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Mutations in Argininosuccinate Synthetase mRNA of Japanese Patients, Causing Classical Citrullinemia

Keiko Kobayashi, Nazma Shaheen, Hiroki Terazono, and Takeyori Saheki

Department of Biochemistry, Faculty of Medicine, Kagoshima University, Kagoshima, Japan

Summary

Citrullinemia is an autosomal recessive disease caused by a genetic deficiency of argininosuccinate synthetase. In order to characterize mutations in Japanese patients with classical citrullinemia, RNA isolated from 10 unrelated patients was reverse-transcribed, and cDNA amplified by PCR was cloned and sequenced. The 10 mutations identified included 6 missense mutations (A118T, A192V, R272C, G280R, R304W, and R363L), 2 mutations associated with an absence of an exon 7 or exon 13, 1 mutation with a deletion of the first 7 bp in exon 16 (which might be caused by abnormal splicing), and 1 mutation with an insertion of 37 bp within exons 15 and 16 in cDNA. The insertion mutation and the five missense mutations (R304W being excluded) are new mutations described in the present paper. These are in addition to 14 mutations (9 missense mutations, 4 mutations associated with an absence of an exon in mRNA, and 1 splicing mutation) that we identified previously in mainly American patients with neonatal citrullinemia. Two of these 20 mutations, a deletion of exon 13 sequence and a 7-bp deletion in exon 16, were common to Japanese and American populations from different ethnic backgrounds; however, other mutations were unique to each population. Furthermore, the presence of a frequent mutation—the exon 7 deletion mutation in mRNA, which accounts for 10 of 23 affected alleles—was demonstrated in Japanese citrullinemia. This differs from the situation in the United States, where there was far greater heterogeneity of mutations.

Introduction

Argininosuccinate synthetase (ASS; E.C.6.3.4.5) catalyzes the conversion of citrulline, aspartate, and ATP into argininosuccinate, AMP, and pyrophosphate. In ureotelic animals such as man, the enzyme is expressed at high levels in the liver, where it functions as part of the urea cycle. The ASS in the kidney and other cells plays a role in the synthesis of arginine, and the activity is lower but detectable in most tissues. The ASS purified from human liver is a cytoplasmic homotetramer with a molecular mass of ~180 kD (O'Brien 1979; Saheki et al. 1983). The cDNA sequence and deduced amino acid sequence for the enzyme with a subunit molecular weight of ~46 kD are known for human (Bock et al. 1983; Kobayashi et al. 1990), rat (Surh et al. 1988), bovine (Dennis et al. 1989), and mouse (Surh et al. 1991). The human expressed gene consists of 16 exons and maps to chromosome 9q34.

Citrullinemia is an autosomal recessive disease caused by a deficiency of ASS. The clinical, biochemical, and molecular aspects of citrullinemia have been reviewed elsewhere (Beaudet et al. 1986; Saheki et al. 1987*b*; Brusilow and Horwich 1989), and heterogeneity is seen clinically, biochemically, and at the molecular level. Saheki et al. (1987*b*) analyzed the enzyme abnormalities in Japanese citrullinemia patients and classified them into three types. Type II citrullinemia is found in most patients with adult-onset citrullinemia in Japan, and ASS deficiency is found specifically in the liver. The most recent study by homozygosity analysis demonstrated that the primary defect of type II citrullinemia is not within the ASS gene locus (Kobayashi et al. 1993). The classical neonatal and/or infantile form was assigned to type I (with abnormal kinetics of the enzyme) and type III (with undetectable or extremely low amounts) and is characterized by hyperammonemia, mental retardation, and early death when untreated. The enzyme defect in the classical form is found in all tissues and/or cells where ASS is expressed and the abnormality is in the ASS gene locus. More recent studies in human neonatal citrullinemia have identified nine single-base missense mutations, four other mutations associated with an absence of an exon in the mRNA, and one mutation with small deletions in the mRNA that result from abnormal splicing (Jackson et al. 1989; Kobayashi et al. 1990, 1991; Su and Lin 1990). These 14 patients, whom we had analyzed previously, were American, except for three alleles from Japanese.

In the present study, reverse transcription of mRNA, amplification of cDNA, and sequencing of cDNA clones were used to characterize mutations in 10 Japanese patients with classical citrullinemia. We have identified five new missense mutations and one novel insertion mutation in mRNA.

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Address for correspondence and reprints: Dr. Keiko Kobayashi, Department of Biochemistry, Faculty of Medicine, Kagoshima University, Sakuragaoka 8-35-1, Kagoshima 890, Japan.

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Patients, Material, and Methods

Patients

Fifteen Japanese patients with classical citrullinemia were included in the present study. Biopsy or autopsy specimens from liver, kidneys, and/or fibroblasts were obtained and were used for analyses of enzyme activity, cross-reactive material (CRM), and mRNA. We diagnosed them as type I (with abnormal kinetics of enzyme) and type III (with undetectable or extremely low amounts), according to criteria that included increased blood ammonia level, high concentrations of serum citrulline, decreased serum arginine level, and decreased ASS activity in the liver, fibroblasts, and/or other tissues (Saheki et al. 1987*b*). The clinical and biochemical findings are summarized in table 1 (Saheki et al. 1985, 1987*a*; Kobayashi et al. 1986, 1987; Imamura et al. 1987; Nukada et al. 1991). Patient A82 is an adult-onset citrullinemia case who suffered from hyperammonemia when she was 25 years old, at the beginning of suckling following delivery. The serum citrulline and arginine levels of the patient were 1,440 (control 35 ± 6) and 37 (control 120 ± 24) nmol/ml, respectively. The ASS activities in the liver and also in the fibroblasts were not detectable under standard assay conditions, and the hepatic enzyme showed abnormal kinetics. Other patients were neonatal and/or infantile variant; in them, onset occurred during the period from birth to age 2 years.

Determination of Enzyme Activity and CRM Analyses

ASS activity given in table 1 was determined as described by Saheki et al. (1981) and Su et al. (1982) and was expressed as a percentage of the age-matched control value. For routine assays, the concentrations of substrates were 5 mM citrulline, 1 mM aspartate, and 1 mM ATP. For the determination of the K_m values for citrulline, the concentrations of citrulline were 0.25–120 mM, with the concentration of the other substrates fixed, at 1 mM aspartate and 1 mM ATP.

For the preparation of anti-ASS antibody, human ASS was expressed in bacteria by cloned ASS cDNA by using pET vector (Studier et al. 1990) and was purified by chromatography on a DE-23 ion-exchange column and fractionation with ammonium sulfate. Anti-ASS polyclonal antibodies were prepared for purified human ASS protein by using rabbit. Western blotting was carried out as follows: liver specimens were homogenized with 0.15 M KCl containing 50 mM Tris-HCl pH 7.5 and then centrifuged at 100,000 *g* for 30 min. The supernatant was subjected to electrophoresis on a 10% SDS-PAGE and was transferred to nitrocellulose membrane. ASS protein was detected by using anti-ASS IgG as a first antibody with anti-rabbit IgG goat Fab'-peroxidase conjugate as a second antibody. The amount of ASS protein was determined by the single-radial immunodiffusion (SRID) method (Saheki et al. 1981) and by enzyme-linked immunosorbent assay (ELISA) (Imamura et al. 1987; Saheki et al. 1987*a*).

Extraction of RNA, Reverse Transcription, and Amplification of cDNA

RNA was isolated from liver specimens or fibroblasts as described by Chomczynski and Sacchi (1987). The synthesis of the first-strand cDNA was carried out according to the supplier's recommendation (Pharmacia LKB Biotechnology), from either total RNA or poly(A)$^+$RNA, by using oligo-$(dT)_{12\text{-}18}$ and M-MuLV reverse transcriptase.

PCR was carried out using the first-strand cDNA without any treatment, as the initial template. The amplification of cDNA was performed by methods described elsewhere (Kobayashi et al. 1990, 1991), with minor modification. The entire reaction from the cDNA synthesis was mixed with 0.5 μM PCR primer in a total volume of 50 μl containing 6.7 mM $MgCl_2$, 67 mM Tris-HCl pH 8.8, 1.5 mM each dATP, dTTP, dCTP, and dGTP, and 1 unit of *Taq* polymerase (Perkin Elmer Cetus). The mixture was initially heated to 94°C for 5 min, and the reactions were subjected to 25 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and DNA polymerization at 72°C for 4 min.

Oligonucleotides SF (−89 to −70 of ASS mRNA; the nucleotides are numbered by denoting the A residue of the initiation codon as +1) and TB (1275 to 1255 of ASS mRNA) as the PCR primers were employed to amplify the entire coding region of the ASS mRNA, as described elsewhere (Kobayashi et al. 1990, 1991). The sequences of oligonucleotide SF and TB are 5′-GCTACTGCAGCTGCCACCGCTGCCCGAGCC-3′ and 5′-CGTAGGATCCAATTGAGGAGGCCCCAGCTCC-3′, respectively. The underlined sequences are identical to the ASS mRNA, and a restriction-enzyme site for cloning was added at the 5′ side of each oligonucleotide, at the *Pst*I site in the SF primer and at the *Bam*HI site in the TB primer.

Cloning and Sequencing of Amplified cDNA

The amplified DNA was digested with *Pst*I and *Bam*HI, was separated by gel electrophoresis, and was directly extracted from the gel bands by using QIAEX (QIAGEN and Funakoshi) prior to subcloning into the M13 vector. For the selection of clones containing cDNA inserts, we used the "C test" for the determination of the orientation of inserts in M13 phage. The addition of an S1 nuclease digestion step to the C test permitted distinction of clones with S1-sensitive regions in cDNA from patients who were compound heterozygotes. These procedures were conducted essentially as described elsewhere (Kobayashi et al. 1990).

Single-stranded DNA cloned into M13 was sequenced by the dideoxy chain-termination method using Sequenase (United States Biochemical) and ^{35}S-labeled dATP (Du Pont New England Nuclear). In addition to universal primer, specific oligonucleotides from the ASS sequence were used to facilitate sequence analysis of the entire cod-

Table 1

Japanese Patients with Classical Citrullinemia Described in Present Paper

Patient (Age; Sex)	ASS Activity[a] (%)	ASS-CRM[b]	K_m for Citrulline[c] (mM)	Type	Reference(s)
	Liver				
N1 (24 d; F)	.7 (11)	NT	20	I	Saheki et al. 1985
N2 (2 years; F)	<.5	−[d]	NT	III	Saheki et al. 1985
N3 (7 d; F)	2.1 (14)	NT	7	I	Saheki et al. 1985
N4 (3 d; F)	<.5	NT	NT	III	Saheki et al. 1985, Kobayashi et al. 1986
N7 (8 mo; F)	<.5	−[d], .62 μg/g liver[e]	NT	III	Saheki et al. 1985, Kobayashi et al. 1986, Imamura et al. 1987, Saheki et al. 1987*a*
N8 (8 mo; M)	3.7	+[d], 120 μg/g liver[e]	1.6	I	Saheki et al. 1985, Imamura et al. 1987
N9 (8 mo; M)	<.5 (17)	+[d]	50	I	Present paper
N10 (1 years, F)	5.2	++[d]	1.1	I	Present paper
N11 (4 d; M)	<.5	1.1 μg/g liver[e]	NT	III	Kobayashi et al. 1987, Saheki et al. 1987*a*, Nukada et al. 1991
N12 (2 mo; F)	<.5	.26 μg/g liver[e]	NT	III	Saheki et al. 1987*a*
N13 (4 d; M)	4.5	8.1 μg/g liver[e]	NT	III	Saheki et al. 1987*a*
N14 (14 d; F)	<.5	.075 μg/g liver[e]	NT	III	Saheki et al. 1987*a*
A82 (25 years; F)	<.5 (5.7)	+[f]	400	I	Present paper
	Fibroblast				
N16 (1 year; F)	<.5	NT	NT		Present paper
RI (3 mo; F)	<.5	8.7 ng/mg protein[e]	NT		Kobayashi et al. 1990, Present paper

[a] Under standard conditions and at high concentration of substrate. Data in parentheses are percent of value in controls.
[b] − = Not detectable; + = ~10%–20% of value in controls; ++ = almost same amount as in controls; and NT = not tested.
[c] Value of ASS in controls was 0.05 mM. NT = not tested.
[d] Measured by SRID test.
[e] Measured by ELISA. Values in control were 650 ± 300 μg/g liver and 144 ± 16.5 ng/mg of protein of fibroblasts.
[f] Measured by western blot analysis.

ing region. At least four clones per allele were analyzed for each patient.

DNA Extraction and Diagnosis of the R304W Mutation

After informed consent was obtained, genomic DNA was isolated from blood, cells, or tissues of 15 patients, from the family of patient RI, and from control individuals, by phenol/chloroform extraction and ethanol precipitation. For amplification of exon 13 from genomic DNA, the oligonucleotide primers were 5′-GCTACTGCAGTTTGGGTTTCATGCG-3′ from intron 12 and 5′-GCTACTGCAGCACTTTGGGATCCCTTGTGAG-3′ from intron 13. The underlined sequences are identical to the ASS gene. Amplification of genomic DNA was as described elsewhere (Saiki et al. 1988), and the PCR product was digested by *Msp*I before agarose gel electrophoresis.

Results

Biochemical Studies

As shown in table 1, all patients with citrullinemia were diagnosed on the basis of deficient activities of ASS in specimens of liver, kidney, and/or cultured fibroblasts. All the cases had enzyme activities <5% of those of normal controls under standard assay conditions. We have already classified them into two types: three patients—N1, N3, and N8—with abnormal kinetics of the enzyme were assigned to type I; and seven patients—N2, N4, N7, N11, N12, N13, and N14—with undetectable or extremely low amounts of the enzyme were assigned to type III (Saheki et al. 1985, 1987*a*; Kobayashi et al. 1986, 1987; Imamura et al. 1987; Nukada et al. 1991). In the present paper, three more patients—N9, N10, and A82—were identified as

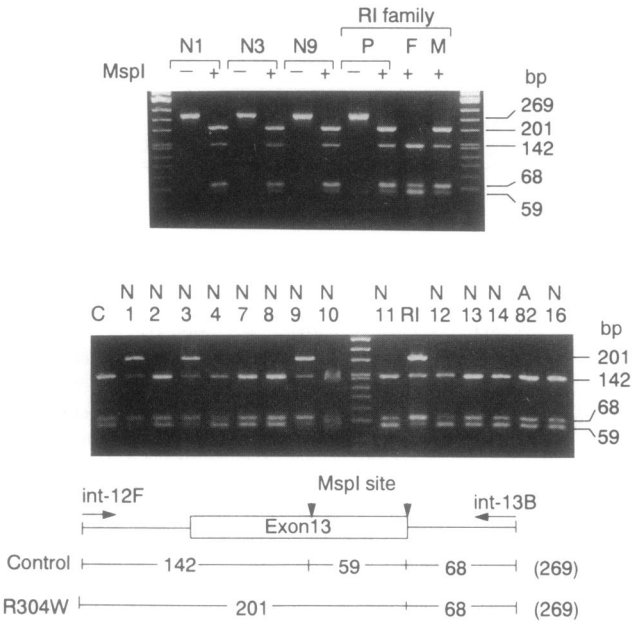

Figure 1 Diagnosis of R304W mutation by amplification of genomic DNA. Exon 13 region was amplified from genomic DNA of 15 patients, from the family of patient RI (P = patient; F = father; and M = mother), and from a control (C), as described in Patients, Material, and Methods. The diagram indicates both the position of the *Msp*I sites and the size of the restriction fragments obtained after *Msp*I digestion. The amplification products in the upper panel were analyzed with (+) and without (−) *Msp*I digestion. All lanes in the lower panel show *Msp*I digests. The sizes of the fragments (in bp) are indicated.

type I citrullinemia because kinetically abnormal ASS was detected in the liver. Two patients—N16 and RI—could not be classified, because we obtained no definitive data on the enzymological properties.

In type I citrullinemia, K_m values for citrulline (table 1) and for aspartate (data not shown) of the enzyme from patients N1, N3, N8, N9, N10, and A82 were 20–4,000 times those of the controls (normal K_m value for citrulline 0.05 mM). ASS protein or ASS-CRM from normal level to ~10% of that in controls was detected in patients N8, N9, and N10 by the SRID method; in patient N8 by ELISA; and in patient A82 by western blot analysis. On the other hand, in type III citrullinemia, no or extremely low ASS activity was detected in the liver, kidney, and/or cultured fibroblasts, even when much higher concentrations of the substrates were used for the assay, whereas a very small amount of ASS-CRM was detected in the liver of patients N7, N11, N12, N13, and N14 by means of a sensitive ELISA. The ASS protein of patient N13 had a specific activity comparable to that of the controls (Saheki et al. 1987*a*), and the hepatic ASS-CRM of patient N11 was found by gel-filtration column chromatography (Kobayashi et al. 1987) to have a molecular weight indistinguishable from that of purified human ASS. These CRMs were detected as a single protein band having a subunit molecular weight the same as that of controls, by SDS-PAGE followed by western blot analysis (Saheki et al. 1987*a*; Nukada et al. 1991).

General Observations of Amplified DNA

The first-strand cDNA was synthesized by reverse transcription of RNA from liver specimens or cultured fibroblasts of 10 Japanese patients with classical citrullinemia. The cDNAs for part or all of the coding region were amplified by PCR using oligonucleotides based on the ASS mRNA sequence (Kobayashi et al. 1990, 1991). In some instances, the size of the PCR product differed from that of controls. This difference was localized in the 5′ half or 3′ half of the molecule. More than one size of PCR product was found in some patients. The PCR product was characterized by direct digestion with individual restriction enzymes known to exist in particular exons of the cDNA and in mutations identified elsewhere (Jackson et al. 1989; Kobayashi et al. 1990, 1991).

Four of nine missense mutations identified previously showed a loss of *Msp*I site in the 3′ half of cDNA (Kobayashi et al. 1990, 1991). In Japanese patients tested in the present study, the loss of the *Msp*I site located in either exon 13 or exon 14 was detected by restriction-enzyme digestion of PCR product either from one allele of patients N1, N3, and N9 or from the other allele of patient N9 (data not shown). On the other hand, the R304W mutation resulting in the loss of the *Msp*I site in exon 13 could be diagnosed by genomic DNA analysis (Kobayashi et al. 1990). As shown in figure 1, the R304W mutation was also observed in 3 (patients N1, N3, and N9) of 14 Japanese patients tested in this study, as well as in patient RI (in whom a previous study [Kobayashi et al. 1990] had observed this mutation).

The cDNA containing the entire coding region was amplified using oligonucleotides SF and TB as PCR primer, in

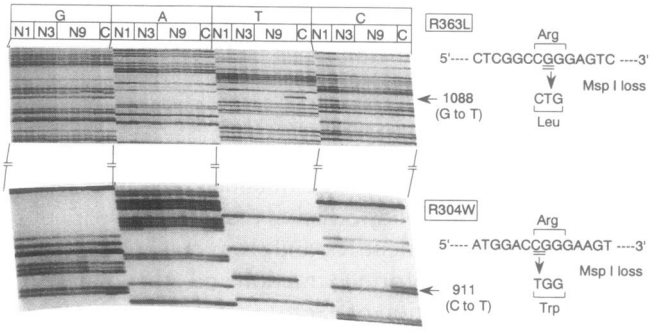

Figure 2 Sequence analysis confirming R304W mutation in three patients—N1, N3, and N9—and identifying a new missense mutation, R363L, in patient N9. Cloning and sequencing of the sense strand was performed for two clones each from patient N1 and N3, four clones from N9, and one clone from a control (C) individual, as described in Patients, Material, and Methods. To facilitate analysis, all reactions for each nucleotide were loaded in adjacent lanes.

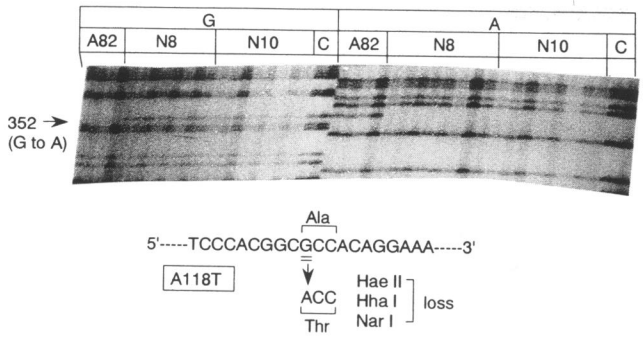

Figure 3 Sequence analysis revealing a G-to-A mutation in patient A82. The nucleotide sequence of the sense-strand cDNA from four clones of patient A82, from eight clones each from patients N8 and N10, and from two clones from a control (C) are shown. The left half of the figure shows lanes containing the dideoxyguanine (G) reactions, and the right half shows lanes containing the dideoxyadenine (A) reactions. The arrow indicates the position of a G-to-A base change at nucleotide 352 in all four clones from the fully S1 nuclease–protective allele of patient A82 (resulting in A118T mutation).

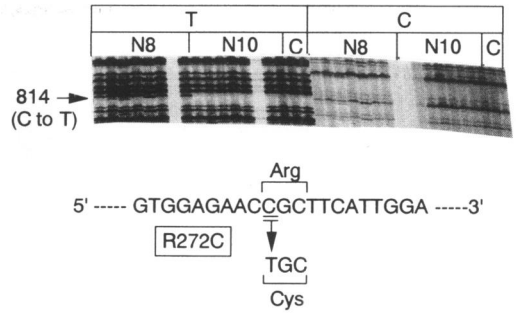

Figure 5 Sequence analysis detecting R272C mutation in patient N8. Sequencing was performed for eight clones each from patients N8 and N10 and for two clones from a control (C). The left half of the figure shows lanes containing the dideoxythymidine (T) reactions, and the right half shows lanes containing the dideoxycytidine (C) reactions. All eight lanes for the fully S1 nuclease–protective allele of patient N8 demonstrate a C-to-T transition at nucleotide 814 (resulting in the R272C mutation).

order to clone the cDNA into M13 vector for sequencing. Individual M13 clones from patients were analyzed by hybridization to M13 clones containing the normal cDNA sequence with antisense strand, and the heteroduplexes were digested with S1 nuclease (data not shown), because many citrullinemia patients have been compound heterozygote (Kobayashi et al. 1990, 1991). For patients N9 and N10, all cloned inserts were fully protected from S1 nuclease digestion. In patients N4, N11, and N13, all clones showed a nuclease-detectable defect in the cDNA, indicating a single class of cDNA clones for each patient, although the defect in patient N11 was different from that in the other two patients. This preliminary information is consistent with previous data, such as the presence of a localized S1 nuclease–detectable defect in patient N11 (Kobayashi et al. 1987; Nukada et al. 1991). For patients N1, N3, N8, and A82, two classes of cDNA clones were identified, one fully protective and one with a nuclease-detectable defect. M13 clones from patient N14 showed two different nuclease-detectable defects.

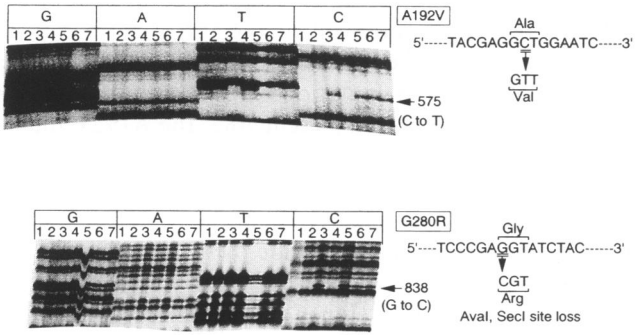

Figure 4 Sequence analysis identifying two different missense mutations in patient N10. Cloning and sequencing were performed for seven clones from patient N10. Counting from the left for each nucleotide, lanes 1, 2, and 4 demonstrate a C-to-T transition at nucleotide 575 (resulting in the A192V mutation), while lanes 3 and 5–7 demonstrate a G-to-C change at nucleotide 838 (resulting in the G280R mutation).

Identification of Single Base Changes

M13 clones from alleles producing fully protective cDNA sequences were thought likely to have single base changes that, because of the amplification and/or cloning process, might be difficult to distinguish from polymerase errors. For this reason, at least four clones per each allele of the patients were sequenced simultaneously, and, for sequencing gels, all lanes for an individual nucleotide from a number of clones were frequently grouped, in order to facilitate rapid identification of mutations. The sequences for fully protective cDNA clones from patients N1, N3, N8, N9, N10, and A82 are shown in figures 2–5.

To confirm and identify the mutations that result in the loss of the *Msp*I site located in either exon 13 or exon 14,

Mutation in ASSmRNA	Type I								Type III			
	N1	N3	N8	N9	N10	A82	RI*	AC*	N4	N11	N13	N14
A118T						+						
A192V					+							
R272C			+									
G280R					+							
R304W	+	+		+			+					
R363L				+								
Ins 37b/Ex15&16						+						
ΔExon7	+	+	+				+	+	++		++	+
ΔExon13										++		
Δ7b/Ex16												+

Figure 6 Summary of mutations in Japanese patients with classical citrullinemia. Mutations in patients RI and AC (indicated by asterisks [*]) have been reported elsewhere (Kobayashi et al. 1990). These two patients were not classified as type I or type III.

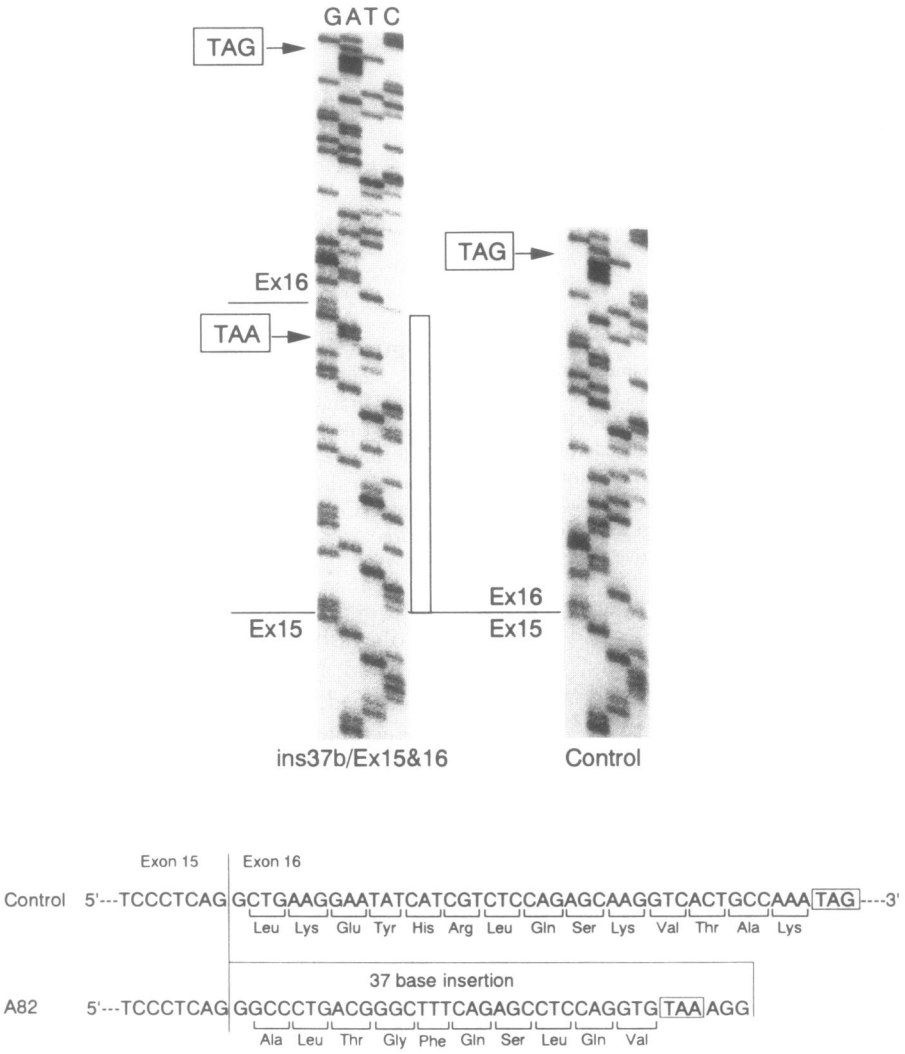

Figure 7 Abnormal cDNA sequence with a 37-bp insertion in patient A82. The partial cDNA sequence at the exon 15/exon 16 junction is shown for the S1 nuclease–detectable allele of patient A82 and for a control. Nucleotides are those of the sense strand. The insertion nucleotide sequence and its deduced amino acid sequence generating a premature stop codon are shown in the lower panel.

two clones from each of patients N1 and N3, four clones from patient N9, and one clone from a control were sequenced (fig. 2). Each allele with the loss of the *Msp*I site in exon 13 from patients N1, N3, and N9 showed exactly the same R304W mutation as had elsewhere been identified in patient RI (Kobayashi et al. 1990). The mutation with the loss of the *Msp*I site in exon 14 found in patient N9 allele was a single base change (G-to-T transversion) at nucleotide 1088. This mutation resulted in the substitution of leucine (CTG) for arginine (CGG) in codon 363 (R363L) and differed from the R363W (C-to-T transition at nucleotide 1087) mutation with the loss of the same *Msp*I site in exon 14, which was found in an American citrullinemia case described elsewhere (Kobayashi et al. 1990). Furthermore, sequence analysis of the entire coding region revealed four additional single-base substitutions at different nucleotide position in the three patients (N8, N10, and A82) studied in the present paper (figs. 3–5); these were the A118T mutation (G-to-A transition at nucleotide 352 in exon 5) in patient A82, the A192V mutation (C-to-T transition at nucleotide 575 in exon 9) and the G280R mutation (G-to-C transversion at nucleotide 838 in exon 12) in patient N10, and the R272C mutation (C-to-T transition at nucleotide 814 in exon 12) in patient N8.

Sequencing of cDNA clones from eight independent alleles with no S1 nuclease–detectable defect allowed the identification of six different single base changes associated with missense mutations (fig. 6). The R304W mutation was identified by cDNA sequencing (fig. 2) and DNA diagnosis (fig. 1) in four Japanese patients including patient RI (Kobayashi et al. 1990). Four of the six single base

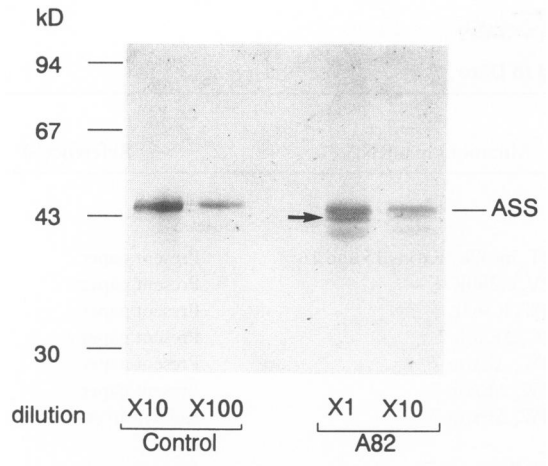

Figure 8 Detection of ASS-CRM in liver extracts from a control and patient A82, by western blot analysis. The supernatant from 10% homogenate of liver specimens was diluted as described. Five microliters of diluted sample was applied to 10% SDS–acrylamide gel. The bands of ASS-CRM were detected by using anti-ASS IgG as a first antibody, as described in Patients, Material, and Methods.

changes altered a restriction-enzyme site, and three of the six mutations involved conversion of a CpG dinucleotide to TpG in one or the other strand of DNA.

Identification of Larger Defects in cDNA Clones

Five mutations resulting in production of mRNA with S1 nuclease–detectable defects had previously been reported to be associated either with the absence of sequences of exons 5, 6, 7, or 13 or with the absence of the first 7 bp of exon 16 in the cDNA (Jackson et al. 1989; Kobayashi et al. 1990). Four mutations from S1 nuclease–detectable alleles of eight Japanese patients have now been shown to be associated with the absence of either exon 7 or exon 13, the absence of the first 7 bp of exon 16, or the insertion of 37 bp within exons 15 and 16 in the cDNA (figs. 6–8). The missing sequence for the allele associated with the absence of either exon 7 or exon 13 corresponds exactly to the sequence contained in each exon (data not shown), and the absence of exon 7 sequence in the cDNA was observed in 10 alleles of Japanese patients, including the two patients (RI and AC) reported elsewhere (Kobayashi et al. 1990). No Southern blot abnormalities have been detected in association with these two (exon 7 and exon 13) deletion mutations when intron probes flanking these exons have been used (Kobayashi et al. 1990). In addition, both patient N13, with the exon 7 deletion mutation, and patient N11, with the exon 13 deletion mutation, showed very low but significant quantities of ASS protein with the same specific activity and/or with the same molecular size as was seen in controls (Kobayashi et al. 1987; Saheki et al. 1987*a*; Nukada et al. 1991). Furthermore, we have shown that the absence of either exon 7 sequence or exon 13 sequence results from an abnormal splicing by a single base change in the intron region of the ASS gene (K. Kobayashi, unpublished data). The 7-bp deletion mutation in exon 16 of patient N14 may be the same abnormality as a G-to-C substitution in the last position of intron 15, resulting in splicing to a cryptic splice site within exon 16, as described elsewhere (Kobayashi et al. 1990; Su and Lin 1990).

Sequence analysis of cDNA from the S1 nuclease–detectable allele of patient A82 demonstrated the insertion of 37 bp within exons 15 and 16 in the cDNA, as shown in figure 7. Because of generation of a stop codon in the additional sequence, translation of the abnormal mRNA would result in the production of an abnormal protein with four amino acid residues shorter than those of the wild type (fig. 7). This abnormal protein molecule may not be labile, since ASS-CRM with smaller size, in addition to ASS protein with normal size, was detected by western blot analysis in liver extracts from patient A82 in whom the other allele was shown to be an A118T mutation (fig. 8).

Discussion

The analysis of mutations causing classical citrullinemia is particularly well suited for ASS cDNA amplification followed by DNA sequencing. Fourteen mutations have been identified in the ASS gene in cell lines derived mainly from American patients (except for three alleles from Japanese), and extreme heterogeneity of mutation has been found (Jackson et al. 1989; Kobayashi et al. 1990, 1991). In the present study, we identified six additional mutations in Japanese patients. These are five new missense mutations (A118T, A192V, R272C, G280R, and R363L) and one

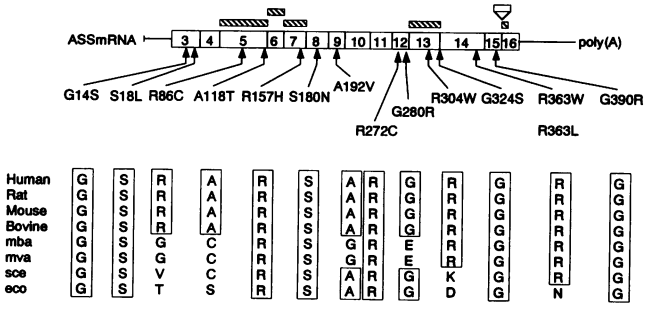

Figure 9 Mutations in the human ASS mRNA that cause classical citrullinemia. Mutations identified to date (Jackson et al. 1989; Kobayashi et al. 1990, 1991; present paper) are summarized. Numbered exons are indicated in boxes representing a coding region of ASS mRNA. Larger defects in cDNA are given in the upper panel: the open box denotes an insertion mutation; and the hatched boxes denote a deletion mutation. The 14 missense mutations known in the human are indicated, and the amino acid residues that each missense mutation located are compared with those from eight organisms. mba = *Methanosarcina barkeri;* mva = *Methanococcus vannielii;* sce = *Saccharomyces cerevisiae;* and eco = *Escherichia coli.*

Table 2

Summary of Mutations in 24 Patients with Classical Citrullinemia Identified to Date

Patient	ASS-CRM[a] (%)	K_m[b]	Mutations in mRNA	Reference(s)
Japanese:				
Type I:				
A82	+[c]	8,000-fold	A118T, ins37b/Exons 15 and 16	Present paper
N10	++[d]	22-fold	A192V, G280R	Present paper
N9	+[d]	1,000-fold	R304W, R363L	Present paper
N8	+[d], 18.5[e]	32-fold	R272C, ΔExon 7	Present paper
N1	NT	400-fold	R304W, ΔExon 7	Present paper
N3	NT	140-fold	R304W, ΔExon 7	Present paper
RI	6.0[e]	NT	R304W, ΔExon 7	Kobayashi et al. 1990
Type III:				
N4	NT	NT	ΔExon 7, Δexon 7	Present paper
N11	.2[e]	NT	ΔExon 13, Δexon 13	Present paper
N13	1.2[e]	NT	ΔExon 7, Δexon 7	Present paper
N14	.01[e]	NT	ΔExon 7, Δ7b/exon 16	Present paper
Japanese American:				
AC	−[f]	NT	ΔExon 7, RNA negative	Kobayashi et al. 1990
American:				
IG	NT	NT	G14S, S180N	Kobayashi et al. 1990
AH	NT	NT	G324S, R363W	Kobayashi et al. 1990
MR	NT	NT	S18L, G390R	Kobayashi et al. 1991
SD	NT	NT	G390R, G390R	Kobayashi et al. 1990
LP	NT	NT	G390R, RNA negative	Kobayashi et al. 1990
BSm	−[f]	NT	G390R, Δ7b/exon 16	Kobayashi et al. 1990
CG	−[f]	NT	Δ7b/Exon 16, Δ7b/exon 16	Kobayashi et al. 1990
CB	−[f]	NT	ΔExon 5, RNA negative	Kobayashi et al. 1990
KSt	+[f]	NT	ΔExon 5, R157H	Kobayashi et al. 1990
GM63	−[f]	2,000-fold	ΔExon 5, R86C	Kobayashi et al. 1990, 1991
BBr	NT	NT	ΔExon 6, RNA negative	Kobayashi et al. 1990
GM1679	−[f]	NT	ΔExon 6, Δexon 13	Kobayashi et al. 1990

NOTE.—All patients had <5% of ASS activity in controls and had neonatal/infantile citrullinemia, except for patient A82, whose age at onset was 25 years.

[a] − = Not detectable; + = ~10%–20% of value in controls; ++ = almost same amount as in controls; and NT = not tested. Data are percent of values in controls.

[b] Data are expressed as increase over values in controls. NT = not tested.

[c] Measured by western blot analysis.

[d] Measured by SRID test.

[e] Measured by ELISA.

[f] Detected by western blot analysis and reported by Su et al. (1982) and Beaudet et al. (1986).

novel insertion mutation in mRNA. Of 23 Japanese alleles that we investigated, 4 had the R304W mutation and 10 had the exon 7 deletion mutation in mRNA. The ASS gene abnormality (IVS-6^{-2} mutation) causing the absence of exon 7 in mRNA has been identified, and patients with the IVS-6^{-2} mutation are detected more frequently in Japanese citrullinemia by DNA diagnostic analysis (K. Kobayashi, unpublished data). Of 20 mutations identified to date, 14 involve single base changes causing missense mutations in the coding sequence of the enzyme, 4 involve the absence of a different exon in the mRNA, 1 causes abnormal splicing, and 1 is the insertion mutation in mRNA, as summarized in figure 9. However, the nonsense mutation has been not identified yet in human citrullinemia, although bovine citrullinemia was caused by a nonsense mutation in codon 86 (Dennis et al. 1989). As mentioned by Eng et al. (1993), reverse-transcription PCR may not detect some nonsense mutations, either because of the instability of the mutant transcripts or because of the alteration of the splice-site selection (Dietz et al. 1993).

Deduced amino acid sequences for ASS from eight species is now available; these species are human (Bock et al. 1983; Kobayashi et al. 1990), rat (Surh et al. 1988), mouse (Surh et al. 1991), bovine (Dennis et al. 1989), *Methanococcus vannielii* (Morris and Reeve 1988), *Methanosarcina barkeri* MS (Morris and Reeve 1988), *Escherichia coli* (Van Vliet et al. 1990), and *Saccharomyces cerevisiae* (Van Vliet et al. 1990). These sequences are ≥93% identical in mammalian taxa, and comparison provides little insight into critical residues. However, a comparison of the more

highly diverged sequences provides a much clearer picture of amino acid residues that are critical to the function of the enzyme (Kobayashi et al. 1991). As shown in figure 9, of the 14 amino acid residues involved in missense mutations, 7 alter residues that are completely conserved among these eight organisms, 4 alter residues that are conserved among at least six species, and only 2 alter residues that are conserved in mammals. It is not known which amino acid residues are essential for the binding of the amino acid substrates by the enzyme. There is evidence that specific arginine residues are involved in the binding of ATP and PPi. Chemical modification studies indicate that arginine residues 153 and/or 157 are involved in the binding of ATP (Isashiki et al. 1989). We reported a mutation, R157H, that affected one of these arginine residues (Kobayashi et al. 1990), and this residue is conserved among the species whereas arginine 153 is not conserved (Kobayashi et al. 1991).

All mutations in 24 patients with classical citrullinemia identified to date are summarized in table 2. We have classified Japanese classical citrullinemia patients with abnormal kinetics of the enzyme as being type I and have identified those with undetectable or extremely low enzyme as being type III. Six patients with type I citrullinemia were all compound heterozygotes involving missense mutation in at least one allele. In contrast, four patients with type III citrullinemia were homozygote or compound heterozygote with deletion mutation but no missense mutation. However, with regard to American citrullinemia patients, there is little information characterizing mutant proteins qualitatively and/or quantitatively, such as kinetic studies and/or determination of ASS-CRM amounts. It has been reported that only one patient (GM63) had abnormal kinetics of enzyme (Kobayashi et al. 1991). The absence of ASS-CRM was demonstrated in the cell lines from five citrullinemia patients (Su et al. 1982; Beaudet et al. 1986); three of them were homozygote or heterozygote with deletion mutation (table 2). Since most patients to date are known to be compound heterozygotes, it is difficult to draw conclusions regarding the effect that the mutations have on either the kinetic parameters or the stability of the ASS protein. We are now analyzing functionally and structurally the abnormal protein, which consists of a homotetramer or heterotetramer from each mutant cDNA, and their combinations expressed in bacterial or mammalian cells.

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