneracy. Sense primers A and B (oligonucleotide 1) are a 2 mix and a 4 mix respectively, and both have a 5′ *Hind* III restriction site within two additional nucleotides. The antisense primer (oligonucleotide 2) is an 8 mix and includes a 5′ *Eco* RI restriction site within two additional nucleotides. The template for these reactions was single stranded cDNA produced from reverse transcription of poly A+ placental mRNA. One-half of each duplicate reaction was subjected to PAGE analysis. Two bands were obvious on the gel: a minor band of 78 bp (the expected size) and a major band of 55 bp (Figure 5c). Bands of 55 bp were often seen on other PCR gels, so this may be an artifact particular to these primers. The gel was electroblotted and after hybridization with an inosine-containing N-terminal internal probe (int A and B, Figure 5b), the minor 78 bp band was strongly positive (Figure 5c). This blot was stripped and reprobed with N-terminal oligos 9, 10, and 1L, all of which hybridized strongly with the 78 bp band.

The remaining one-half of the reactions were digested with *Hind* III and *Eco* RI, isolated from a polyacrylamide gel, and subcloned into m13mp18. Transformants were plated on agar plates and filters were screened using the N-terminal oligos int A and B, and 9. Positive plaques were purified and sequenced. Of 16 putative positive m13 clones, three were of particular interest. One, which had an insert which hybridized with the specific N-terminal ASB probe, was colinear with the N-terminal peptide of ASB, while the other two, which had been isolated with the codon usage probe oligo 9, encoded a sequence of 77 bp which was very similar to but distinct from the ASB clone (Figure 6). Presumably the longer probe allowed mismatches to occur.

In summary, the MOPAC procedure successfully amplified a 78 bp sequence from the N-terminal region of ASB and facilitated the synthesis of oligonucleotide probes which were used for the isolation of cDNAs encoding ASB.

Figure 6 Colinearity of the ASB MOPAC product with the N-terminal peptide of human ASB. Starred bases are those for which the sequence differs between this PCR product and the ASB cDNA. Two other ASB-related clones, also amplified by MOPAC, are shown for comparison.

Colinearity of the ASB MOPAC Product

P P H L V F L L A D D L G W N D V G F H G	
CCC CCG CAC CTG GTG TTC CTN CTG GCT GAC GAC CTG GG$^{T}_{C}$TGG AAC GAC GTG GGC TTC CAC GG	ASB MOPAC Clone
CCG CCG CAT CTG GTG TTC CTN CTG GCG GAT GAC CTG GGG GG AAC GAT GTC GGC TTT CAC GG	ASB-Related MOPAC Clone 1
CCG CCG CAT CTG GTG TTC CTN CTG GCG GAT GAC CTG GGG GG AAC GAT GTC GGC TTC CAC GG	ASB-Related MOPAC Clone 2

D. Isolation of cDNAs Encoding Human ASB

Two probes, a 17-mer (ASB-1) and a 35-mer (ASB-2), were synthesized based on the N-terminal ASB MOPAC sequence (Figure 7). These probes were used to screen a λgt11 placental library. Five clones were obtained which weakly hybridized with ASB-1, ASB-2, and oligos 3L, 6, and 8A and B, but not with oligos 2A and B. After subcloning the longest and the shortest of these clones into puc19 and m13mp18, respectively, sequencing was initiated. These clones, and a third one later sequenced, proved to encode portions of the B1 chain of laminin. No obvious colinearity between the probes and B1 laminin sequence was seen, although only one of the clones was sequenced entirely. The repeat of Southern analyses with all six of the above probes gave ambiguous results, so these clones were not pursued any further. Because of these results, a longer probe was built for additional cDNA library screenings. Again, the core of this sequence was based upon the sequence which had been generated by MOPAC, but additional flanking sequence was added based upon the N-terminal sequences for ASA and ASC (see below), and on the mammalian codon usage table (70). This 66-mer (ASB-3) is also shown in Figure 7.

When this probe was used to screen a hepatoma cell library, four putative positives were obtained. Three of these clones (#22, #35, and #38) strongly hybridized to probes ASB-3, 2A and B, 3L, and 6 on Southern blots. Therefore, sequencing was begun on single-stranded DNA rescued from the smallest clone, #35. Colinearity with ASB peptide 84 was seen immediately and double and single-stranded sequencing of clone 35 was continued. Although this clone contained 559 bp of 5′untranslated

Figure 7 N-terminal probes used in the isolation of ASB cDNAs. The corresponding portion of the ASB N-terminal peptide is shown at the top of the figure. Probes ASB 1 and ASB 2 were derived from the MOPAC product, and probe ASB 3 was derived from the MOPAC product, from the table of mammalian codon usage according to Lathe (70), and from the nucleotide sequences of arylsulfatases A and C.

ASB N-terminal Probes

A S R P P H L V F L L A D D L G W N D V G F H G S

GCT GAC GAC CTG GG$^{T}_{C}$ TG **ASB-1 (17 mer, 2 mix)**

TTC CTN CTG GCT GAC GAC CTG GG$^{T}_{C}$ TGG AAC GAC GT **ASB-2 (35 mer, 8 mix)**

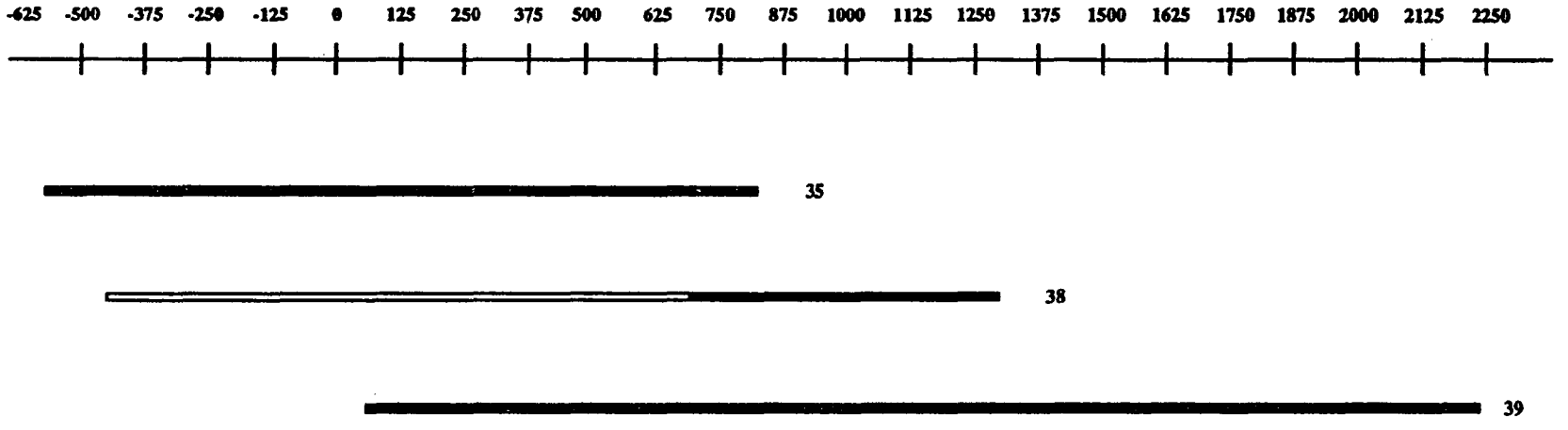
CGT CCG CCC CAC CTG GTG TTC CTG CTG GCT GAC GAC CTG GGC TGG AAC GAC GTG GGC TTC CAC GGG **ASB-3 (66 mer)**

sequence and 810 bp of coding sequence, it lacked some 3′ coding sequence. This was demonstrated by the fact that it did not contain sequence encoding two of the five microsequenced peptides or a 3′ untranslated sequence. The next clone sequenced, #38, provided 465 bp of additional 3′ sequence, but the nucleotides encoding the two microsequenced peptides were still not present. Clone 22 contained about 200 bp from the middle portion of ASB, but the 5′ sequence of this clone differed totally from clones 35 and 38. Therefore, it was set aside. It is rather unusual to obtain clones missing so much of the 3′ end from an oligo dT-primed library. It is possible that the reverse transcriptase used to generate both strands of the cDNA from the oligo dT-primed poly A+ RNA template failed to complete the second strand of the cDNA. Subsequent use of S1 nuclease to degrade the 5′ hairpin loop would have then permitted digestion of single stranded 3′ end sequences, thus generating 3′ truncated clones.

Clone 35, which had been totally sequenced, was then used as the probe to search for a more complete cDNA clone. Rescreening of the λgt11 placental library failed to produce any positive clones. However, screening of a λgt11 testis library yielded two putative positives, one of which hybridized with probes 1L, 6, and 8A and B as well as #35 on Southern blots. This clone, 39, was subcloned and sequenced by single and double-stranded methods. This ASB clone contained a 2,203 bp insert which encoded all 112 microsequenced amino acids, as well as 644 bases of 3′ untranslated sequence and a polyadenylation signal. The relationship of clones 35, 38, and 39 is shown schematically in Figure 8. The 2,802 bp full length sequence of ASB, compiled from clones 35, 38, and 39, is shown in figure 9. ASB has a 5′ untranslated

Figure 8 A schematic of the human ASB cDNAs and their region of overlap. cDNAs #35 and #38 are from a hepatoma library, and cDNA #39 from a testis library. Clone 39 is missing 34 bp of 5′ coding sequence including the initiating methionine codon.

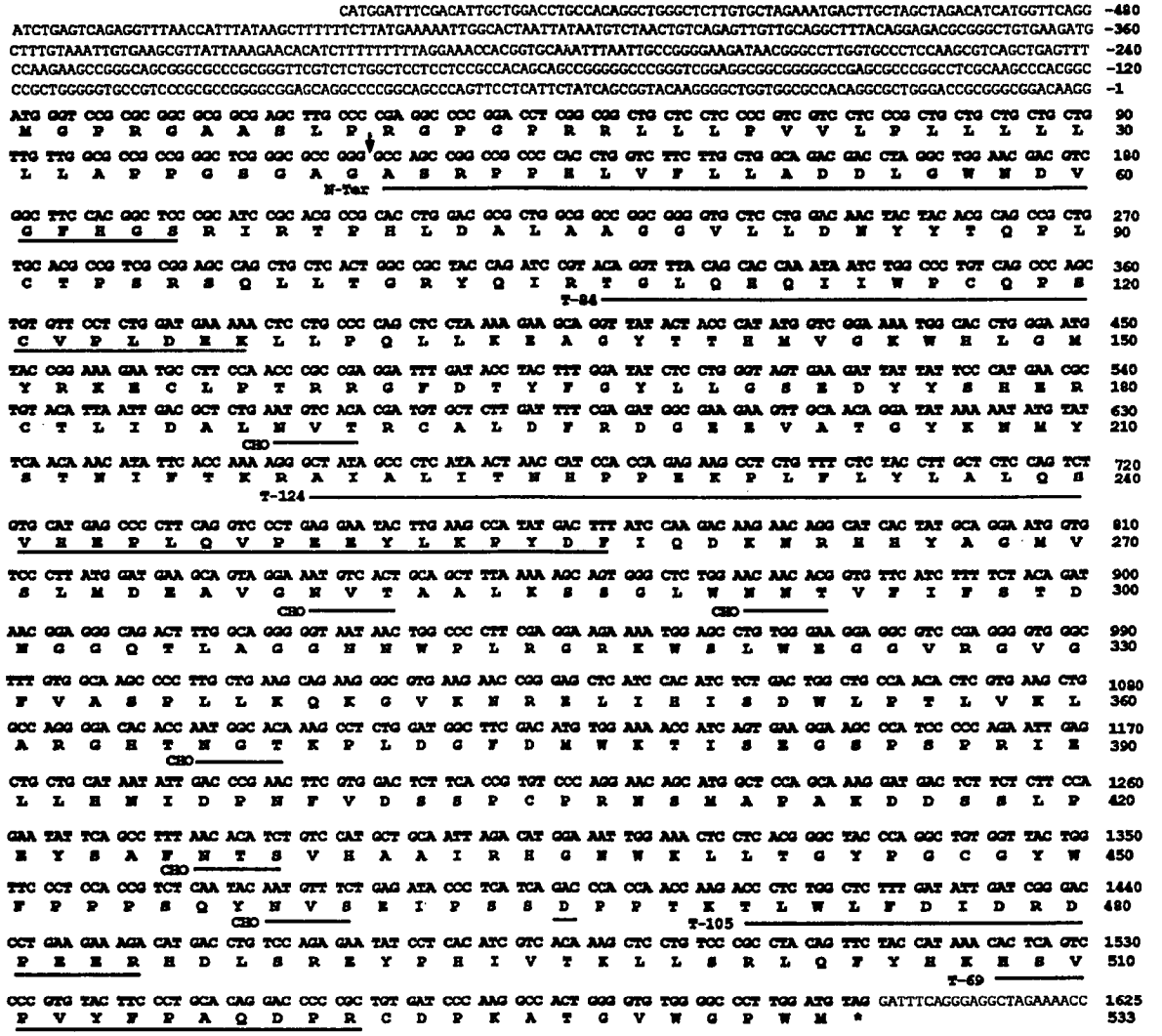
Schematic of hASB cDNA Clones



Area shown in white has not been sequenced

Figure 9 The full-length cDNA sequence of human ASB. The microsequenced polypeptides are underlined, as are the six potential *N*-glycosylation sites and the N-terminal amino acid of the smaller ASB polypeptide. The signal cleavage site (N-terminal amino acid by microsequencing) is indicated by an arrow.

NUCLEOTIDE SEQUENCE OF hASB



region of 559 bp, a coding sequence of 1,599 bp coding for 533 amino acids, a 3′ untranslated region of 644 bp including a polyadenylation signal, and six potential *N*-glycosylation sites. The von Heijne weight matrix method (147) was used to predict the signal peptidase cleavage site for ASB. Although the amino acid microsequencing had identified ^{41}Ala as the N-terminal amino acid, the weight matrix method predicted that the signal peptidase cleavage site was ^{39}Ala (score 14.1) or ^{40}Gly (score 13.4). This indicated either that the protein is N-terminally processed, or that the protein sent for microsequencing was slightly degraded. However, the latter possibility is unlikely since the N-terminal peptide has been microsequenced twice, from two different liver preparations, and the same N-terminal amino acid was predicted both times. Litjens *et al.* have directly sequenced the amino terminus of the ASB 43 kDa subunit, and report that the N-terminal amino acid is ^{40}Gly. Their results also indicate that ASB has a staggered N-terminus, and they suggest that limited proteolysis produces the mature ASB, which begins at ^{41}Ala (75).

Notably, two differences were found in the overlapping region of hepatoma clone 38 and testis clone 39. One is a silent mutation at position 1191 (A to G), and the other, at position 1126 (G to A), codes for a methionine (hepatoma) to valine (testis) base change. The N-terminal amino acid of the smaller ASB polypeptide (D 466), as reported by Wilson *et al.* (156), is underlined.

After the sequencing of these ASB cDNA clones had been completed, an interesting observation was made. Oligonucleotide ASB-3, the 66-mer which was used for the screening of the hepatoma library, differed from ASB clone 35 at 3 of the 28

bases which had been amplified between the MOPAC PCR primers (see starred bases, Figure 6). The amino acid sequence was conserved despite these base changes. While this may be due to polymerase error or polymorphisms, it is also possible that the ASB specific primer was not built from ASB sequence at all, but from the sequence of a closely related gene. As is discussed in the following section, notable N-terminal homology exists between Arylsulfatases A, B, and C.

Peters *et al.* have also cloned the cDNA for ASB. Their full-length construct has 6 bp of 5′ untranslated sequence and 622 bp of 3′ untranslated sequence. The reported coding region for ASB is identical to that of the cDNA reported in this thesis. Position 1126 in their cDNA is a "G", which will code for valine and not methionine in the ASB protein, and position 1191 (the silent change) is an "A", similar to ASB cDNA #38 (hepatoma) (99).

Further molecular characterization of ASB has been recently accomplished. Litjens *et al.* have isolated and characterized a partial genomic clone for ASB. A 1.35 kb *Hind* III fragment from two overlapping genomic clones was sequenced. This clone included "exon 1" of ASB, which consisted of 450 bp of 5′ untranslated sequence and 312 bp coding for the first 104 amino acids of ASB. The remainder of the sequence was intronic. Restriction mapping of the genomic clones indicated that the first intron was at least 14 kb in length (75). Peters *et al.* have also isolated a partial 5′ genomic ASB clone (99), and our laboratory has isolated a partial 3′ genomic clone.

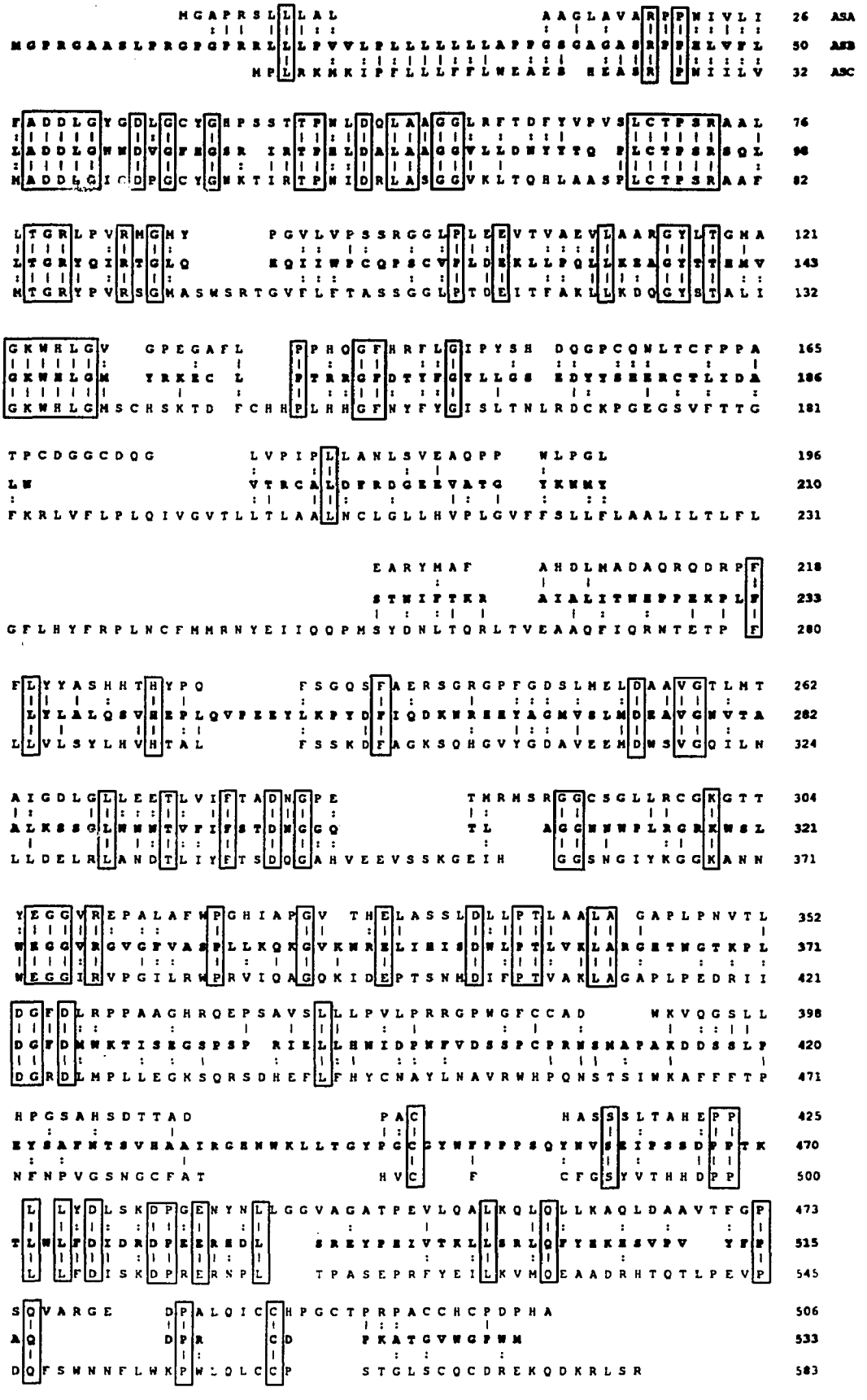
Four point mutations causing MPS VI have also been identified. Jin *et al.* in this laboratory have identified three point mutations. A patient with severe MPS VI

was found to be homoallelic for a T to C transition at nucleotide 349, which causes a cysteine to arginine change at amino acid 117. A second patient with a mild MPS VI phenotype was heteroallelic, with a T to C transition at nucleotide 707 causing a leucine to proline change at amino acid 236, and a G to A transition at nucleotide 1214 producing a cysteine to tyrosine change at amino acid 405. These mutations were not found in three other MPS VI patients or in 120 alleles from normal individuals (59). Wicker *et al.* have reported a point mutation causative for the intermediate type of MPS VI. This G to T transversion at nucleotide 410 of the ASB cDNA causes a substitution of valine for glycine at amino acid 137. The patient, a product of a consanguineous mating, was homoallelic for this mutation (154).

E. Amino Acid Homology Between the Sulfatases

The amino acid similarities between Arylsulfatases A, B, and C are shown in Figure 10. Using the "Bestfit" program (University of Wisconsin Genetics Computer Group Software) (28), significant N-terminal homology is seen between these sequences. Over the 110 amino-acid region between amino acid residues 40 and 150 (with respect to ASB), there is 47% amino acid identity between ASA and ASB, and 45% identity between ASC and ASB. On the nucleotide level in this same area, there is 65% identity between Arylsulfatases A and B and 52% identity between Arylsulfatases B and C. Within this region, there are three conserved amino acid blocks, one of five amino acid residues, ADDLG, and two of six amino acid residues, LCTPSR and GKWHLG. Similarities in these areas are also seen in sea urchin

Figure 10 Amino acid comparison of arylsulfatases A, B, and C. The ASB sequence is in bold type. Residues conserved in all three proteins are boxed. A solid line indicates identity, and a broken line a conservative amino acid change as determined by the University of Wisconsin GCG program "Bestfit" (28).



arylsulfatase, although they are less marked (112).

ASA is a soluble lysosomal enzyme responsible for hydrolysis of sulfate groups from cerebroside sulfate. cDNA and genomic clones encoding ASA have been isolated by Stein *et al.* and Kreysing *et al.*, respectively (128,64). The ASA genomic clone is 3.2 kb and includes eight exons. The ASA gene has been mapped to chromosome 22. Deficiency of ASA is the cause of the neurodegenerative disorder, Metachromatic Leukodystrophy (MLD) (128). ASC, or steroid sulfatase, is a microsomal enzyme. It maps to the X chromosome, and the cDNA for ASC has been cloned by three laboratories (4,129,158). The ASC gene has only been partially sequenced, is estimated to be very large (146 kb), and includes 10 exons (119). Deficiency of ASC is the cause of X-linked icthyosis (110,1,89).

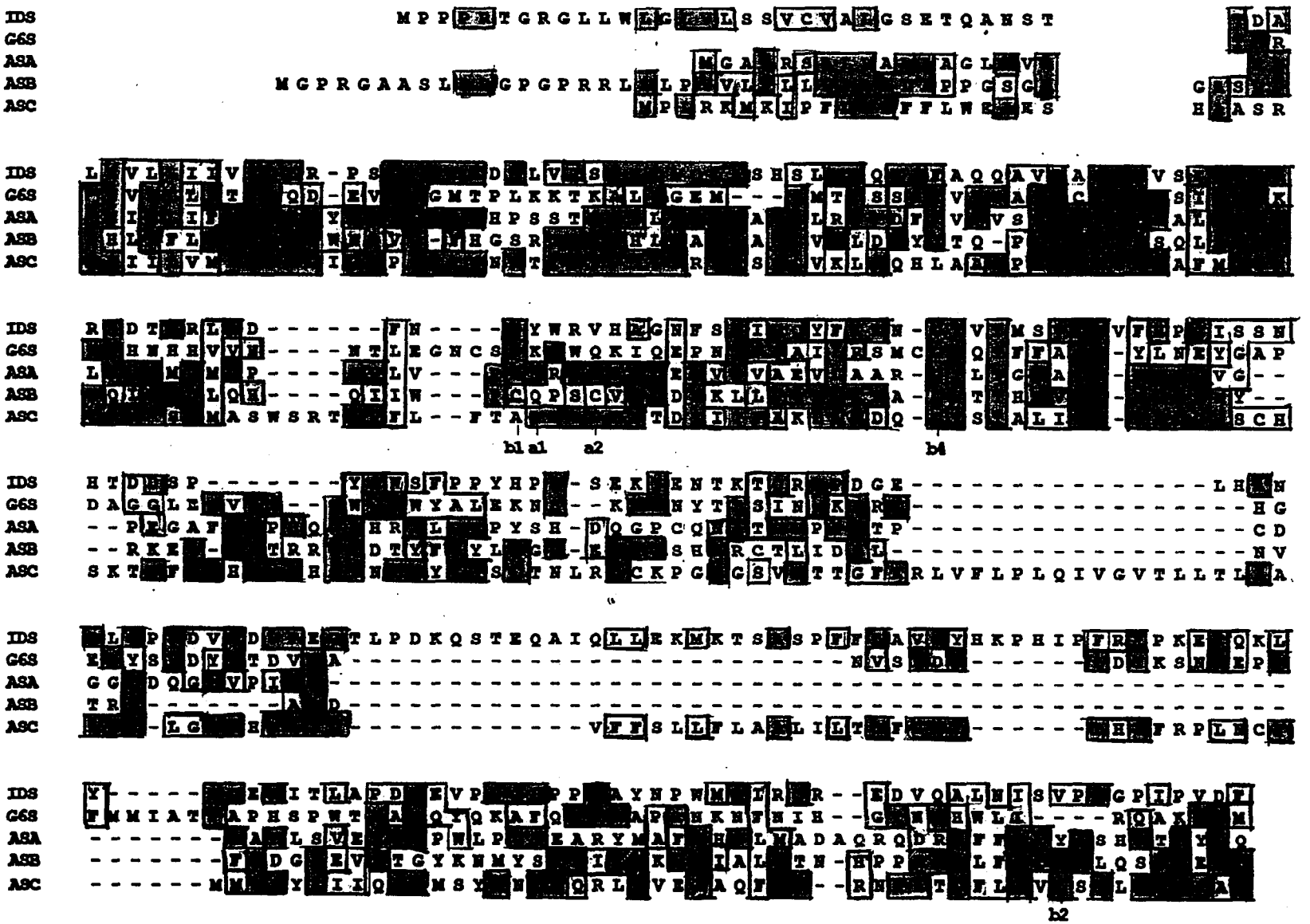
The amino acid residues conserved in the three enzymes may be related to their catalytic function as sulfatases. Alternatively, these sites might be related to the common deficiency of these three enzymes, and of at least four other sulfatases, in the severe neurodegenerative disorder known as Multiple Sulfatase Deficiency (MSD). The cDNAs for two of these other sulfatases, iduronate 2-sulfatase (IDS) and glucosamine 6-sulfatase (G6S), have also been isolated (156,106), while those for *N*-acetylglucosamine 4-sulfatase and heparan *N*-sulfaminidase have not yet been cloned. Additionally, a 1.2 megabase YAC contig which spans the entire IDS gene has been isolated (95). MSD patients have clinical features of both Metachromatic Leukodystrophy and MPS disorders. MSD patients can be classified into two groups- Group I, with all sulfatase activities $<10\%$ of normal, and Group II, which may have

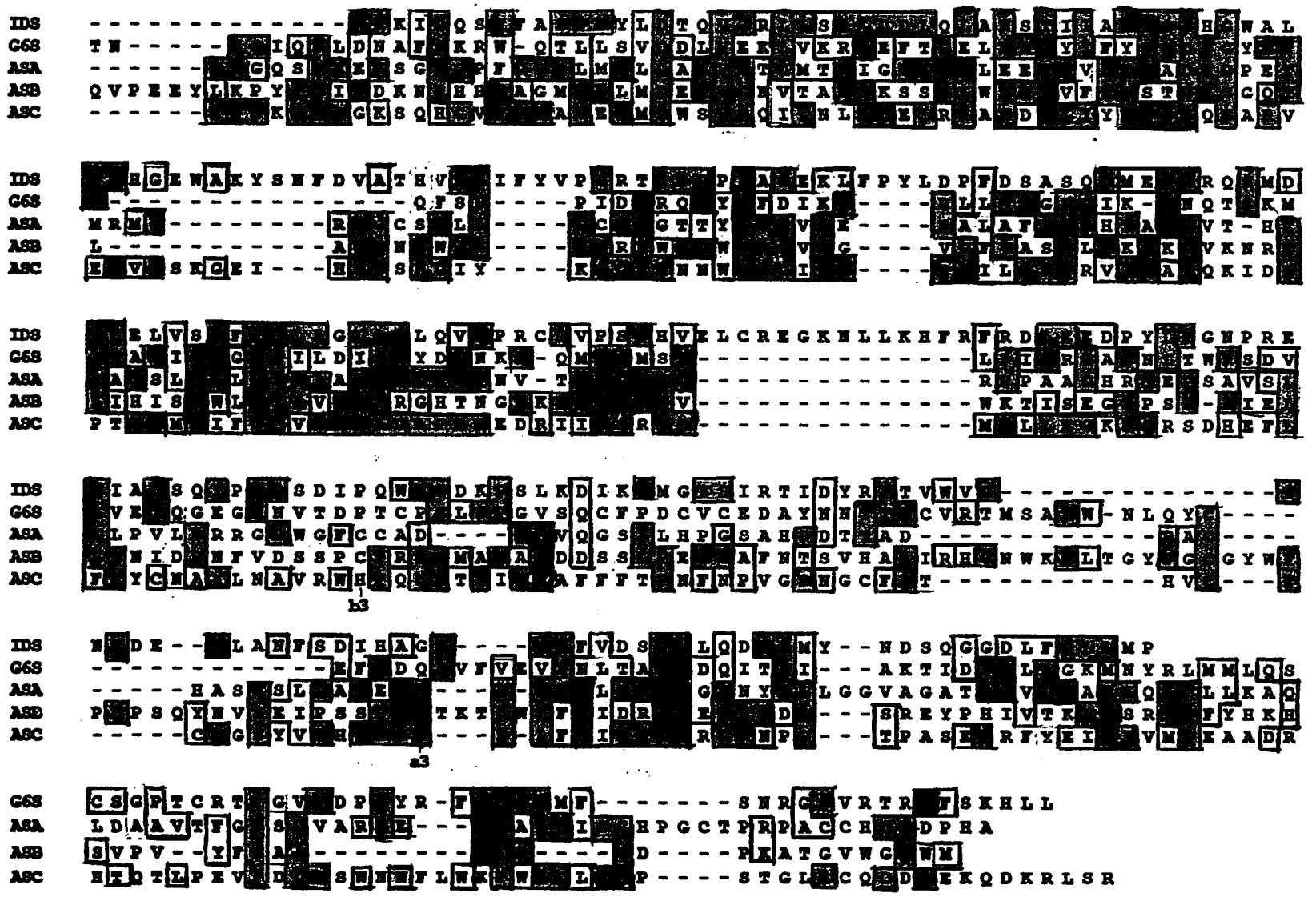
sulfatase activities up to 90% of normal. Group I patients have a more severe, neonatal form of MSD (61).

Studies of the synthesis and maturation of ASA, ASB, and ASC in Group I and II MSD cell lines have shown that the rate of synthesis and the catalytic activity of these sulfatases may be affected in MSD. Often, the half-lives of these enzymes are decreased (135,55,126,20) Chang *et al.* have performed cross correction studies with MLD and MSD cell lines. These studies were the first to show that MLD and MSD are clearly separate disorders, and that a sulfatase inhibitor is not responsible for the MSD phenotype (18). There are two currently held theories on the nature of the "MSD factor". The first is that there is a stabilizing factor common to all of the sulfatases, and the second is that a specific posttranslational modification is required for their activity. The modifying enzyme would likely be localized in the ER, since ASC is a microsomal enzyme. Studies performed by Steckel *et al.* seem to favor the second hypothesis. In these experiments, metabolically labelled ASA and ASB secreted by MSD or normal fibroblasts was added to normal and MSD fibroblast cell lines in culture. The MSD fibroblast-synthesized enzyme was far less stable than the normal enzyme after uptake by either type of cell. Stability of three other lysosomal enzymes (non-sulfatases) from either source was similar. This indicates that ASA and ASB synthesized by MSD cell lines is abnormal, and that there is no factor diffusible in the lysosomes which is able to stabilize these proteins (127).

Figure 11 shows the homology between the five cloned human sulfatases. It is interesting that some amino acids of the N-terminal region are conserved in all five

Figure 11 Homology between the human sulfatases. Regions of amino acid identity are boxed and shaded in purple; regions of amino acid homology (conservative change) are boxed and shaded in yellow. Point mutations causative for ASA or ASB deficiency are designated as a1-a3 and b1-b4. Gieselman *et al.* characterized a1 (40), Kondo *et al.* a2 (62), Polten *et al.* a3 (102), Jin *et al.* b1-b3 (59), and Wicker *et al.* b4 (154). The sulfatase alignment is based on Wilson *et al.* (156).


IDS
G6S
ASA
ASB
ASC
b1
a1
a2
b4
b2


IDS
G6S
ASA
ASB
ASC
b3
a3

sulfatases. Perhaps they are involved in a posttranslational modification common to the sulfatases, or they are essential residues in the active sites of these enzymes. Previous studies have indicated that there is at least one histidine residue essential to the catalytic activity of rabbit liver ASA (72), and that there are at least two arginine residues essential to the activity of human liver ASA (56). There is a histidine residue present in the third arylsulfatase conserved box, but this residue is only conserved in four of the five sulfatases (not in G6S). In the second N-terminal conserved box, there is an arginine residue conserved in all five sulfatases, and near to this, an arginine conserved in all except G6S (in which there is a conservative change at this position). Perhaps these arginine residues are important in the activity of the sulfatases. There is less homology between ASB and G6S and IDS than between ASB and the two other arylsulfatases. Overall, the percent identity and similarity between ASB and the other sulfatases is: ASA, 54% similarity and 31% identity; ASC, 52% similarity and 30% identity; G6S, 51% similarity and 22% identity; IDS, 50% similarity and 27% identity. When the N-terminal region between amino acids 40 and 150 (with respect to ASB) is compared between these proteins, ASB and ASA are 47% identical, ASB and ASC 45% identical, ASB and IDS 33% identical, and ASB and G6S 26% identical. The availability of the cDNA and deduced amino acid sequences for five of the seven MSD-involved sulfatases may be very useful in determining the site of action of the MSD factor, and even in the isolation and characterization of this factor. Additionally, comparison of the amino acid sequences and identification of common residues provides some obvious choices for the beginning of mutational analysis to study the structure and

function of these enzymes.

The known point mutations which cause ASA or ASB deficiency are indicated in Figure 11. Ninety percent of the ASC mutations are deletions; no point mutations have yet been identified (119). No point mutations causative for IDS or G6S deficiency have yet been reported. One ASB point mutation found by Wicker *et al.* (b4) occurs at a glycine residue which is conserved in all five sulfatases. Metabolic labelling studies on a cell line expressing this transfected mutant cDNA and on fibroblasts from the homoallelic patient showed that very little of the mutant ASB reached the lysosomes, and most was likely degraded before reaching the trans-Golgi network (154). As discussed previously (see Background), this mutation seems to affect the stability of ASB and not its activity. The conservation of the glycine at this position may indicate that this is a residue important to the structure of all of these sulfatases. Gieselman *et al.* characterized a C to T transition in exon 2 of the ASA gene which caused a ^{96}Ser to Phe change (a1) (40), and Kondo *et al.* characterized a G to A transition which caused a ^{99}Gly to Asp change (a2) (62). No expression of either of these mutants was seen in transient expression systems. Polten *et al.* characterized two common ASA mutations found to account for one half of the alleles of 68 MLD patients. One mutation changed a splice donor site, and the other was a C to T transition coding for a ^{426}Pro to Leu change (a3). This mutant expressed at very low residual activity in BHK cells (102). It is interesting that four of these mutations cluster in the N-terminal conserved area of the sulfatases, and that mutation a3 occurs at a proline residue conserved in the arylsulfatases.

F. Analysis of a Human ASB Polymorphism

Instead of expressing both methionine and valine containing full-length ASB cDNAs, it was decided to determine if this difference between cDNA clones 35 and 38 was due to a polymorphism in the population, or if the change was due to a cloning artifact. PCR was performed on genomic DNA from 20 normal individuals, and the amplification products were analyzed by agarose gel electrophoresis and ethidium bromide staining. Gels were blotted onto Biotrace membrane and these Southern blots were hybridized with allele specific oligonucleotides (ASOs) encoding Met or Val. All forty alleles hybridized only with the Val probe. Wicker *et al.* have also described this same "Met" allele, and in fact have expressed full-length hASB cDNAs with either A or G (Met or Val) at position 1126 (154). It is likely, then, that the A nucleotide which generates a methionine at amino acid 376 is a genuine polymorphism, with a low frequency in the population. Two other polymorphisms have been reported in the ASB sequence. Jin *et al.* in this laboratory have reported a polymorphism at cDNA nucleotide 1072, a G to A transition causing a valine to methionine change at amino acid 358. The frequencies of the alleles are .62 and .38, respectively, in the Caucasian population (58). Additionally, the silent change in the cDNA sequence (A to G) at position 1191 has also been observed by Wicker *et al.* (154).

Interestingly, the genomic PCR product amplified for this study was slightly larger than the expected product. When run on a high percentage agarose gel alongside PCR-amplified ASB cDNA, the genomic product was 60-70 bp larger than the cDNA product. Therefore, this region of the ASB gene may contain a small intron.

G. Expression of Full-length Human ASB cDNAs

Two hASB cDNA constructs were made for transient expression studies. These full-length cDNAs are depicted in Figure 12. hASB 1, which was constructed by joining the *Sma* I/*Aat* II fragment from clone 35 to the *Aat* II/*Stu* I fragment from clone 39, includes 167 bp of 5′ untranslated sequence and 48 bp of 3′ untranslated sequence. hASB 2, which was derived from hASB 1, was digested at the *Sac* II site at -12 and a vector polylinker site to remove additional 5′ untranslated sequence. Due to T4 polymerase digestion to remove the *Sac* II digest 3′ overhang, this cDNA has 10 bp of 5′ untranslated sequence, with the same 48 bp of 3′ untranslated sequence as hASB 1.

Both of the cDNAs were entirely sequenced and subcloned into the *Eco* RI site of expression vector p91023(B) (158), for transient expression studies in COS-1 cells (42). The p91023(B) vector has several features which allow very efficient expression of a foreign protein. These features include the powerful adenovirus major late promotor, the adenovirus tripartite leader and the adenovirus virus-associated (VA) genes, which serve to increase translation efficiency, and the SV40 origin of replication which allows the plasmid to replicate to a high copy number in the SV40-transformed cell line COS-1. Several other lysosomal cDNAs have been effectively expressed in this system (13,148).

COS-1 expression results for hASB 1 are outlined in Table 1. Initial experiments included the α-galactosidase A (α-gal A) expression construct as a positive control. ASB activity in COS-1 cells transfected with hASB 1 clone #15 averaged 2.8 fold above the negative control. Further experiments, which were performed using

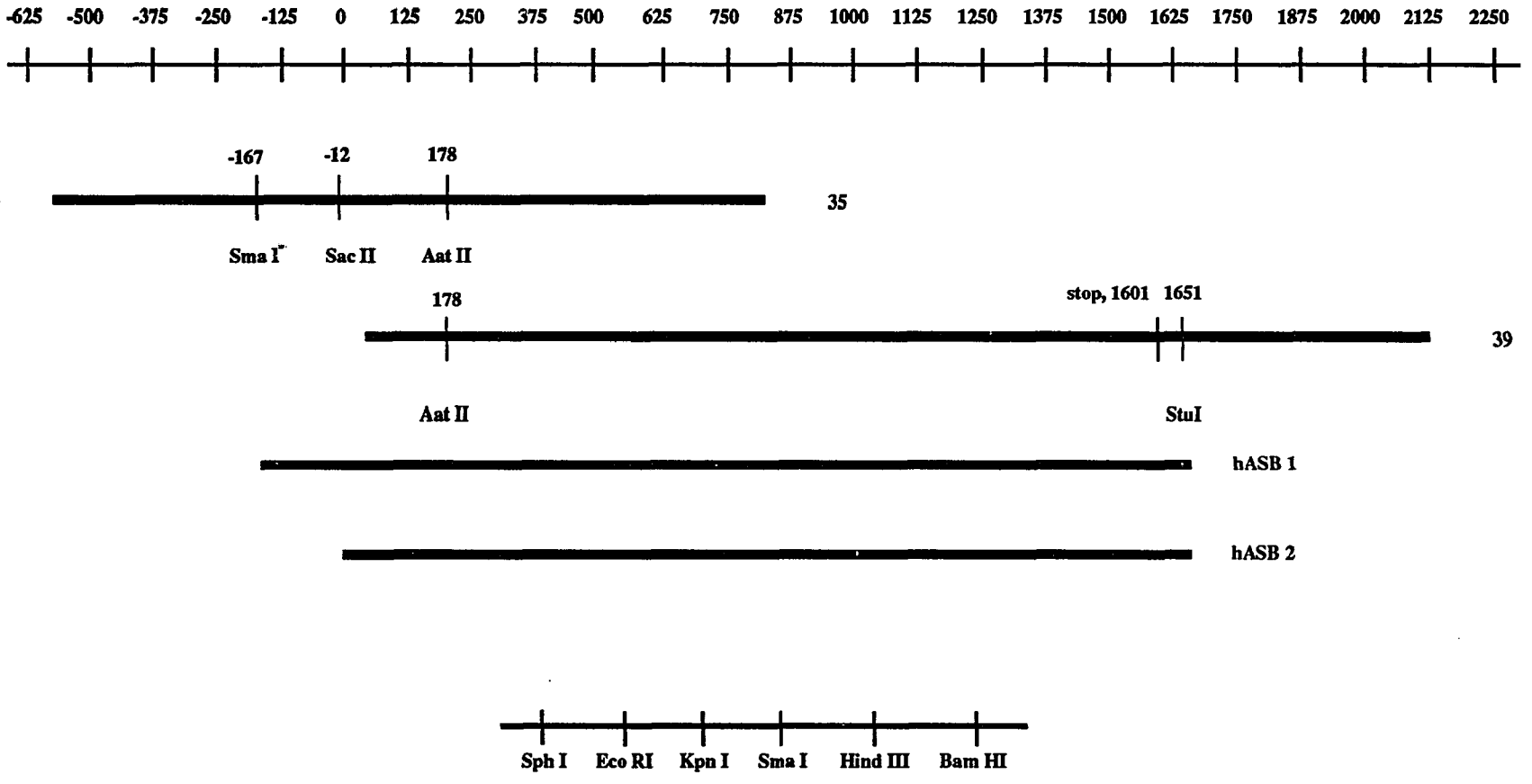
colchicine to enhance expression (103), were more convincing. hASB 1 clones #11 and #15 expressed ASB an average of 6.3 and 9.0 fold over background. Thus, the reconstructed hASB 1 full-length cDNA is a functional ASB cDNA.

The COS-1 expression results for hASB 2 are shown in Table 2. hASB 1 #15 was the positive control for this set of experiments. hASB 1 #15 expressed ASB 3.8 fold above background. The two sense constructs, hASB 2 #2 and #5, expressed ASB an average of 5.3 and 4.6 fold above background, respectively. The seeming increase in hASB 1 #15 expression in this series of experiments (2.8 vs. 3.8 fold average) may be due to use of a healthier batch of COS-1 cells, or because this set of assays was performed under conditions more favorable to the ASB enzyme. Previously, it was necessary to lyse the cells in saline for the α-gal A and fluorescamine assays. With the use of an ASB rather than an α-gal A construct as a positive control, it was possible to lyse the cells in 10 mM Tris-HCl, pH 7.5. Chloride ions may somewhat inhibit ASB in the assay with the artificial substrate NCS.

It seems that the activity of the hASB 2 construct may be slightly higher than that of the hASB 1 construct in the p91023(B)/COS-1 expression system. The hASB 2 construct was made especially for inclusion in retroviral vectors, where it is known that 5′ and 3′ untranslated sequences may interfere with the efficiency of expression. Of course, one cannot directly compare expression in COS-1 cells and in retroviral systems, but the results are encouraging, and this hASB 2 cDNA may prove to be important in expression of ASB in retroviral vectors.

Figure 12 Construction of full-length human ASB cDNAs. Thirty-five and 39 are partial cDNAs, and hASB 1 and 2 are the completed full length cDNAs. A portion of the pGEM-7 polylinker is included at the bottom of the figure as a reference.

Construction of Full-Length hASB cDNAs



Construct	Colchicine	α-gal A (U/mg prot)	ASB (U/mg prot)	ASB fold increase
no DNA (n=2)	-	290	551	1
α-gal A (n=2)	-	2,865	568	1.0
hASB 1 #15 (n=4)	-	411	1,563	2.8
no DNA (n=2)	+	108 (n=1)	254	1
α-gal A (n=1)	+	2,462	334	1.3
hASB 1 #15 (n=2)	+	-	2,277	9.0
hASB 1 #11 (n=3)	+	148 (n=1)	1,595	6.3

Table 1 Transient expression of hASB 1 in COS-1 cells. n= number of trials, units of ASB are nmol of NCS hydrolyzed per hour per ml sample. ASB expression in the transfectants with no DNA were used as the baseline level for comparison.

Construct	ASA (U/mg prot)	ASB (U/mg prot)	ASB fold increase
no DNA (n=2)	372	391	1
hASB 1 #15 (n=2)	459	1,501	3.8
hASB 2 #2 (n=2)	444	2,082	5.3
hASB 2 #5 (n=2)	276	1,804	4.6

Table 2 Transient expression of hASB 2 in COS-1 cells. ASA and ASB units are nmol of NCS hydrolyzed per hour per ml of sample. ASB expression in the transfectants with no DNA was the baseline level for comparison. n= number of trials.

H. Northern Analysis of Human ASB

To determine the length of the ASB transcripts, Northern analyses were performed. The first Northern included 50 μg of total lymphoblast RNA, with random-primed partial cDNA #39 as probe. After a two week exposure, bands were seen at 4.8 kb, 3.8 kb, and 2.2 kb. The strongest of these bands was at 3.8 kb. The second Northern included 10 μg of lymphoblast total RNA, and 10 and 25 μg of fibroblast total RNA. After a two week exposure bands were seen at 3.7 and 2.2 kb. Again, the stronger band was at 3.7 kb. Since this blot had less RNA than the first and a weaker signal, it is possible that the large 4.8 kb band was not visible.

These results differ slightly from those reported by Peters *et al.*, who observed bands of 4.8, 2.5, and 1.8 kb on Northerns from fibroblast poly A^+ RNA (99). Since no naturally occurring full-length hASB cDNA has ever been isolated, it is difficult to be certain which of these mRNAs may represent a functional ASB transcript. The two longest ASB cDNAs isolated, one in this lab and one by Peters *et al.*, were both about 2.2 kb and were almost identical in sequence. Both cDNAs were isolated from a Clontech λgt11 testis library. It is possible that 5′ truncated cDNAs of this type are representative of the 2.2 kb Northern transcripts. Possibly, then, the abundant 3.7-3.8 kb transcript is the functional ASB transcript. The molecular nature of the 4.8 kb transcript is uncertain.

I. Retroviral Constructs

The hASB 1 and 2 cDNAs were subcloned into the DCTK retroviral vector (44),

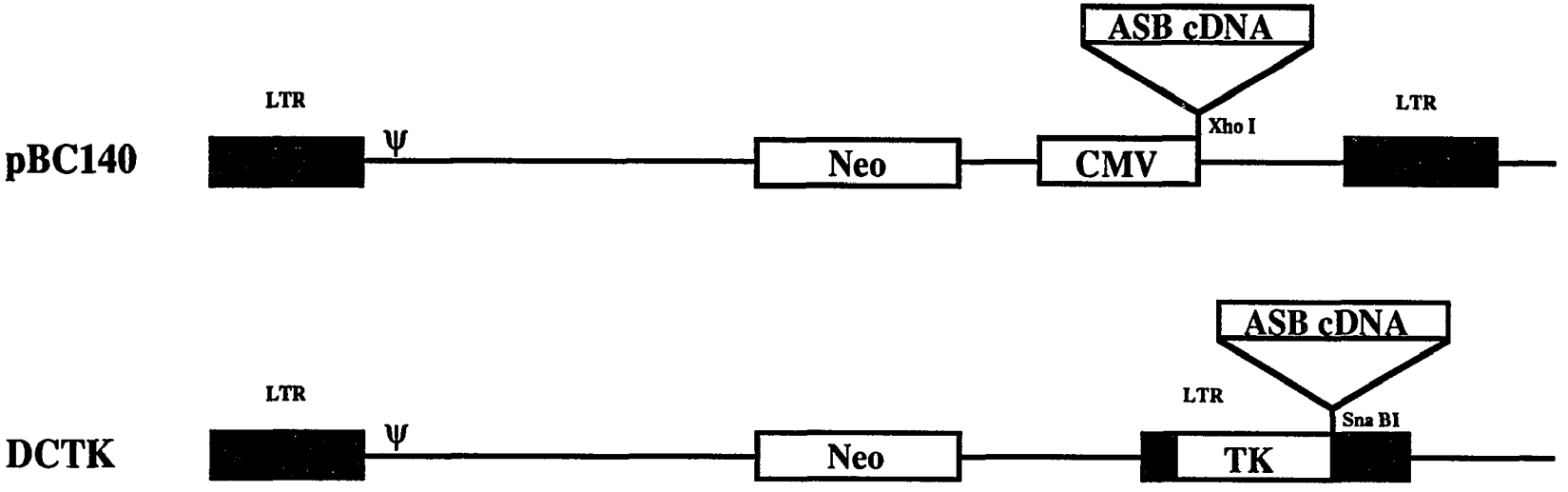
and the hASB2 cDNA was subcloned into the pBC140 vector (150). These vectors both have the neomycin resistance (neo^r) gene for selection with G418. Two important steps in the development of a good gene therapy system are the generation of high-titer virus to infect host cells, and the construction of vectors which express the introduced gene at a high level in the host. The pBC140 and DCTK vectors both produce high titers of virus, and both have been successful in gene transfer studies (44,150,134). It is often necessary to try several retroviral constructs to optimize expression in a particular host cell. In the pBC140 vector, the gene of choice is subcloned 3′ of the neo^r gene, and expression is driven off the human cytomegalovirus (CMV) immediate early promotor. The CMV promotor is downstream of the viral 5′ LTR promotor, which may cause decreased expression off the CMV promotor. The DCTK vector, however, avoids this problem because the gene of interest is inserted after the herpes simplex virus thymidine kinase (TK) promotor in the 3′ LTR. Because the 3′ LTR serves as the template for the 5′ LTR when infectious virus is made, the virus will have two copies of the gene of interest. The gene in the 5′ LTR precedes the viral promotor, and will be outside any possible inhibitory effects of this promotor, and the gene in the 3′ LTR might be expressed as well. The hASB 2/DCTK construct has been transfected into packaging cell line GP + *env* AM12 (78), and two clones produced titers over 10^4/ml, as determined by titering in NIH 3T3 cells. ASB assays performed on the transduced NIH 3T3 cells showed that ASB was expressed 2 and 5 fold over background; however, the ASB background is very high in NIH 3T3 cells. If these fold expression levels are compared to expression in normal human fibroblasts, these

clones express 8 and 21 fold increased ASB activities, respectively. Thus, the hASB 2/DCTK construct clearly expresses active ASB.

Peters *et al.* have corrected the ASB deficiency in an MPS VI fibroblast cell line by infection with an ASB cDNA-containing retroviral construct. The vector used in this experiment was the N2-derived pXT1 vector, which has the neo^r gene for G418 selection. Expression of ASB in this construct is driven off an internal herpes simplex virus TK promotor. The ASB cDNA subcloned into this vector was a chimeric construct coding for the first 29 amino acids of the signal peptide of acid phosphatase, and amino acids 37-533 of ASB. The completed pXT-ASB construct was packaged in the amphotropic packaging line PA317. MPS VI fibroblasts infected with the retrovirus expressed ASB at levels up to 36 fold above the ASB activity in normal fibroblasts. The ASB expressed in the infected fibroblasts was processed normally as demonstrated by pulse-chase studies, and localized in lysosomes as demonstrated by immunofluorescence and Percoll density gradient centrifugation (100).

Figure 13 ASB retroviral constructs in vectors DCTK and pBC140.

ASB RETROVIRAL CONSTRUCTS



Chapter Two

Molecular Genetic Studies of Feline Arylsulfatase B

Abstract

In order to begin characterization of the feline model for MPS VI, cDNAs encoding feline ASB (fASB) were isolated. Two cDNAs were isolated from a feline liver cDNA library using the human partial cDNA #39 as a probe. The first cDNA, f12, was 1,440 bp long and included 3 bp of 5′ untranslated sequence and 1437 bp of the coding region of fASB. The second cDNA, f27, was 289 bp and consisted entirely of 3′ untranslated sequence. The fASB coding sequence was 90% identical to the hASB cDNA. PCR was performed on first strand cDNA to complete the coding sequence of fASB. The complete fASB cDNA is 1,939 bp long and includes 3 bp of 5′ untranslated sequence, 331 bp of 3′ untranslated sequence, and a 1,605 bp open reading frame encoding 535 amino acids. There are five potential *N*-glycosylation sites. A full-length fASB cDNA was constructed from partial cDNA f12 and a 3′ PCR product. To demonstrate the functional integrity of this cDNA, it was transiently expressed in the human embryonic kidney line 293. The cDNA sequence is now available to characterize the mutations in the fMPS VI model, which will facilitate heterozygote detection in the fMPS VI colony, and for retroviral studies if needed. Additionally, the fASB gene was mapped to feline chromosome A1 by PCR analysis of somatic cell hybrid panels.

Experimental Procedures

A. Isolation of Feline ASB cDNAs

Two feline cDNA libraries were screened in order to isolate a feline ASB cDNA. Both libraries were constructed by Dr. Naoya Yuhki (National Cancer Institute, Fredericksburg, MD). These feline spleen and liver libraries were constructed in the λZAP II vector. The probe used for the library screening was the human partial cDNA #39. The libraries were plated at a density of 2 X 10^4 pfu/150mm plate using host cell strain BB4. Prehybridization and hybridization were at 57 °C, and washing, also performed at 57 °C for 2 hours, was in 2X SSC, 0.1% SDS. Pure positives from the liver library screening were rescued (see Chapter One, Experimental Procedures), and analyzed by *Eco* RI digestion of the plasmid DNA.

B. Completion of the Full-Length Feline cDNA by PCR

Feline total RNA was purified from 6 flasks of normal feline fibroblasts as described in Chapter One, Experimental Procedures. First-strand cDNA was made from 10 μg of total RNA using a BRL cDNA synthesis kit according to the manufacturer's instructions, and as described in Chapter One. The cDNA was resuspended in 20 μl T:E buffer, pH 8.

A schematic for the completion of the feline cDNA is shown in Figure 1. Sense primer CS2 (5′-GGAATTCCGATACCCTCATCAGACC-3′) was constructed from the

3′ end of feline cDNA 12, and antisense primer CA2 (5′-GGAATTCCCAGAAA CAAGAGAAGGA-3′) from the 5′ end of fASB cDNA 27. These primers include *Eco* RI restriction sites for subcloning. PCR was performed for 30 cycles of 94 °C denaturation for 1 min., 50 °C annealing for 2 min., and 72 °C extension for 1 min. Reactions contained 1 μM each primer, 2.5U Promega *Taq* Polymerase, Promega buffer, and 330 μM dNTPs. The authenticity of the PCR product was demonstrated by hybridization to a mix of two human internal oligos from the homologous area of the human cDNA. The 340 bp PCR product was isolated from a polyacrylamide gel, digested with *Eco* RI, and subcloned into the *Eco* RI site of the pGEM-7 vector. Sequencing was done on mini prep DNA.

C. Construction of a Full-Length Feline ASB cDNA

The construction of a fASB full-length cDNA is depicted in Figure 3. To generate the 3′ coding region of the cDNA, PCR was performed on normal cat first strand cDNA using sense primer CS6 (5′-GCCGCCAAGCTTGCACCAAGCCTC TGGATGGCTT-3′) and antisense primer CA6 (5′-GCCGCCGAATTCGACACGA GATGCAGGAGTAAAG-3′). Primer CS6 includes a *Hind* III restriction site, and primer CA6 includes an *Eco* RI restriction site. The PCR conditions were similar to those used to complete the feline cDNA sequence, but each cycle consisted of 94 °C denaturation for 1 min., 65 °C annealing for 1 min., and 72 °C extension for 1 min. The 580 bp PCR product was gel purified, digested with restriction endonucleases *Eco* RI and *Hind* III, and subcloned into the *Eco* RI and *Hind* III digested vector pGEM-7.

The subcloned product was sequenced completely in both directions to ensure that no PCR errors had occurred.

Feline ASB cDNA 12 was linearized at nucleotide 1301 with the restriction endonuclease *Sph* I. In order for this digestion to work, it was necessary to purify the f12 plasmid by the protocol of W.P.C. Stemmer (130). Briefly, 10-25 μg DNA is taken up in 250 μl of dH_2O. Fifteen μl of 10 mg/ml ethidium bromide and 140 μl of 7.5 M ammonium acetate are added, the solution is mixed, and shielded from UV light. The solution is then extracted with 420 μl of phenol/chloroform, and the aqueous phase precipitated with 2 volumes 100% ethanol. To construct the full-length cDNA, a 390 bp piece was excised from the PCR product with *Sph* I, which cleaves both in the coding sequence and in the pGEM-7 polylinker. This fragment was gel purified and ligated into the linearized f12 plasmid. *Eco* RI restriction sites are present upstream of the f12 cDNA in pBluescript, and downstream of the PCR product in the antisense primer. Full-length sense constructs were identified from mini-preps by *Eco* RI excision of a 1.7 kb cDNA insert from pBluescript. A full-length cDNA was sequenced entirely in both directions to ensure its integrity. This cDNA is referred to as fASB 1.

D. Transient Expression of a Full-Length Feline ASB cDNA

The 1.7 kb fASB 1 insert was isolated by *Eco* RI digestion of a large scale plasmid prep DNA. The insert was gel purified and ligated into the expression vector p91023(B) as described for the human cDNAs. The cDNA orientation in p91023(B)

was determined by sequential digestion with restriction endonucleases *Bgl* II and *Bst* XI.

Electroporation and expression trials of three feline sense p91023(B) constructs were conducted as described for hASB expression in COS-1, with the hASB 1 (15) or hASB 2 (2) constructs used as the positive control. All lysates were assayed for ASA as well as ASB activity.

Optimal electroporation parameters for primary feline fibroblasts were determined by a series of trial electroporations of cells only. Optimal electroporation occurs at those conditions which produce about 50% cell death. For feline fibroblasts, this occurred at a setting of 350V, 25 μF capacitance. Human embryonic kidney 293 cells (ATCC, Bethesda, MD) were electroporated at a setting of 250V, 25 μF.

E. Mapping of the Feline ASB Gene

Genomic DNA from the feline/murine (17T) and feline/hamster (49C) somatic cell hybrids was the gift of Dr. Stephen O'Brien (National Cancer Institute, Fredericksburg, MD). PCR sense primer CS2 (GGAATTCCGATACCCTCATCAG ACC) and antisense primer CA2 (GGAATTCCCAGAAACAAGAGAAGGA) amplify a 355 bp product from the feline cDNA. These primers proved to amplify a similar size sequence from feline genomic DNA. Both primers include an *Eco* RI site and a one base pair spacer flanking the recognition site. PCR was performed on 100 ng of genomic DNA from mouse/cat (17T) or hamster/cat (49C) somatic cell hybrids. One μm of each primer was used. Each reaction was performed for 30 cycles consisting of 1 min. denaturation at 94 °C, 1 min. annealing at 65 °C, and 1 min. extension at 72

°C. Following PCR, one half of each PCR product was analyzed by agarose gel electrophoresis on 1.85% agarose gels. Gels were blotted by standard methods onto either nitrocellulose or Zetaprobe membranes. Blots were hybridized with a 5′ end-labelled feline-specific internal oligonucleotide (GAAGAAAGACATGACCT). Hybridizations were performed overnight at 42 °C in 6X SSPE, 0.5% SDS, and 5X Denhardt's. The blots were washed 20 min. at room temperature, then 1 hr. at 42 °C in 6x SSC, 0.5% SDS. Blots were analyzed after overnight exposure.

Results and Discussion

A. Isolation of Feline ASB cDNAs

In order to begin characterization of feline ASB and the feline model of MPS VI, cDNAs encoding feline ASB have been isolated. Two feline cDNA libraries were screened in order to isolate fASB cDNA clones. Screenings were performed using the partial human cDNA #39 at a reduced stringency, in order to allow for mismatches in isolating a cDNA across species. No authentic fASB cDNAs were isolated from the feline splenic library, but three positives were isolated from the feline liver library. Because the library had been constructed in the λZAP II vector, it was necessary to rescue the Bluescript plasmid which harbored the cDNA clones from the phage vector. Rescue from λZAP II has been discussed in Chapter One. One of the feline clones, 21, resisted rescue through six separate attempts, performed under varying conditions. However, the remaining two positives were easily rescued and characterized. One of

these clones, f12, contained a 1440 bp insert which, upon sequencing, was 90% identical at the nucleotide level to the hASB cDNA. By comparison with the hASB cDNA, the feline sequence had only 3 bp of 5′ untranslated sequence, and contained most of the coding region. The open reading frame began at +1 and remained open throughout the clone; no stop codon was seen. The second clone characterized, f27, was 289 bp long. This clone was completely sequenced and when compared with the hASB cDNA, proved to be 83% identical at the nucleotide level to a portion of the hASB 3′ untranslated sequence.

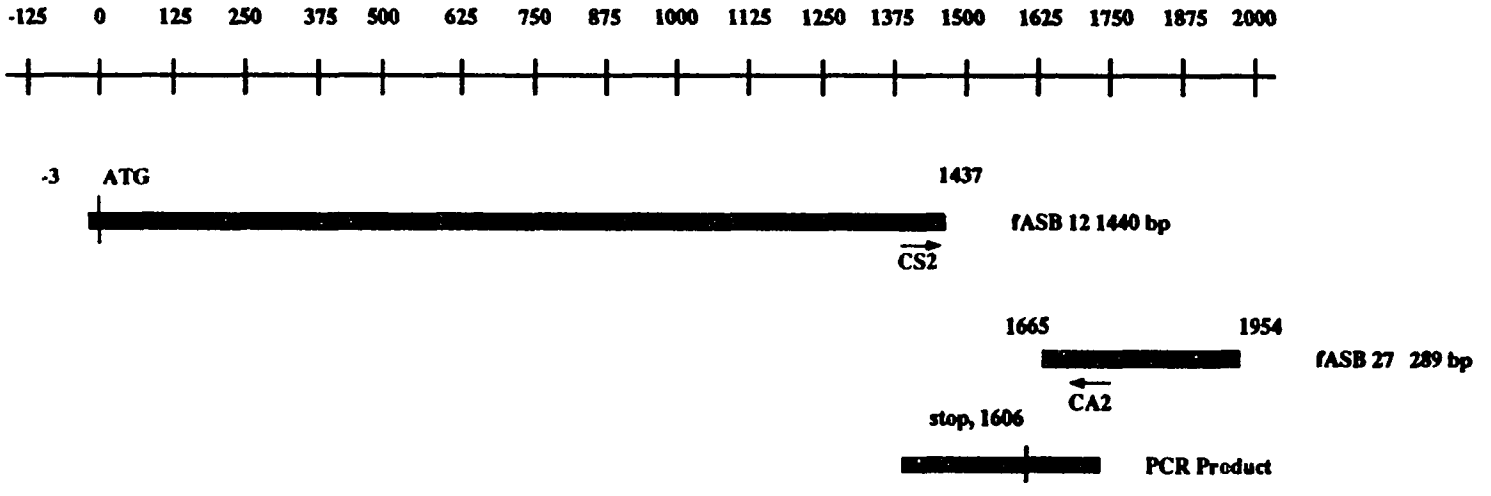
B. Completion of the Full-Length Feline ASB Sequence

Because of the great homology between feline and human ASB cDNAs, it was possible to infer that most likely, only 200 bp were missing from the 3′ coding region of fASB. PCR primers were chosen from the 3′ end of the f12 cDNA and from the 5′ end of the f27 cDNA, as shown in Figure 1. The antisense primer, CA2, was chosen from an area of reduced homology to the hASB sequence in order to guard against PCR contamination with human DNA. The missing feline sequence was PCR-amplified from feline first strand cDNA. The 355 bp product was subcloned into the *Eco* RI site of the pGEM-7 vector, and was sequenced on both strands. The PCR product differed from the 3′ sequence of the f12 cDNA at the last 20 bp. When both 20 bp sequences were compared with the hASB cDNA, the 20 bp from the PCR product were much more homologous to the hASB cDNA than were the f12 20 bp. Thus, it was likely that the last 20 bp of the f12 cDNA were anomalous, and the PCR

product included the authentic fASB sequence for this region. Further sequencing of PCR amplified cDNAs from several different cats has demonstrated that this is the case.

Figure 1 Completion of the feline ASB cDNA sequence by PCR. Partial cDNAs f12, which covered the majority of the fASB coding region, and f27, which consisted of 3′ untranslated sequence, are shown. The PCR product included the rest of the 3′ coding sequence of fASB, the stop codon, and additional 3′ untranslated sequence.

Completion of Feline ASB cDNA Sequence by PCR



C. The Feline ASB cDNA Sequence and Comparison of Human and Feline ASB

The full-length feline ASB cDNA sequence, as compiled from f12, f27, and the PCR product, is depicted in Figure 2. Overall, the cDNA sequence is highly homologous to hASB (89%), even in the 3′ untranslated sequence (81%). Feline ASB has five potential *N*-glycosylation sites, which are conserved between man and cat; a sixth glycosylation site at human amino acid 366 is not conserved in the cat. The fASB cDNA is 1,939 bp in length with 3 bp of 5′ untranslated sequence and 331 bp of 3′ untranslated sequence. The coding region includes 1,605 bp coding for a protein of 535 amino acids. The signal cleavage site as predicted by von Heijne (147) is before ^{42}Gly. The amino acid sequence comparison of hASB and fASB is shown in Figure 3. The overall identity between the two sequences is 91%, and the homology is 94%.

ASB is the first cDNA encoding a metabolic protein to be isolated in both humans and cats. The only other cDNAs which have been isolated from both man and cat are the islet amyloid polypeptide, an 89 amino acid precursor of a potential insulin regulating hormone (88,111), and c-sis, a cellular oncogene closely related to the β chain of platelet derived growth factor (141,105). Over a stretch of 89 amino acids, human and feline *(F. catus)* islet amyloid polypeptide are 91% similar and 81% identical, and over a stretch of 210 amino acids, human and feline *(F. silvestris)* c-sis are 93% similar and 89% identical. From this data it may seem the homology between human and feline ASB is remarkable, but more cDNA sequences will have to be isolated in both humans and cats before this is certain. Human ASB is 533 amino acids and fASB is 535 amino acids in length. Considering how similar these two enzymes

are, it is perhaps surprising that fASB functions as a dimer and hASB as a monomer. It is known from studies of normal and mutant fASB that a cysteine residue may be important in the dimeric structure of fASB (146). Interestingly, hASB has eight cysteine residues while fASB has nine. The eight cysteines are conserved in position, and the extra cysteine in the cat is at amino acid 451, which is a tyrosine in the comparable position in hASB.

Figure 2 Full-length cDNA sequence of feline ASB. The five potential *N*-glycosylation sites are underlined, and the signal cleavage site as determined by the weight matrix method (147) is shown by an arrow.

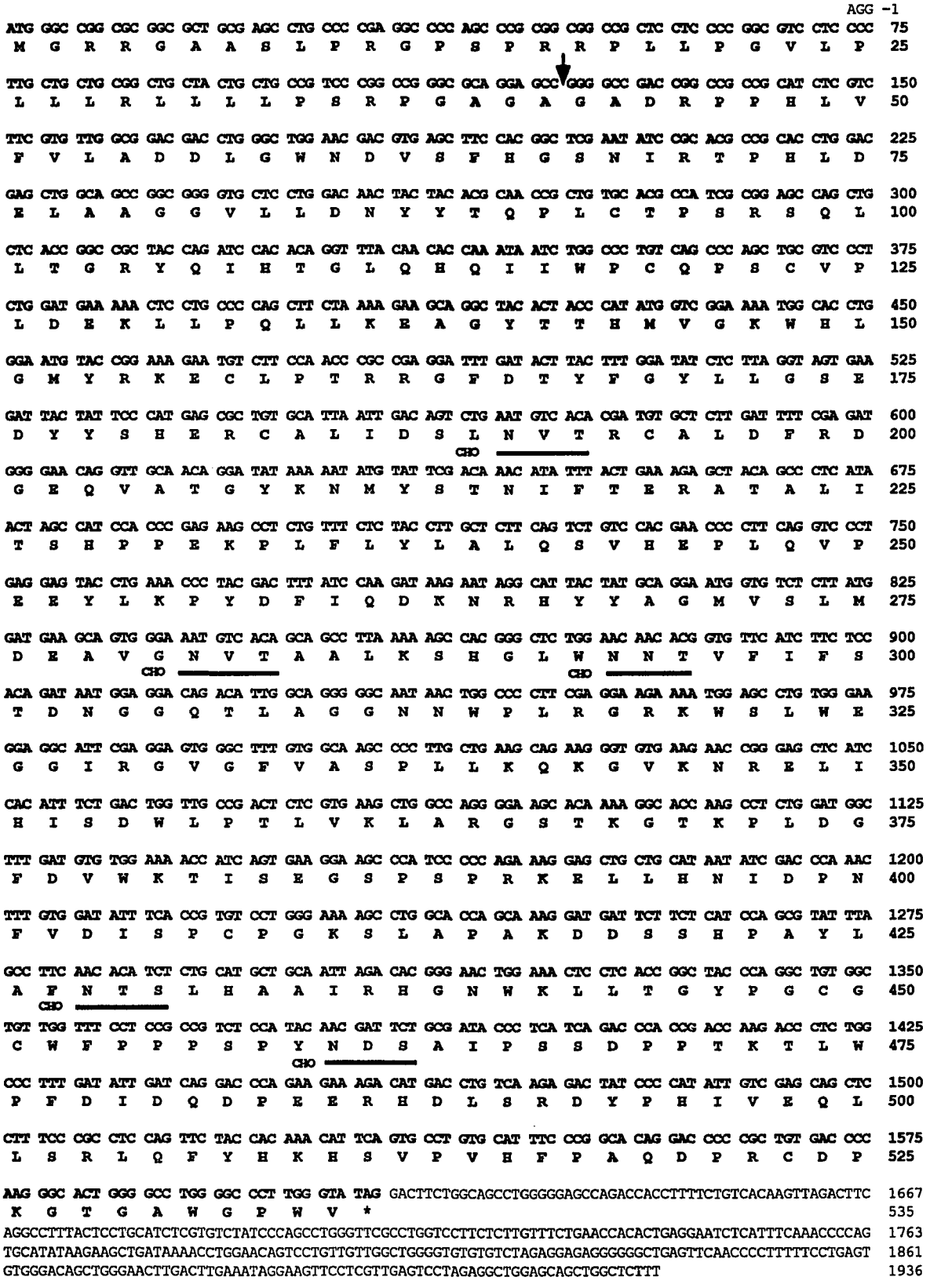

AGG -1
ATG GGC CGG CGC GGC GCT GCG AGC CTG CCC CGA GGC CCC AGC CCG CGG CGG CCG CTC CTC CCC GGC GTC CTC CCC 75
M G R R G A A S L P R G P S P R R P L L P G V L P 25
TTG CTG CTG CGG CTG CTA CTG CTG CCG TCC CGG CCG GGC GCA GGA GCC GGG GCC GAC CGG CCG CCG CAT CTC GTC 150
L L L R L L L L P S R P G A G A G A D R P P H L V 50
TTC GTG TTG GCG GAC GAC CTG GGC TGG AAC GAC GTG AGC TTC CAC GGC TCG AAT ATC CGC ACG CCG CAC CTG GAC 225
F V L A D D L G W N D V S F H G S N I R T P H L D 75
GAG CTG GCA GCC GGC GGG GTG CTC CTG GAC AAC TAC TAC ACG CAA CCG CTG TGC ACG CCA TCG CGG AGC CAG CTG 300
E L A A G G V L L D N Y Y T Q P L C T P S R S Q L 100
CTC ACC GGC CGC TAC CAG ATC CAC ACA GGT TTA CAA CAC CAA ATA ATC TGG CCC TGT CAG CCC AGC TGC GTC CCT 375
L T G R Y Q I H T G L Q H Q I I W P C Q P S C V P 125
CTG GAT GAA AAA CTC CTG CCC CAG CTT CTA AAA GAA GCA GGC TAC ACT ACC CAT ATG GTC GGA AAA TGG CAC CTG 450
L D E K L L P Q L L K E A G Y T T H M V G K W H L 150
GGA ATG TAC CGG AAA GAA TGT CTT CCA ACC CGC CGA GGA TTT GAT ACT TAC TTT GGA TAT CTC TTA GGT AGT GAA 525
G M Y R K E C L P T R R G F D T Y F G Y L L G S E 175
GAT TAC TAT TCC CAT GAG CGC TGT GCA TTA ATT GAC AGT CTG AAT GTC ACA CGA TGT GCT CTT GAT TTT CGA GAT 600
D Y Y S H E R C A L I D S L N V T R C A L D F R D 200
CHO
GGG GAA CAG GTT GCA ACA GGA TAT AAA AAT ATG TAT TCG ACA AAC ATA TTT ACT GAA AGA GCT ACA GCC CTC ATA 675
G E Q V A T G Y K N M Y S T N I F T E R A T A L I 225
ACT AGC CAT CCA CCC GAG AAG CCT CTG TTT CTC TAC CTT GCT CTT CAG TCT GTC CAC GAA CCC CTT CAG GTC CCT 750
T S H P P E K P L F L Y L A L Q S V H E P L Q V P 250
GAG GAG TAC CTG AAA CCC TAC GAC TTT ATC CAA GAT AAG AAT AGG CAT TAC TAT GCA GGA ATG GTG TCT CTT ATG 825
E E Y L K P Y D F I Q D K N R H Y Y A G M V S L M 275
GAT GAA GCA GTG GGA AAT GTC ACA GCA GCC TTA AAA AGC CAC GGG CTC TGG AAC AAC ACG GTG TTC ATC TTC TCC 900
D E A V G N V T A A L K S H G L W N N T V F I F S 300
CHO CHO
ACA GAT AAT GGA GGA CAG ACA TTG GCA GGG GGC AAT AAC TGG CCC CTT CGA GGA AGA AAA TGG AGC CTG TGG GAA 975
T D N G G Q T L A G G N N W P L R G R K W S L W E 325
GGA GGC ATT CGA GGA GTG GGC TTT GTG GCA AGC CCC TTG CTG AAG CAG AAG GGT GTG AAG AAC CGG GAG CTC ATC 1050
G G I R G V G F V A S P L L K Q K G V K N R E L I 350
CAC ATT TCT GAC TGG TTG CCG ACT CTC GTG AAG CTG GCC AGG GGA AGC ACA AAA GGC ACC AAG CCT CTG GAT GGC 1125
H I S D W L P T L V K L A R G S T K G T K P L D G 375
TTT GAT GTG TGG AAA ACC ATC AGT GAA GGA AGC CCA TCC CCC AGA AAG GAG CTG CTG CAT AAT ATC GAC CCA AAC 1200
F D V W K T I S E G S P S P R K E L L H N I D P N 400
TTT GTG GAT ATT TCA CCG TGT CCT GGG AAA AGC CTG GCA CCA GCA AAG GAT GAT TCT TCT CAT CCA GCG TAT TTA 1275
F V D I S P C P G K S L A P A K D D S S H P A Y L 425
GCC TTC AAC ACA TCT CTG CAT GCT GCA ATT AGA CAC GGG AAC TGG AAA CTC CTC ACC GGC TAC CCA GGC TGT GGC 1350
A F N T S L H A A I R H G N W K L L T G Y P G C G 450
CHO
TGT TGG TTT CCT CCG CCG TCT CCA TAC AAC GAT TCT GCG ATA CCC TCA TCA GAC CCA CCG ACC AAG ACC CTC TGG 1425
C W F P P P S P Y N D S A I P S S D P P T K T L W 475
CHO
CCC TTT GAT ATT GAT CAG GAC CCA GAA GAA AGA CAT GAC CTG TCA AGA GAC TAT CCC CAT ATT GTC GAG CAG CTC 1500
P F D I D Q D P E E R H D L S R D Y P H I V E Q L 500
CTT TCC CGC CTC CAG TTC TAC CAC AAA CAT TCA GTG CCT GTG CAT TTC CCG GCA CAG GAC CCC CGC TGT GAC CCC 1575
L S R L Q F Y H K H S V P V H F P A Q D P R C D P 525
AAG GGC ACT GGG GCC TGG GGC CCT TGG GTA TAG GACTTCTGGCAGCCTGGGGGAGCCAGACCACCTTTTCTGTCACAAGTTAGACTTC 1667
K G T G A W G P W V * 535
AGGCCTTTACTCCTGCATCTCGTGTCTATCCCAGCCTGGGTTCGCCTGGTCCTTCTCTTGTTTCTGAACCACACTGAGGAATCTCATTTCAAACCCCAG 1763
TGCATATAAGAAGCTGATAAAACCTGGAACAGTCCTGTTGTTGGCTGGGGTGTGTGTCTAGAGGAGAGGGGGGCTGAGTTCAACCCCTTTTTCCTGAGT 1861
GTGGGACAGCTGGGAACTTGACTTGAAATAGGAAGTTCCTCGTTGAGTCCTAGAGGCTGGAGCAGCTGGCTCTTT 1936

Figure 3 The amino acid sequence comparison of human and feline ASB. This alignment was done by the "Bestfit" program of the University of Wisconsin GCG package (28). Amino acid identity is shown as a solid line, conservation as two dots, and similarity as one dot. Overall, the identity between these two proteins is 91%, and the similarity is 94%. The ninth cysteine residue in the feline sequence, which is not conserved between man and cats, is underlined.

AMINO ACID SEQUENCE COMPARISON OF HUMAN AND FELINE ARYLSULFATASE B

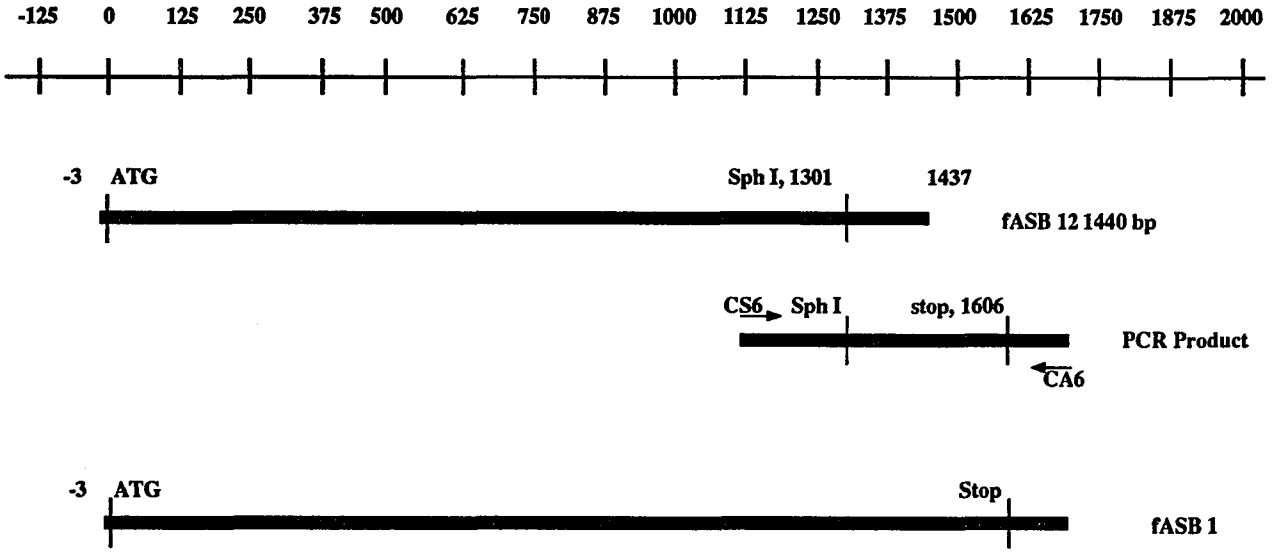
```
HASB   1 MGPRGAASLPRGPGPRRLLLPVVLPLLLLLLLAP..PGSGAGASRPPHLV
         ||.|||||||||||:|||  |||.||||||  |||  |   ||.||||.|||||||
FASB   1 MGRRGAASLPRGPSPRRPLLPGVLPLLLRLLLLPSRPGAGAGADRPPHLV

      49 FLLADDLGWNDVGFHGSRIRTPHLDALAAGGVLLDNYYTQPLCTPSRSQL
         |:|||||||||||:|||||.|||||||.|||||||||||||||||||||||||||
      51 FVLADDLGWNDVSFHGSNIRTPHLDELAAGGVLLDNYYTQPLCTPSRSQL

      99 LTGRYQIRTGLQHQIIWPCQPSCVPLDEKLLPQLLKEAGYTTHMVGKWHL
         |||||||:|||||||||||||||||||||||||||||||||||||||||||||||
     101 LTGRYQIHTGLQHQIIWPCQPSCVPLDEKLLPQLLKEAGYTTHMVGKWHL

     149 GMYRKECLPTRRGFDTYFGYLLGSEDYYSHERCTLIDALNVTRCALDFRD
         |||||||||||||||||||||||||||||||||||||||||.|||.|||||||||||
     151 GMYRKECLPTRRGFDTYFGYLLGSEDYYSHERCALIDSLNVTRCALDFRD

     199 GEEVATGYKNMYSTNIFTKRAIALITNHPPEKPLFLYLALQSVHEPLQVP
         ||:|||||||||||||||||.||.||||.|||||||||||||||||||||||
     201 GEQVATGYKNMYSTNIFTERATALITSHPPEKPLFLYLALQSVHEPLQVP

     249 EEYLKPYDFIQDKNRHHYAGMVSLMDEAVGNVTAALKSSGLWNNTVFIFS
         |||||||||||||||||.|||||||||||||||||||  ||||||||||||
     251 EEYLKPYDFIQDKNRHYYAGMSVLMDEAVGNVTAALKSHGLWNNTVFIFS

     299 TDNGGQTLAGGNNWPLRGRKWSLWFGGVRGVGFVASPLLKQKGVKNRELI
         ||||||||||||||||||||||||||||||||:|||||||||||||||||||
     301 TDNGGQTLAGGNNWPLRGRKWSLWFGGIRGVGFVASPLLKQKGVKNRELI

     349 HISDWLPTLVKLARGHTNGTKPLDGFDMWKTISEGSPSPRIELLHNIDPN
         |||||||||||||||  |.|||||||||:|||||||||||  ||||||||||
     351 HISDWLPTLVKLARGSTKGTKPLDGFDVWKTISEGSPSPRKELLHNIDPN

     399 FVDSSPCPRNSMAPAKDDSSLPEYSAFNTSVHAAIRHGNWKLLTGYPGCG
         |||  ||||  .|:|||||||||  |.|  ||||||:|||||||||||||||||||
     401 FVDISPCPGKSLAPAKDDSSHPAYLAFNTSLHAAIRHGNWKLLTGYPGCG

     449 YWFPPPSQYNVSEIPSSDPPTKTLWLFDIDRDPEERHDLSREYPHIVTKL
         :|||||||.||  |.|||||||||||||  ||||.|||||||||||:||||||..|
     451 CWFPPPSPYNDSAIPSSDPPTKTLWPFDIDQDPEERHDLSRDYPHIVEQL

     499 LSRLQFYHKHSVPVYFPAQDPRCDPKATGVWGPWM
         ||||||||||||||||.|||||||||||||:||.||||:
     501 LSRLQFYHKHSVPVHFPAQDPRCDPKGTGAWGPWV
```

D. Construction of a Full-Length Feline ASB cDNA

In order to determine if the fASB cDNA encoded a functional protein, it was necessary to construct a full-length cDNA for expression studies. The full-length fASB cDNA was constructed from the f12 cDNA and a 3′ PCR product (see Figure 4). A 580 bp product was generated by PCR using first strand cDNA as the template. This product was digested in its PCR primers with restriction enzymes *Eco* RI and *Hind* III, subcloned into the pGEM-7 vector, and sequenced on both strands. An *Sph* I fragment from this product was ligated to *Sph* I digested f12 cDNA. A full length fASB cDNA could be isolated from this construct by *Eco* RI digestion. The complete *Eco* RI-ended construct, referred to as fASB 1, was entirely sequenced to ensure the integrity of the sequence.

Figure 4 Construction of a full-length fASB cDNA. Partial cDNA 12 is shown above, and the 3′ PCR product from first strand cDNA is shown below. The fASB 1 construct includes 3 bp of 5′ untranslated sequence and 87 bp of 3′ untranslated sequence.

Construction of a Full-Length Feline ASB cDNA



E. Transient Expression Studies of Feline ASB

The fASB 1 cDNA was isolated from the vector by *Eco* RI digestion, and subcloned into the *Eco* RI site of expression vector p91023(B). Two expression trials with three separate fASB 1 sense constructs were performed in COS-1 cells, as described in Chapter One. Positive controls for these trials were the hASB 1 and hASB 2 constructs. Although the human constructs expressed ASB at levels 3.8 to 5.3 fold above background in COS-1 cells, all three feline sense constructs failed to express ASB at any noticeable level. Expression of the feline constructs was then attempted in two different cell lines: primary feline fibroblasts and the human embryonic kidney line 293. Although 293 cells are not SV40 transformed and do not have the SV40 large T antigen required to replicate p91023(B) to a high copy number, they are adenovirus transformed and should express well off of the adenovirus major late promotor. Primary fibroblasts proved to grow too poorly for the experiment and were abandoned after one electroporation attempt. In the first expression trial using 293 cells, with hASB 2 #2 as positive control, a very slight level of expression was seen in the human and all three of the feline sense constructs. Human ASB was expressed 1.4 fold, and the best feline construct, fASB 1 #6, 1.3 fold. Expression studies in 293 cells were pursued using colchicine to enhance expression. The results of these experiments are seen in Table 1. Background ASB levels are determined from transfection with buffer only. The negative control is human ASB antisense construct 10. As seen in the table, antisense construct 10 expresses ASB at 1.2 fold above background. Perhaps the slight elevation in ASB expression is due to an increase in lysosomes or lysosomal activity

in order to degrade the introduced DNA. Human ASB expresses well above this level- an average 3.6 fold, and fASB, an average 5.2 fold. It seems likely then that the fASB cDNA is functional, and simply could not be expressed in COS-1 cells. Perhaps the enzyme did not dimerize properly. There is a precedent for a lysosomal enzyme, glucocerebrosidase, which cannot be expressed in COS-1 (Dr. Marie Grace, personal communication).

Construct	ASB (U/mg prot)	ASB fold increase
no DNA (n=2)	104	1
hASB 2 #2 (n=2)	371	3.6
hASB ans 10 (n=2)	124	1.2
fASB 1 #6 (n=2)	539	5.2

Table 1 Transient expression of feline ASB in human embryonic kidney 293 cells. n= number of trials; hASB 10 is an antisense construct. ASB units are nmol NCS hydrolyzed per hour per ml of sample. Background level of expression was taken from the transfection with no DNA.

F. Assignment of the Feline ASB Gene to Chromosome A1.

To extend the molecular characterization of feline ASB and the feline model for MPS VI, the feline ASB gene was mapped to feline chromosome A1 by PCR. An ethidium bromide stained agarose gel showing the PCR amplified products from rodent, feline, or hybrid cell lines is shown in Figure 5. A 355 bp product is specifically amplified only in those reactions containing feline DNA, and not hamster or mouse genomic DNA. This product hybridizes to a specific feline internal oligonucleotide probe in the Southern hybridization shown in Figure 5.

Because the PCR conditions allowed for specific amplification of feline sequence on a hamster or mouse background, it was possible to screen two separate somatic cell hybrid panels, a cat/mouse panel (17T) and a cat/hamster panel (49C). Figure 6 shows the results of the PCR and hybridization screening of the cat/hamster panel, and Figure 7 shows the screening results of the cat/mouse panel. Percent concordance for the combined results of both panels is shown in Figure 8. It can be seen that fASB shows 97% overall concordance with feline chromosome A1. The discordance in 1 of 38 somatic cell hybrids may be due to the great sensitivity of PCR (29). Each hybrid in the panel is scored negative if less than 2% of the cells contain a particular chromosome, and it is well within the range of PCR sensitivity to detect chromosomal DNA present at this frequency (109). All other chromosomes have a discordance greater than or equal to 34%. A clear assignment was made to feline chromosome A1. ASB is the fifth locus in the cat to be mapped to chromosome A1. Two of these mapped loci are endothelial cell growth factor (ECGF) and colony stimulating factor

receptor (CSF-R) which, like ASB, map to chromosome 5q in humans. This strengthens the indication that synteny of human 5q may be conserved on feline A1. Eight other feline lysosomal enzymes have been mapped (41,113,90) and each of these is also located on the feline homologue (as determined by linkage) of the human chromosome to which the corresponding human gene maps.

Figure 5 Specific amplification of feline ASB sequences in feline/rodent somatic cell hybrids. NC, negative control with no DNA; C, cat genomic DNA; H, hamster genomic DNA; M, mouse genomic DNA; +, positive hybrid (17T2) ; +, positive hybrid (49C10); -, negative hybrid (49C11). This gel was blotted and hybridized with a feline specific internal oligonucleotide.

SPECIFIC AMPLIFICATION OF FELINE ARYLSULFATASE B SEQUENCES IN FELINE/RODENT SOMATIC CELL HYBRIDS

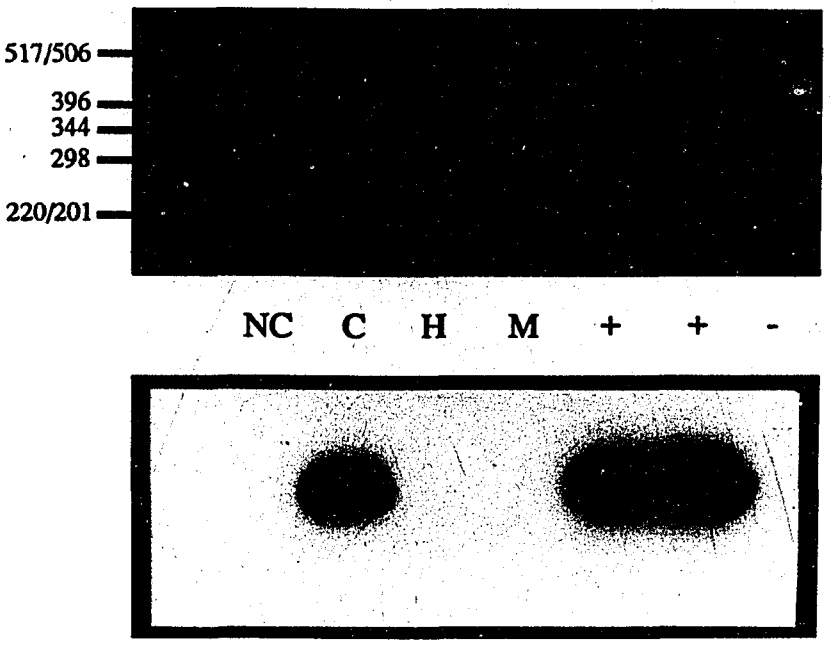


Figure 6 Segregation of ASB and feline chromosomes in feline/hamster somatic cell hybrids. Hybrids were scored negative for a chromosome if <2% of the cells contained the chromosome, and positive if >5% included the chromosome. u= uncharacterized.

Cat/Hamster Cell Hybrids	FELINE CHROMOSOMES																			
	A1	A2	A3	B1	B2	B3	B4	C1	C2	D1	D2	D3	D4	E1	E2	E3	F1	F2	X	ASB
49C1E	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	+	-
49C3E	-	-	-	+	+	-	+	-	-	-	+	+	+	-	-	-	-	+	-	-
49C4B	+	-	+	+	+	+	-	-	+	+	+	-	-	-	+	+	-	+	+	+
49C5E	-	+	-	-	-	+	-	+	-	-	-	+	+	-	-	-	-	+	+	-
49C7B	-	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-
49C9C	+	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	+	+
49C10B	+	-	-	-	+	-	-	-	+	-	-	+	-	-	-	-	-	+	+	+
49C11C	-	-	-	+	+	-	-	-	+	+	-	+	+	-	-	-	-	+	+	-
49C12D	+	-	-	-	-	-	+	-	+	+	+	+	+	-	-	+	-	-	+	+
49C13E	+	-	+	+	+	+	+	-	+	+	+	-	+	-	-	+	-	+	+	+
49C14D	-	-	+	+	-	+	+	-	+	-	-	-	-	-	-	+	-	-	+	-
49C15E	-	+	+	-	+	+	+	+	+	+	-	+	+	-	u	-	-	+	+	-
49C16F	-	+	-	+	+	+	+	+	+	+	-	+	+	-	-	-	-	+	+	-
49C18F	-	+	+	-	-	+	-	-	+	-	-	-	-	-	-	+	-	-	+	-
49C19E	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	+	-	-
49C20D	-	+	+	-	-	+	-	-	+	-	u	-	-	-	-	-	-	-	+	-
49C21D	-	-	+	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	+	-
49C24I	-	+	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	+	-
%Concordant	100	33	56	61	56	39	56	56	44	67	82	56	56	72	76	72	72	50	44	

Figure 7 Segregation of ASB and feline chromosomes in feline/murine somatic cell hybrids. Hybrids were scored negative for a chromosome if $<2\%$ of the cells contained the chromosome, and positive if $>5\%$ included the chromosome.

Cat/Mouse Cell Hybrids	FELINE CHROMOSOMES																			
	A1	A2	A3	B1	B2	B3	B4	C1	C2	D1	D2	D3	D4	E1	E2	E3	F1	F2	X	ASB
17T1G	+	-	-	+	+	+	+	-	+	-	-	-	-	-	-	-	-	+	-	+
17T2F	+	-	-	+	+	+	+	-	+	-	-	-	-	-	-	-	-	+	+	+
17T3E	+	-	+	-	-	+	+	+	+	-	+	-	+	+	-	-	-	+	-	+
17T4E	+	-	-	+	+	-	-	-	+	+	+	-	+	-	-	+	-	+	+	+
17T5H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
17T7E	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	-	-
17T8F	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	+	+	-	-	-
17T9D	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
17T10C	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	-	+	-	+
17T11D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
17T12G	+	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	-	+	+	+
17T26D	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-
17T27D	-	-	-	-	-	-	+	-	+	-	-	-	+	-	-	-	-	+	-	-
17T28E	-	-	-	-	-	-	-	+	-	-	+	-	+	-	-	+	-	+	-	+
17T29D	+	-	-	-	+	-	-	-	+	-	-	-	-	+	-	+	-	+	+	+
17T30C	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
17T33E	+	-	-	-	+	-	-	-	+	-	-	-	-	-	-	+	-	+	+	+
17T34D	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	+	-	+
17T36B	+	-	+	-	-	+	+	-	+	+	-	-	+	+	-	+	-	+	+	+
17T37D	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	-	-	+	-
%Concordant	95	35	50	60	65	70	55	40	85	50	50	40	45	50	40	55	25	80	55	

Figure 8 Overall concordance of feline ASB and feline chromosomes in feline/rodent somatic cell hybrids.

OVERALL CONCORDANCE OF ASB AND INDIVIDUAL FELINE CHROMOSOMES IN FELINE-RODENT SOMATIC CELL HYBRIDS

	FELINE CHROMOSOMES																		
	A1	A2	A3	B1	B2	B3	B4	C1	C2	D1	D2	D3	D4	E1	E2	E3	F1	F2	X
% Concordant	97	34	53	61	63	55	55	47	66	58	65	47	50	61	62	63	47	66	50

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