

Two Exon-Skipping Mutations as the Molecular Basis of Succinic Semialdehyde Dehydrogenase Deficiency (4-Hydroxybutyric Aciduria)

Ken L. Chambliss,¹ Debra D. Hinson,¹ Flavia Trettel,³ Patrizia Malaspina,³ Andrea Novelletto,³ Cornelis Jakobs,⁴ and K. Michael Gibson^{1,2,5}

¹Institute of Metabolic Disease, Baylor University Medical Center, and ²Department of Neurology, University of Texas Southwestern Medical Center, Dallas; ³Dipartimento di Biologia, Università di Roma "Tor Vergata," Rome; ⁴Department of Clinical Chemistry and Pediatrics, Free University Hospital, Amsterdam; and ⁵Department of Molecular and Medical Genetics, Oregon Health Sciences University, Portland

Summary

Succinic semialdehyde dehydrogenase (SSADH) deficiency, a rare metabolic disorder of 4-aminobutyric acid degradation, has been identified in ~150 patients. Affected individuals accumulate large quantities of 4-hydroxybutyric acid, a compound with a wide range of neuropharmacological activities, in physiological fluids. As a first step in beginning an investigation of the molecular genetics of SSADH deficiency, we have utilized SSADH cDNA and genomic sequences to identify two point mutations in the SSADH genes derived from four patients. These mutations, identified by standard methods of reverse transcription, PCR, dideoxy-chain termination, and cycle sequencing, alter highly conserved sequences at intron/exon boundaries and prevent the RNA-splicing apparatus from properly recognizing the normal splice junction. Each family segregated a mutation in a different splice site, resulting in exon skipping and, in one case, a frameshift and premature termination and, in the other case, an in-frame deletion in the resulting protein. Family members, including parents and siblings of these patients, were shown to be heterozygotes for the splicing abnormality, providing additional evidence for autosomal recessive inheritance. Our results provide the first evidence that 4-hydroxybutyric aciduria, resulting from SSADH deficiency, is the result of genetic defects in the human SSADH gene.

Introduction

Succinic semialdehyde dehydrogenase (SSADH; E.C. 1.2.1.24 [MIM 271980]) deficiency is a defect in the 4-aminobutyric acid (GABA) degradative pathway that results in the organic aciduria 4-hydroxybutyric aciduria (Gibson et al. 1994) (fig. 1). The enzyme deficiency causes not only an increase of the neurotransmitter GABA but also significant accumulation of 4-hydroxybutyric acid (GHB) in physiological fluids, presumably through the NAD(P)H-linked reduction of succinic semialdehyde by one or more GHB dehydrogenases (Cash et al. 1979). These observations suggest that SSADH deficiency is a unique disorder, since two neuropharmacologically active compounds accumulate.

4-Hydroxybutyric aciduria was first reported in 1981 (Jakobs et al. 1981) in a child presenting with neurological abnormalities. Since then, ~150 patients with SSADH deficiency have been identified, with considerable variability in phenotype, ranging from mild retardation in mental, motor, and language development to more-severe neurological defects associated with hypotonia, ataxia, and seizures (Gibson et al. 1997). Consanguinity in many families and demonstration of intermediate enzyme activity in the parents of affected patients suggest that SSADH deficiency is inherited in an autosomal recessive fashion. Molecular cloning of the human gene, as well as the mapping of it to chromosome 6p22 (Trettel et al. 1996), suggests that the mode of inheritance for this disorder is autosomal recessive.

Tentative identification of SSADH deficiency is achieved through detection of elevated levels of GHB in physiological fluids (Jakobs et al. 1990). Verification of the diagnosis is achieved by enzymatic assay of SSADH in the patient lymphocytes, lymphoblasts, or fibroblasts (Gibson et al. 1991, 1994), and prenatal diagnosis of 4-hydroxybutyric aciduria has been reported in several cases (Jakobs et al. 1993; Gibson et al. 1994). In affected pregnancies, GHB is elevated in amniotic fluid, and SSADH activity is absent from cultured amniocytes and,

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Address for correspondence and reprints: Dr. K. Michael Gibson, Department of Molecular and Medical Genetics, Oregon Health Sciences University, Baird Hall, Room 2029, Mail Code L473, 3181 S.W. Sam Jackson Park Road, Portland, OR 97201-3098. E-mail: gibsonm@ohsu.edu

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in one case, from autopsied fetal brain, liver, and kidney (Chambliss et al. 1993).

Although it is highly likely that defects in the SSADH gene are the cause of SSADH deficiency, thus far there has been no evidence to support this hypothesis. Recently, we reported cDNAs encoding rat and partial human SSADH (Chambliss et al. 1995). Cosmid clones were employed to localize the gene to human chromosome 6p22 (Trettel et al. 1996). An extremely high G+C content in the 5' end of the human SSADH transcript prohibited isolation of full-length human cDNA clones. In the present article, we have used recently obtained genomic cosmid clones to derive the remainder of the cDNA structure of human SSADH, including the putative mitochondrial leader sequence. A composite human cDNA expresses enzymatically active SSADH protein. We also report, in four patients (two families), two mutations in the SSADH gene that lead to exon skipping, and we document that these mutant alleles exist at the cDNA and genomic level. These alleles have been labeled "ALDH4A1*1" (G→T transversion, first base of intron 9) and "ALDH4A1*2" (G→A transition, first base of intron 5), according to the nomenclature proposed by Nebert and coworkers (Vasiliou et al., in press). In the consanguineous matings, inheritance was confirmed by allele analysis in family members. These exon-skipping mutations result in near-complete absence of SSADH activity, representing the first demonstration that structural defects in the SSADH gene can be the cause of SSADH deficiency (4-hydroxybutyric aciduria).

Material and Methods

Lymphoblastoid Cell Lines and Enzyme Assay

Lymphocytes isolated from blood samples of patients, family members, and controls were used to assay SSADH activity, as has been reported elsewhere (Gibson et al. 1991), as well as to establish lymphoblastoid cell lines by standard procedures using Epstein-Barr virus. Cells were grown, maintained, and assayed as reported else-

Table 1

SSADH-Specific Primers for RT-PCR and Genomic PCR

| Primer | Sequence | Orientation |
|--------|-------------------------|-------------|
| 1 | TCTGGGCATGGTAGCCGACTG | Forward |
| 2 | CTTTTCACAGAGTTTGCTGC | Reverse |
| 3 | GCATCTAAATTTAGGAACACTGG | Forward |
| 4 | CAAAGAATCCTACAAGCCCC | Reverse |
| 5 | CCTATATATGTAGATAAGTCTCC | Reverse |
| 6 | CTGAGTTATAACGGGGAATGGT | Forward |
| 7 | TGCACCTTCTTCTTCCCACA | Reverse |
| 8 | GTAATTTGTTGGCACATGTTTG | Forward |
| 9 | TGGTGATCAGGATGAAATAG | Reverse |

where (Gibson et al. 1997). The four patients studied were from consanguineous marriages, and clinical findings have been reported in earlier articles (Haan et al. 1985; Gibson et al. 1997).

Nucleic-Acid Isolation, Amplification, and Sequencing

DNA and total RNA were isolated from lymphoblast cell lines by means of established procedures (Chambliss et al. 1995). Reverse transcription (RT) of ~10 µg of total RNA was performed at 42°C for 1 h, by means of Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco BRL) with a gene-specific antisense primer (primer 5; table 1). Subsequent PCR amplification was performed with Expand High Fidelity thermostable polymerase (Boehringer Mannheim), either with an exon 6 forward primer (primer 3) and exon 10 reverse primer (primer 4), to examine exon 9 deletions, or with an exon 1 forward primer (primer 1) and an exon 6 reverse primer (primer 2), to examine exon 5 deletions (for exon structure, see fig. 2).

For amplification of exon 9 from genomic DNA, 100 ng of genomic DNA from lymphoblast cell lines was used in PCR amplifications using primers 6 and 7. Primers 8 and 9 were used to amplify exon 5 and its flanking regions. Amplification products were excised from agarose gels and were purified by means of the Qiaex II gel-extraction kit (Qiagen). Direct sequencing of purified

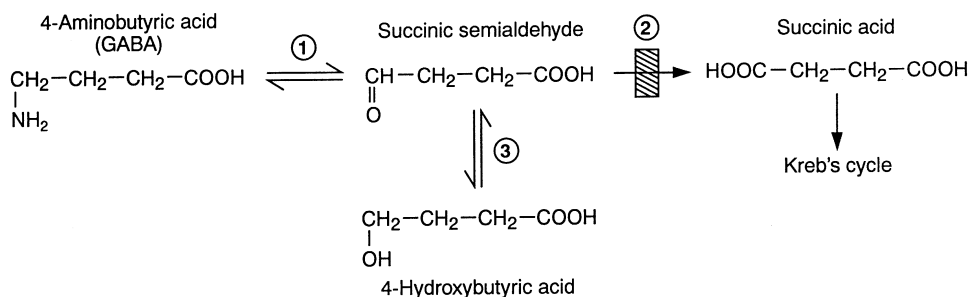


Figure 1 GABA degradative pathway. 1 = GABA-transaminase; 2 = SSADH; and 3 = one or more 4-hydroxybutyrate dehydrogenases. The cross-hatched box denotes the site of the defect in patients with SSADH deficiency.

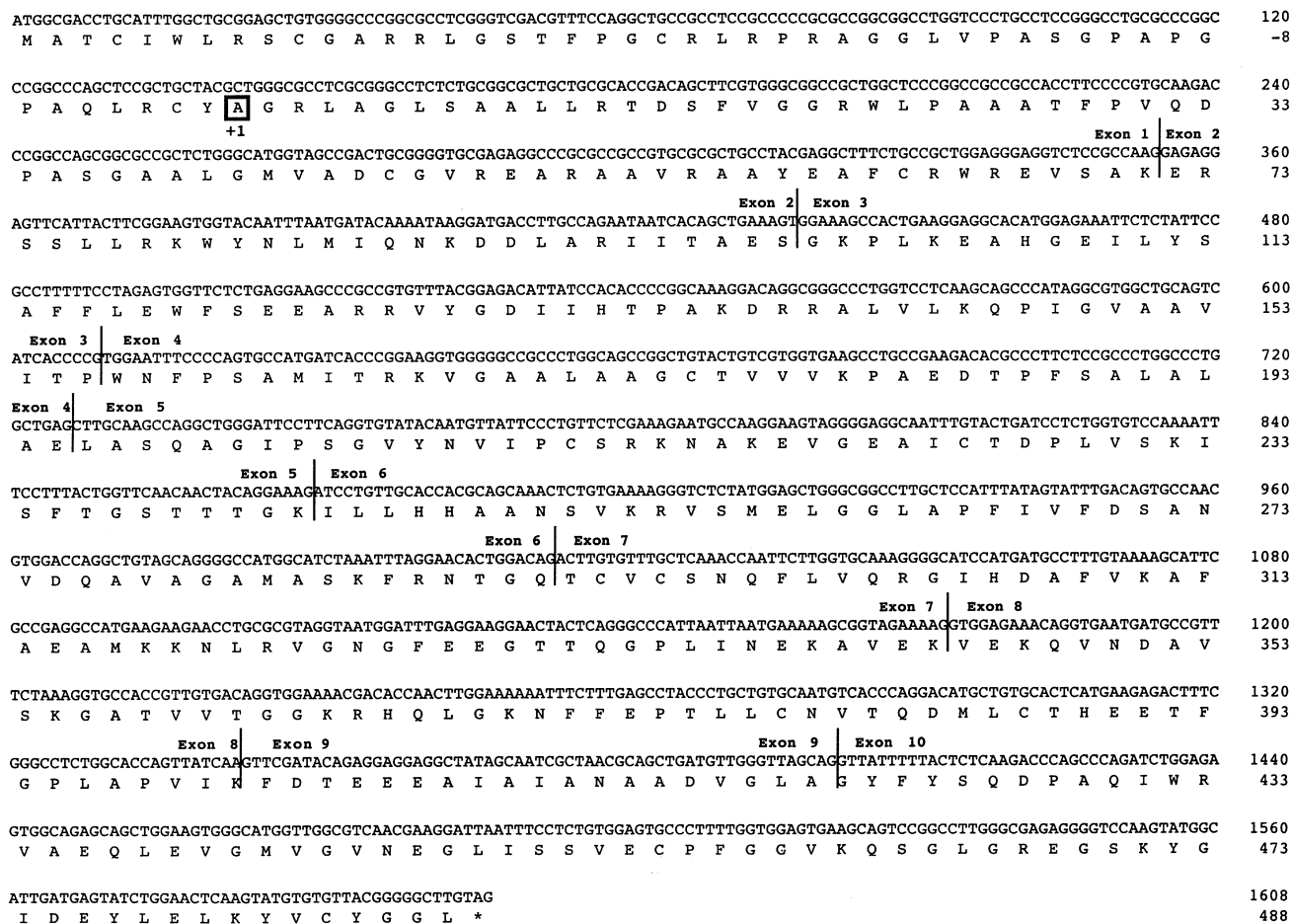


Figure 2 Nucleotide and deduced amino acid sequence of human SSADH. Nucleotides and amino acid residues are numbered to the right, with the first residue of the mature protein being boxed and numbered as “+1.” The positions of the nine introns that split the coding region were determined by comparison of cDNA and genomic clones and are indicated by vertical lines. This sequence has been deposited in GenBank (accession number Y11192).

products was performed by means of Thermo Sequenase DNA polymerase and Redivue [³³P]-labeled terminators (Amersham).

An intermediate product of the amplification of RNAs from individuals heterozygous for the exon 5 deletion was shown to be produced during PCR reactions in which both wild-type and exon 5–deletion templates were present and independent of any cellular RNA. The wild-type and exon 5–deficient RT-PCR products were purified extensively by multiple agarose gels and subsequent Qiaex purification. Individual amplification of purified wild-type or exon 5–deficient PCR products yielded a single product identical to the starting template; however, simultaneous amplification of the two templates produced not only the two starting templates but also an intermediate product, seen in the heterozygotes (data not shown); this suggests that this PCR product, intermediate between the wild-type and exon 5–de-

ficient amplicons, is due to heteroduplex formation due to reannealing of one strand containing exon 5 and one strand deficient in exon 5.

Expression of Recombinant Human SSADH

The human SSADH-expression clone was constructed by first subcloning the most full-length cDNA (GenBank accession number R20294; I.M.A.G.E. consortium, Lawrence Livermore National Laboratory) (Lennon et al. 1996) into the bacterial-expression vector pGEX-4T3 (Pharmacia). This construct lacked the DNA region encoding the first 21 amino acid residues of the mature protein. A segment from an SSADH genomic subclone encoding the additional 21 residues plus 47 amino acid residues of the putative mitochondrial targeting sequence was ligated into the proper position of the expression clone by means of *Xma*CI and *Not*I restriction

sites. Bacterial-cell lines, growth conditions, and enzymatic assay of bacterial extracts were those used in protocols reported elsewhere (Chambliss et al. 1995).

Results

Completion and Expression of the Human SSADH cDNA

We recently isolated and characterized a 1,091-bp human liver SSADH cDNA that encoded ~66% of the mature human SSADH protein (Chambliss et al. 1995). A search of the GenBank database revealed three additional expressed sequence tag (EST) cDNAs (R20294, H06675, and H46643; I.M.A.G.E. consortium, L.L.N.L) (Lennon et al. 1996) that overlapped the partial human SSADH cDNA and that extended the sequence farther upstream, to include all but the N-terminal 21 amino acids of the mature human protein (fig. 2). The remainder of the human SSADH cDNA coding region can be inferred from human SSADH genomic cosmid clones from chromosome 6 (Trettel et al. 1996). The genomic clones contain sequences that overlap the most full-length cDNAs and extend through the mature N-terminus coding region, which was verified by amino acid sequencing of the purified protein (Chambliss et al. 1995). The genomic clones also extended beyond the putative start codon 47 amino acid residues upstream of the mature N-terminus. Several observations suggest that this AUG is the translation-initiation site. First, there is a single AUG codon in reasonable proximity to (and in the proper reading frame with) the mature N-terminus; the next closest AUG is 477 bp upstream from the mature N-terminus and has two stop codons shortly downstream. Second, this AUG is within a Kozak consensus translational start motif (Kozak 1991). Last, the 47 amino acids from the N-terminal methionine to the mature N-terminus are recognized, by computer analysis (PROSITE), as a mitochondrial targeting sequence with a predicted cleavage site exactly at the point of the mature N-terminus (Gavel and von Heijne 1990).

Despite these largely confirmatory observations, we cannot exclude the possibility that there is another intron somewhere within this region, since it is relatively common for some or most of a leader peptide to be on an independent exon. Thus far, multiple attempts at RT-PCR and RACE, with a wide variety of primer pairs derived from genomic DNA sequence, have been unsuccessful in producing a PCR product from this region to verify that it is indeed within the cDNA sequence. This most likely relates to the extremely high G+C content and probable secondary structure within this region. Once a PCR product is obtained, it will be of value to investigate precursor translocation and proteolytic-pro-

cessing in isolated mitochondria, in addition to transfection into patient lymphoblasts.

As a first step in verification that the isolated sequence is human SSADH, a composite construct was made, which encoded the full-length human SSADH protein (with putative leader) fused to glutathione-S-transferase. Bacterial extracts overexpressing the protein were assayed for SSADH activity, with the following results: rat composite cDNA (without leader), 1,345–1,390 nmol/min/mg protein; human expression clone (with putative mitochondrial leader), 60–65 nmol/min/mg protein; and expression vector only, 15–18 nmol/min/mg protein (in each case, the results are those of two independent expression studies). Although the recombinant human enzyme was considerably less active (22-fold less) than recombinant rat SSADH, the human protein had significant SSADH activity, compared with that in controls expressing glutathione-S-transferase only. It is unclear why the human enzyme was less active than the rat enzyme; however, comparable results were obtained during purification of SSADH from human brain and rat brain. In those studies, the final specific activity of purified rat-brain SSADH was 21.39 $\mu\text{mol}/\text{min}/\text{mg}$ protein, whereas that for the human brain enzyme was 2.74 $\mu\text{mol}/\text{min}/\text{mg}$ protein (Chambliss and Gibson 1992). Although the presence of the mitochondrial targeting sequence in the recombinant fusion protein may have negatively impacted enzyme activity, the recombinant fusion protein still had four times the background activity, indicating that we indeed were measuring SSADH activity.

Mutation of the SSADH Gene in Four Patients

Knowledge of the entire coding region of human SSADH enabled us to examine the gene of patients with 4-hydroxybutyric aciduria, for the presence of possible molecular defects. Four previously reported patients from two families, along with available family members, were investigated. The patients were two siblings, OD and PD, and their cousin, ZZ, from one family and a single child, patient AC, from a second, unrelated family (Haan et al. 1985; Jakobs et al. 1993). All patients were from consanguineous matings, with ZZ and AC being from a first-cousin marriage and with OD and PD being from a second-cousin marriage. SSADH enzyme activities in extracts of cells from one of the families (three patients and their family members) are presented in table 2. Residual activity in patient cells was very low and, in most cases, nearly undetectable. Intermediate activities were observed in parents' and siblings' cell lines. Enzyme activities for both patient AC and his parents have been reported elsewhere and are consistent with those reported here for members of the family of patients OD and PD. The very low SSADH activities in extracts of cells from members of the family of patient ZZ are in-

Table 2**SSADH Enzyme Activities of SSADH-Deficient Patients and Their Family Members**

| Cell Type and Subject ^a | SSADH Specific Activity ^b (nmol/min/mg protein) |
|-------------------------------------|---|
| Lymphoblasts: ^c | |
| Family of ZZ: | |
| ZZ | 0 [0%] |
| Father | .09 [8%] |
| Mother | .18 [16%] |
| Male sibling | .11 [10%] |
| Female sibling | .11 [10%] |
| Controls for family (<i>n</i> = 5) | 1.15 ± .27 [NA] |
| Family of OD and PD: | |
| OD | .013 [1%] |
| PD | .022 [1%] |
| Father | 1.467 [79%] |
| Mother | 1.247 [67%] |
| Sibling (male) | .836 [45%] |
| Controls for family (<i>n</i> = 4) | 1.85 ± .43 [NA] |
| Lymphocytes: | |
| Family of OD and PD: | |
| OD | .015 [7%] |
| PD | .000 [0%] |
| Father | .107 [53%] |
| Mother | .065 [32%] |
| Sibling (male) | .099 [49%] |
| Controls for family (<i>n</i> = 5) | .20 ± .06 [NA] |

^a Patients ZZ, OD, and PD are related (Jakobs et al. 1990).

^b Percentages are percentage of control values; NA = not applicable. Control values are the average of assays from four or five parallel control cell lines.

^c Analyses of patient ZZ and of patients OD and PD were performed on separate occasions.

triguing, since they were considerably below both the values expected in heterozygotes and the values detected in members of the family of patients OD and PD (who carry an identical mutation). Unfortunately, additional members of the family of patient ZZ (i.e., individuals who did not harbor the splicing defect and could be assayed for SSADH activity in lymphoblasts) were not available; lymphoblasts from these individuals would be more appropriate as controls than are random lymphoblasts from unrelated, unaffected individuals. The data suggest the possibility that another gene (or gene product) affects SSADH activity and is different between these two families, although, at present, there are no data to support this possibility.

We performed RT followed by PCR (RT-PCR) analysis of RNA isolated from lymphoblasts of the aforementioned SSADH-deficient patients (ZZ, OD, and PD), their parents and siblings, and two unrelated SSADH-deficient patients (SM and IA). Analysis of the RT-PCR products on agarose gels revealed shortened amplicons for all patients except SM and IA, compared with those in controls (figs. 3 and 4). Direct sequencing of the amplicons in OD, PD, and ZZ revealed an exact deletion

of the 59-bp exon 9 (Trettel et al. 1996) (fig. 5). The parents of OD and PD and the parents of ZZ were heterozygous for the exon 9 deletion, as were a male and female sibling of ZZ and a male sibling of OD and PD. All controls and unrelated patients were homozygous for the presence of exon 9. The net effect of the missing exon is a frameshift after amino acid residue 401 (of a total of 488 amino acids in the mature protein), followed by 52 nonsense residues before a stop codon is reached. Of the missing 87 amino acid residues, 26 are conserved between human, rat, and bacterial SSADH (Chambliss et al. 1995). Of these 26 residues, 11 are highly conserved among many aldehyde and semialdehyde dehydrogenases, from mammals to bacteria, including 6 invariant residues at N413, G441, N446, G459, S463, and G466 (Hempel et al. 1993; Chambliss et al. 1995).

Examination of the genomic region surrounding exon 9 was performed to determine why the exon sequence was missing in the cDNA. Intron-specific primers on either side of exon 9 were employed in PCR amplifications using genomic DNA isolated from the lymphoblasts of patients OD, PD, and ZZ, of their family members, and of controls. All samples yielded the expected 471-bp product. Direct sequencing of the products revealed that exon 9 sequence was present in all individuals tested; however, patients OD, PD, and ZZ were homozygous for a G→T transversion at the first base of intron 9 in the splice-donor site (fig. 6). The parents and siblings of these patients proved to be heterozygous for the transversion, a finding that was consistent both with the RT-PCR results and the enzymatic assay; none of the controls exhibited the base change. Thus, the three patients in this family are homozygous for an SSADH allele that leads to a splicing defect (skipping of exon 9), whereas the unaffected parents and siblings are heterozygous for this allele.

Direct sequencing of the single RT-PCR product from patient AC (fig. 4) revealed an exact deletion of the 144-bp exon 5 (fig. 7). In RT-PCR, the parents of AC exhibited four amplified products. The top two bands are present in control samples. The more prominent of these two bands—the lower band (632 bp)—displayed wild-type sequence on direct sequencing, whereas the less intense of the two bands—the upper band—was a mixture of sequences containing wild-type SSADH and another, unidentified sequence. This upper band appears to be a PCR artifact. The smallest RT-PCR product (488 bp) in the parents proved to be the same 144-bp deletion of exon 5 as was seen in the patient. Direct sequencing of the RT-PCR product intermediate between the wild-type DNA band and exon 5-deficient DNA band shows a mixture of sequences consistent with both the wild-type product and the exon 5-deficient product. Further experimentation (see Material and Methods) indicated that

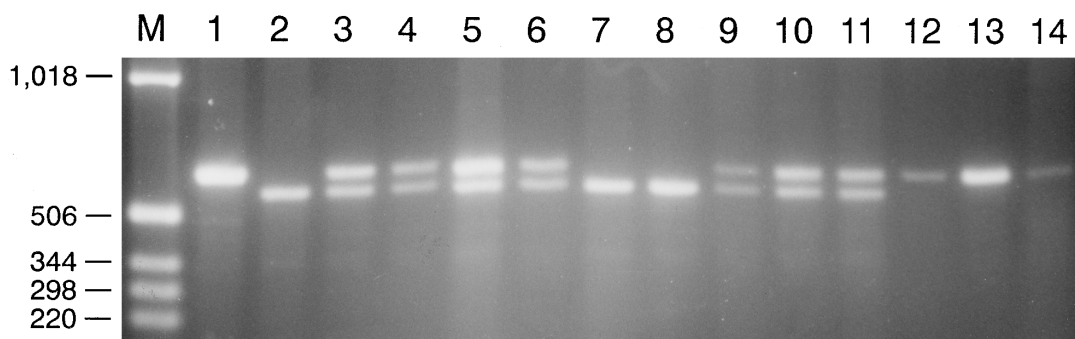


Figure 3 RT-PCR products from five patients with 4-hydroxybutyric aciduria, their family members, and a control, analyzed on an ethidium bromide-stained agarose gel. The amplicon from a normal SSADH cDNA is 638 bp. Three of the patients exhibit a visibly smaller product, whereas their parents and unaffected siblings have both RT-PCR products, indicating heterozygosity. Two unrelated patients have the wild-type-size amplicon. Lane M, Molecular-weight size standards. Lane 1, Control. Lane 2, Patient ZZ. Lane 3, Father of ZZ. Lane 4, Mother of ZZ. Lane 5, Brother of ZZ. Lane 6, Sister of ZZ. Lane 7, Patient OD. Lane 8, Patient DP (sister of OD). Lane 9, Father of OD and PD; Lane 10, Mother of OD and PD. Lane 11, Brother of OD and PD. Lane 12, Unrelated patient SM. Lane 13, SM mother. Lane 14, Unrelated patient IA.

this product was an artifact most likely due to heteroduplex formation between strands with or without exon 5 sequence. The result of the exon 5 deletion is an in-frame excision of 48 amino acid residues (196-242), 17 of which are conserved between human, rat, and bacterial SSADH (Chambliss et al. 1995); 11 of these 17 residues are highly conserved among other mammalian and bacterial aldehyde dehydrogenases with the two invariant glycines G237 and G242 (Hempel et al. 1993).

Examination of the genomic DNA sequence around exon 5 was performed by PCR amplification using gene-specific intron primers flanking exon 5 (fig. 8). The sequence of patient AC revealed a G→A transition at the first base of intron 5 in the splice-donor site, whereas the parents were heterozygous for this base change. These findings are consistent both with RT-PCR results and with the results of previously reported enzymatic assays. Thus, the patient in this family is homozygous for an SSADH allele that leads to the skipping of exon 5, whereas the healthy parents are heterozygous for the allele.

The remaining coding regions of patients OD, PD, ZZ, and AC were amplified by RT-PCR, sequenced, and shown to have no changes from the published wild-type sequence. Additionally, to examine the possibility that one or both of these mutations may be common in other SSADH-deficient patients, genomic DNA from lymphoblasts of 11 individuals with SSADH deficiency in 10 families, from 9 family members from 3 of the families, and from 5 control cell lines was used to amplify the regions around exons 5 and 9, and the amplification products were sequenced. Neither of these mutations was observed in any other patients or controls (data not shown).

Discussion

We have characterized human SSADH cDNAs and have completed the identification of the regions encoding the putative mitochondrial leader sequence and mature protein, as a first step in beginning an investigation of the molecular genetics of SSADH deficiency. Recombinant human protein encoded by a composite cDNA clone and expressed in *Escherichia coli* was shown to have SSADH activity. By using the SSADH cDNA and genomic sequence, we have identified, in the SSADH genes of four patients, two point mutations that alter the highly conserved sequences at intron/exon boundaries and that prevent the RNA splicing apparatus from properly recognizing the normal splice junction. The re-

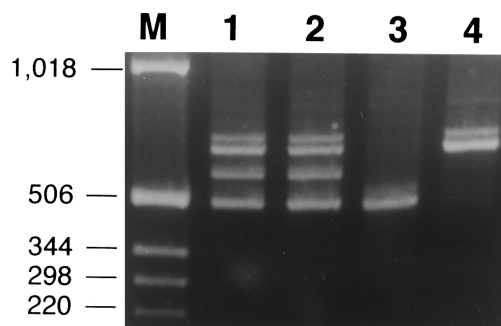


Figure 4 RT-PCR products from patient AC (lane 3), his parents (lanes 1 and 2), and a control (lane 4), analyzed on an agarose gel. Lane M, Size standards. The dominant product obtained from the control sample is 632 bp, whereas the single product from PCRs of the patient is 144 bp smaller (i.e., 488 bp). The intermediate-size product was shown to be artifactual and independent of RNA template.

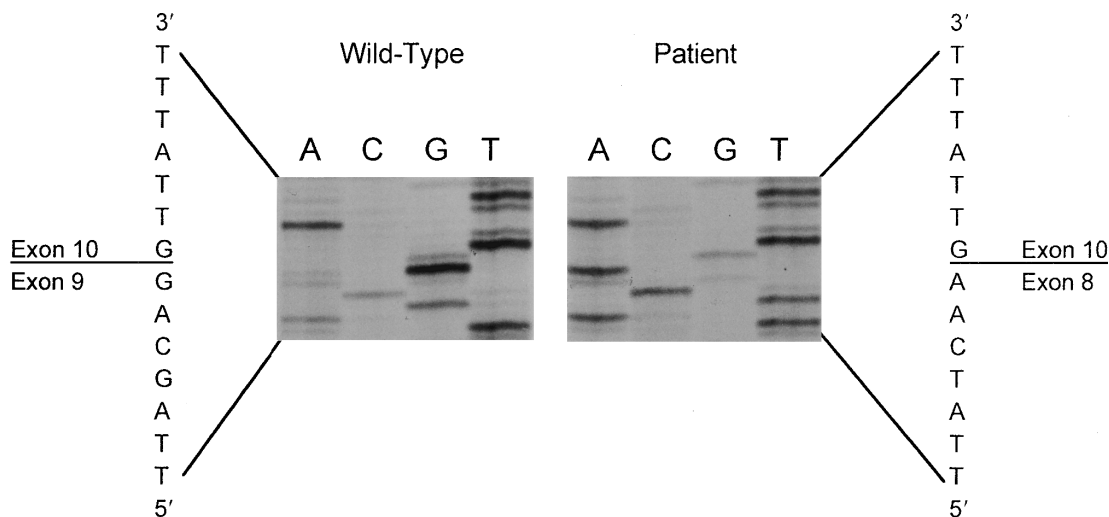


Figure 5 DNA sequence of representative RT-PCR products from the gel in figure 3. The larger band consistently contained the wild-type SSADH cDNA sequence, whereas the smaller amplicon consistently exhibited a complete deletion of the 59-bp exon 9.

sulting RNA splicing errors result in both SSADH deficiency, as demonstrated by enzymatic assay of patient lymphocytes and lymphoblasts, and 4-hydroxybutyric aciduria, as established by previous biochemical investigations of patient blood and urine. Our results provide the first demonstration of genetic defects in the SSADH gene that lead to SSADH deficiency, and they confirm the autosomal recessive nature of the disorder.

It has been estimated that, of all genetic diseases caused by point mutations, 15% are in RNA consensus splice sites (Krawczak et al. 1992). Mutations at the first base of an intron at the invariant G of the splice-donor

site are involved in the majority of 5' splice-site mutations. The most common mutation at this position is a G→A transition, which was observed in patient AC. Other genetic disorders documented to have been caused by a G→A change at the same site include retinoblastoma, acute porphyria, phenylketonuria, Ehlers-Danlos syndrome VII and IV, and many others (Krawczak et al. 1992). Although not as frequently reported, G→T transversions, as seen in patients OD, PD, and ZZ, also have been documented in a number of genetic diseases, including androgen insensitivity, cystic fibrosis, hemophilia B, and retinoblastoma (Krawczak et al. 1992). In

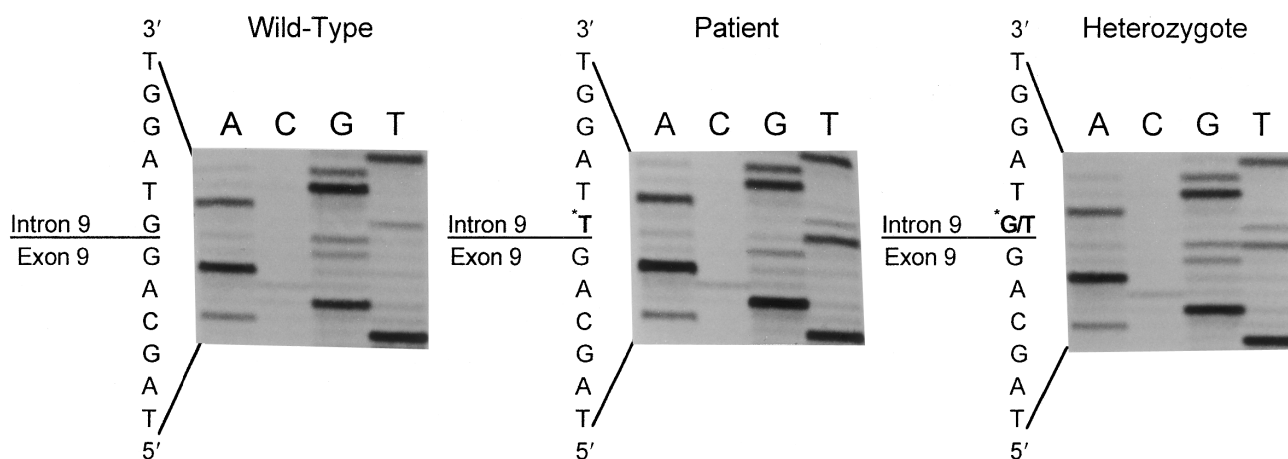


Figure 6 Representative DNA sequence of PCR products amplified from genomic DNA of wild-type controls (*left*), patients (*center*), and family members of patients (*right*). The three patients, (ZZ, OD, and PD) all contained a G→T mutation at the first base of intron 9, which alters the 5' splice site. Family members of patients who exhibited both RT-PCR products proved to be heterozygous for this mutation.

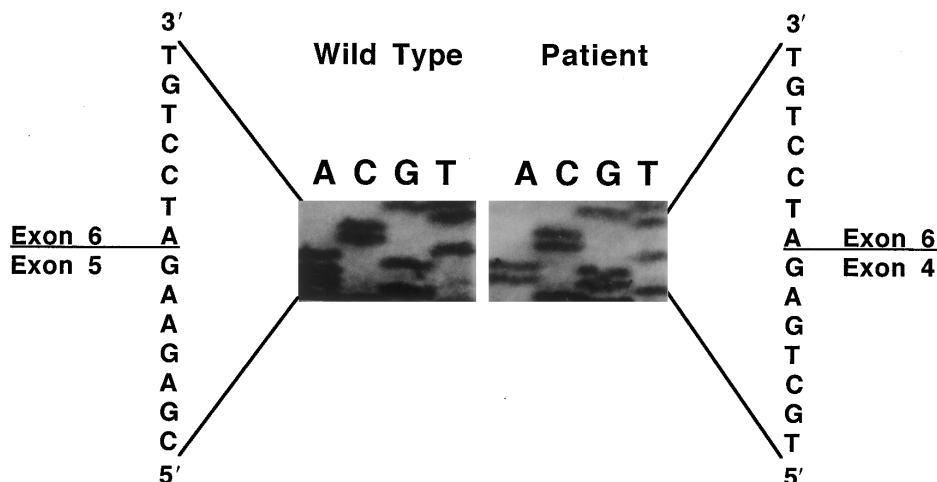


Figure 7 DNA sequence of representative RT-PCR products from the gel in figure 4. All lower bands were missing the 144-bp exon 5, whereas the predominant upper band contained wild-type sequence. The less predominant upper band and the intermediate band were shown to be PCR artifacts (see text).

the aforementioned disorders, the consensus 5' splice-site mutations lead to a skipping of the exon preceding the mutation, as seen in both mutations in the present report, and/or to the utilization of a cryptic splice site somewhere within the intron.

SSADH is clearly a member of the aldehyde dehydrogenase superfamily, sharing 36%–38% homology to mammalian general aldehyde dehydrogenases. Comparison of SSADH with other aldehyde and semialdehyde dehydrogenases, in both prokaryotes and eukaryotes, reveals amino acid residues with important enzymatic

function, as evidenced by their strict conservation (Hempel et al. 1993; Chambliss et al. 1995). The exons missing in patients described in the present report remove several of these key—and, in some cases, invariant—residues. The ideal situation in which to examine the effects of such changes on the enzyme activity is represented by the lymphoblast cell lines derived from the homozygous patients. These patients represent an in vivo system in which only the mutant form of SSADH is produced. It is apparent that these mutations abolish SSADH activity. Further corroboration of the effects of

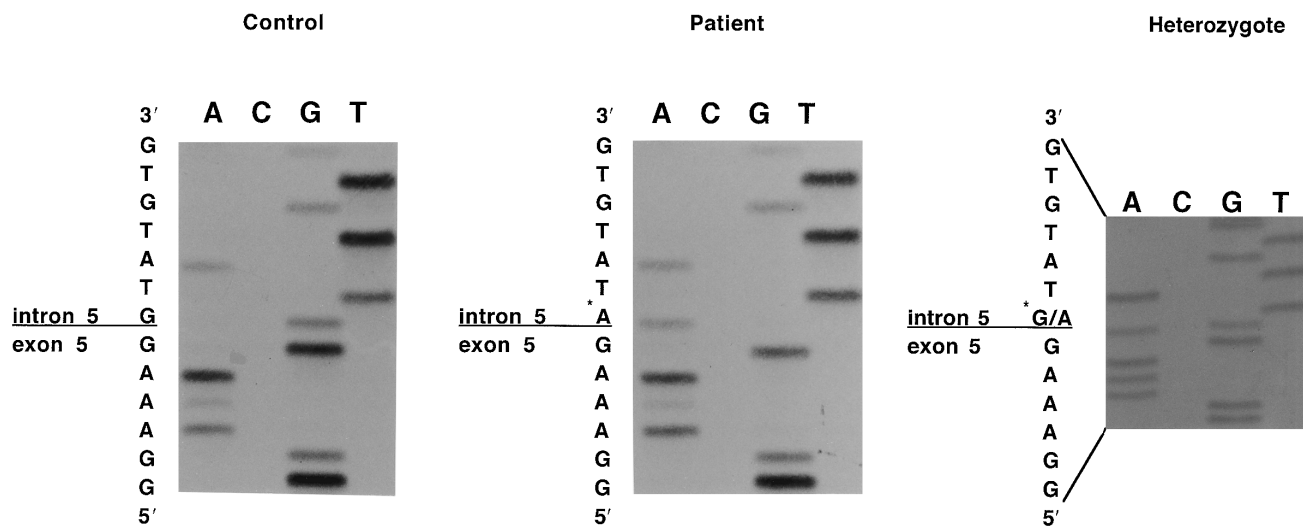


Figure 8 Genomic DNA sequence around exon 5 in controls (left), patient AC (center), and patient AC's parents (right), examined by PCR. The single, 271-bp amplicon was directly sequenced. The patient was homozygous for a G→A base change at the first base of intron 5. His parents were heterozygous for the normal G at that position and the mutant A. Controls showed wild-type sequence.

the mutations is obtained in the patients' heterozygous parents and siblings, who have intermediate activity, compared with the homozygous mutant patients and the unaffected controls.

The clinical phenotype of 4-hydroxybutyric aciduria varies from mild to severe and is nonspecific in presentation. Most patients exhibit some degree of neurological deficit, although speech delay, hypotonia, ataxia, and seizures vary from patient to patient, even within the same family. The patients in the present study are moderately affected, exhibiting developmental and speech delays, hyporeflexia, and behavioral problems, including mild autism. Patients AC, OD, and PD exhibit hypotonia, and patients OD and PD manifest ataxia. Patient ZZ is the first of only two reported cases of 4-hydroxybutyric aciduria in an adult and was 23 years of age at the time of examination, in 1990. At the initial presentation, all patients had increased GHB in blood and urine. For all patients, including those in the present study, residual SSADH activity measured in extracts of cultured cells is <5% of control values.

Thus far, correlation between either GHB levels or residual SSADH activity and clinical symptoms has not been demonstrated. Similar levels of GHB in patient physiological fluids have been seen in both mildly and severely affected patients. One previously reported patient (Gibson et al. 1997) presented with only mild oculomotor problems, truncal ataxia, and no other major neurological sequelae, and this patient already has developed a vocabulary that is nearly age appropriate. Ironically, this patient has almost no residual enzyme activity. Other patients, with comparable levels of residual enzyme activity, have suffered a devastating clinical course, with severe psychomotor retardation leading to a persistent vegetative state (DeVivo et al. 1988). Whether this heterogeneity is the result of mutation severity, tissue expression and/or penetrance, modifier genes, environmental influences, or other factors remains to be determined. However, a first step in trying to unravel this phenotypic heterogeneity is a careful analysis of the molecular genetics of SSADH deficiency.

The prevalence of SSADH deficiency in the general population is not known and is difficult to estimate. Patients are identified only when primary-care physicians or metabolic specialists submit samples to laboratories that analyze organic-acid patterns. The major difficulty is the nonspecific phenotypic presentation of SSADH deficiency, and many patients have received a differential diagnosis of autism, fragile-X syndrome, or idiopathic mental retardation. We suggest that many patients presenting in neurology clinics are undiagnosed because organic-acid analysis is not requested. Additionally, GHB in urine is an unstable compound that could be missed if laboratory staff who analyze organic acids are inexperienced. Assuming that, because spe-

cialized assays are required, SSADH deficiency is only rarely detected and noting that the number of patients identified (~150) is relatively large, we could speculate that the disorder would occur as frequently as some of the other rare metabolic disorders, such as medium-chain acyl-CoA dehydrogenase deficiency, the frequency of which has been estimated to be 1/6,400–1/46,000, with 1%–2% of the population estimated to be heterozygous for a disease-causing allele (Roe and Coates 1995).

Once 4-hydroxybutyric aciduria is diagnosed on the basis of organic-acid profiling, a common treatment is use of the antiepileptic vigabatrin (gamma-vinyl GABA; Sabril) (Gibson et al. 1995). Pharmacologically, the mode of action of this drug is an irreversible inhibition of GABA-transaminase, leading to accumulation of free and total GABA in brain. The results of this therapy have been encouraging in some patients and of little value in others. Patients for whom vigabatrin has shown clinical efficacy had improvement in ataxia (when present), increased alertness, improved attention span, and better manageability. Other patients had no improvement in clinical signs, and at least two patients experienced seizures during vigabatrin intervention (Gibson et al. 1989; Matern et al. 1996). There is an obvious need for better therapeutic agents for treatment of SSADH-deficient patients. We are currently pursuing the production of a murine transgenic model of the disease, which will be of great use in examination of other currently available drugs that may provide better therapeutic efficacy.

Carrier screening of the general population for SSADH deficiency is currently impossible and unfeasible. Although lymphocyte SSADH activity of heterozygous individuals is generally intermediate between that in patients and that in controls, the range of carrier enzyme activity overlaps both patient and control values. It is not yet known whether there will be a wide variety of mutations in the SSADH gene or whether shared alleles will be found to account for a high percentage of patients. As the genetic defects in other documented patients are identified, it may become possible to use molecular tests to screen high-risk populations (i.e., those in which there is a high incidence of consanguineous marriages), for the presence of shared disease-causing alleles. To achieve this, we will need to have as complete a picture as possible of the molecular abnormalities responsible for SSADH deficiency; these studies are in progress.

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Electronic-Database Information

GenBank, <http://www.ncbi.nlm.nih.gov/Web/Genbank> (for EST cDNAs [accession numbers R20294, H06675, and H46643])

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/omim> (for SSADH; E.C.1.2.1.24 [MIM 271980])

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