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Messenger RNA Coding for Argininosuccinate Synthetase in Citrullinemia

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SUMMARY

Messenger RNA coding for argininosuccinate synthetase (ASS), extracted from the livers of some patients with citrullinemia, was analyzed using a cell-free translation system and dot and Northern blot hybridization with cDNA probe for ASS.

In patients with quantitative-type citrullinemia, called type II here, previous studies have demonstrated that the hepatic content of the enzyme was about 10% of the control value, whereas the translatable mRNA level for the enzyme was similar to that of control livers. Here, we confirmed that the type II liver contained an almost normal amount of mRNA coding for ASS, judged by the dot-blot hybridization technique with cDNA. Northern blot hybridization of RNA indicated that there was hybridizable mRNA of approximately normal size (about 1.7 kilobase [kb]) in each, suggesting that large structural gene deletions had not occurred. These results indicate that in type II citrullinemia, the decrease in the enzyme protein is due either to increased degradation of the enzyme or to decreased or inhibited translation in the liver.

Another type of citrullinemia was found and classified as type III. It is characterized by no detectable enzyme activity for ASS or translation activity for ASS mRNA. However, a smaller amount of RNA molecule hybridized for ASS cDNA was detected.

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INTRODUCTION

Citrullinemia (21570 in McKusick [1]), first described by McMurray et al. [2], has been considered to be a rare hereditary metabolic disorder of neonates and early infants caused by the decreased activity of argininosuccinate synthetase (E.C.6.3.4.5; ASS) in the urea cycle [3]. In Japan, however, many cases of adult (20–50 years old) citrullinemic patients have been reported. We have already analyzed the enzyme abnormalities in the adult patients and classified them into two types: qualitative and quantitative [4–6]. Recently, following analysis of abnormal ASSs in eight neonates and infants with citrullinemia, we found another type of enzyme abnormalities, so that we roughly classified for convenience citrullinemia into the following three categories [7].

The first is a qualitative type (type I), in which ASS has abnormal kinetic properties such as higher K_m values for the substrates and abnormal cooperative properties. Kinetically abnormal ASSs are formed in the liver, kidney, and cultured skin fibroblasts of patients with type I citrullinemia, presumably caused by some abnormal structure of the enzyme derived from some point mutation in the exon of the gene. The second is a quantitative type (type II), in which the decrease in ASS activity in the liver is due to a decrease in the amount of the kinetically normal enzyme. Most adult citrullinemic patients in Japan belong to type II, in which the ASS abnormality is found in the liver, but not in the kidney or cultured skin fibroblasts.

On the other hand, type III enzyme abnormality is characterized by no detectable ASS activities in the liver, kidney, or cultured skin fibroblasts, even when much higher concentrations of the substrates are used for the assay. Only a very small amount of the immune crossreactive material, however, could be detected in the liver of type III with a sensitive enzyme immunoassay (Imamura et al., manuscript in preparation).

To date, we have analyzed enzyme abnormalities in 50 cases of citrullinemia in Japan and found that among 39 adult cases one to two belonged to type I and 37 to type II and that five, two, and four of 11 cases in neonatal and/or infantile belonged to type I, II, and III, respectively.

To understand the molecular basis of the pathogenesis in citrullinemia, it is important to determine the ASS mRNA contents in type II and III in which the amount of enzyme protein is decreased. In previous studies of the translation activity of mRNA coding for ASS with the cell-free translation system, we observed that in two of the type II (adult quantitative type) cases, the actual enzyme quantity was reduced, but the translatable ASS mRNA activities were comparable to those of the control [8]. Here, however, we report that in one case of type III no translation activity of ASS mRNA was detected in the liver using the cell-free translation system.

The availability of a specific, cloned ASS cDNA probe [9, 10] offers the opportunity for more detailed analysis of the citrullinemia mutation at the nucleic acid levels. The human genome contains one expressed ASS gene, which has been mapped to chromosome region 9q34→qter by somatic cell hybridization [11–13], and about 14 pseudogenes, which are dispersed to at

least 11 human chromosomes including X and Y [14–16]. DNA sequence analysis of citrullinemia mutants will require an extensive effort because the expressed ASS gene with multiple introns (at least 13 exons) is large (63 kb) [16] and there are numerous dispersed pseudogenes. In this study, pAS1, one of the ASS cDNA clones [9, 10], was used as a hybridization probe to measure the ASS mRNA levels in the liver derived from type II and III citrullinemic patients. The results of the hybridization experiments are compared with those of the specific mRNA translational system.

MATERIALS AND METHODS

Patients

The patients included in this report are seven adults (19–66 years old) and three neonates or infants (3 days–12 years old) whose surgical or autopsy liver specimens were sent to this laboratory. Age, sex, and other biochemical data of the patients have already been reported by Sase et al. [8] for adult patients (AP) nos. 17 and 18 and by Saheki et al. [7] for neonatal or infantile patients (NP) nos. 4, 6, and 7. Clinical data on AP nos. 21, 24, 27, 29, and 30 will be reported elsewhere. The serum citrulline levels of type II (all adults and NP no. 6) and type III (NP nos. 4 and 7) patients were 200–650 and 1,700–3,950 nmol/ml, respectively (control level: 20–40 nmol/ml).

Control livers were obtained within 3 hrs of death by autopsy from patients who died of various causes such as extrahepatic cancer, heart failure, or car accidents, and by biopsy during operations of extrahepatic cancer. All specimens were stored at −80°C for further use.

Determination of Enzyme Activities

Procedures for homogenizing and determining enzyme activities were performed as described [4]. Aldolase B activity was measured spectrophotometrically by the rate of decrease in absorbance at 340 nm [17]. Protein concentrations were determined by the method of Lowry et al. [18], using crystalline bovine serum albumin as the standard.

RNA Extraction

Total RNA was extracted from frozen liver specimens (0.24–1 g liver) with guanidine-HCl [8, 19] or guanidine-thiocyanate [20]. The yield of RNA extraction was about 2–4 mg/g liver in the control and 0.8–3 mg/g liver in the experimental cases, except NP no. 6 (0.2 mg/g liver). In some of those, poly(A)$^+$RNA was isolated by chromatography on oligo(dT)-cellulose column [21] and the yield of poly(A)$^+$RNA was about 8%–10% of total RNA. The concentration of RNA was estimated by using an extinction coefficient of $E_{260nm}^{1\%} = 200$ [22].

The liver specimens from AP nos. 18, 21, 24, and 27 and NP nos. 4 and 6 were obtained at autopsy and the rest of the specimens by open biopsy. In our experience, it seems that the mRNAs prepared from the latter specimens were more stable. It is important to assess whether mRNA extracted from liver specimens obtained under various conditions is intact or not; however, it is difficult to measure individual mRNA content in crude samples. Therefore, we used mRNA coding for aldolase B as an internal standard for translational and dot-blotting analysis, because aldolase B, which is a cytosolic enzyme, as is ASS, with a molecular weight similar to that of ASS, exhibits activity in the citrullinemic liver comparable to that in the control liver.

Translational Analysis

Cell-free translation programmed with mRNA was performed with nuclease-treated rabbit reticulocyte lysates and [^{35}S]methionine [23, 24]. The conditions for translation,

analysis of translational products, and immunoprecipitation of ASS and aldolase B have been described [8].

Nick-Translation

Cloned human ASS (pAS1) and aldolase B (pHABL120-3) cDNA inserts were purified on gel electrophoresis after digestion of recombinant plasmid using restriction enzymes (*Pst*I and *Pvu*II, respectively). The corresponding purified inserts were labeled with [α-^{32}P]dCTP by nick-translation [25], with a specific radioactivity of about 1–2 $\times$ 10^8 cpm/μg, and were used as probe.

Dot and Northern Blot Hybridization Analysis

For dot-blot hybridization, total RNA was suspended in 7% formaldehyde, containing 1 M NaCl and 30 mM NaH_2PO_4, pH 6.8, and incubated at 55°C for 15 min [26]. The denatured RNA was applied to a nitrocellulose filter that had been pretreated with 20 $\times$ SSC [27]. The filter was baked at 80°C for 2 hrs, and then prehybridization, hybridization, and washing were carried out according to the method of Thomas [27]. To measure specific mRNA concentration by dot-blot analysis, 0.5–4 μg of total RNA were dotted on nitrocellulose filters, the hybridization being performed in the presence of 2 $\times$ 10^5 cpm/cm^2 (5.6–5.8 $\times$ 10^6 cpm/ml) of labeled purified inserts. After autoradiographic exposure, each dot was cut and its radioactivity was counted by liquid scintillation. Values were corrected for the background by subtracting the radioactivity of contiguous areas of the same filter not containing bound RNA.

For Northern blot analysis, RNA samples were treated with glyoxal and dimethyl sulfoxide and subjected to electrophoresis in 1% agarose gel [27]. After electrophoresis, one side of the gel was cut off and stained with ethidium bromide (0.5 μg/ml in H_2O) to measure the migration of 28S and 18S rRNA. The separated RNAs were transferred to a nitrocellulose filter and baked for 2 hrs at 80°C. Before prehybridization, the nitrocellulose filters were treated with 20 mM Tris-HCl, pH 8.0, at 100°C for 5 min to remove the glyoxal adduct and increase the efficiency of hybridization of the RNA [28].

Materials

Complementary DNA probes from human ASS and aldolase B were gifts from Dr. A. L. Beaudet, Baylor College of Medicine, and Dr. T. Mukai, Saga Medical School, Japan, respectively. [α-^{32}P]dCTP (−3,000 Ci/mmol), [^{35}S]methionine (1,000–1,300 Ci/mmol, translation grade), and nuclease-treated rabbit reticulocyte lysates were purchased from Amersham International plc (Amersham, U.K.). DNA polymerase I was from New England BioLabs (Beverly, Mass.); nitrocellulose filters (Trans-Blot, transfer medium) was from Bio-Rad Laboratories (Richmond, Va.); salmon testis DNA and oligo(dT)-cellulose (type 7) were obtained from P-L Biochemicals (Milwaukee, Wisc.). *Pst*I, *Pvu*II, and DNase I were products of Takara Shuzo (Kyoto, Japan). X-ray film was obtained from Fuji Photo Film (Kanagawa, Japan).

RESULTS

Enzyme Activity and K_m *Values of ASS*

A specific decrease of ASS activity was found in the urea-cycle enzymes in the liver of the citrullinemic patients presented here (table 1). In the case of type II, the K_m values for the substrates were comparable to those of controls (table 1), and the values of ASS activity in the kidney and/or the cultured skin fibroblasts were the same as those of controls as reported [5, 6]. The decreases in ASS activity in the type II liver were parallel to the decreases in the enzyme protein estimated from a single radial immunodiffusion method [4, 6].

TABLE 1

ACTIVITIES OF UREA-CYCLE ENZYMES AND THE K_m VALUES OF ASS WERE DETERMINED IN THE LIVERS OF CONTROL AND TYPES II AND III CITRULLINEMIA

	Enzyme activity (mU/mg protein)					K_m values of ASS (mM)		
	CPS	OTC	ASS	ASase	Arginase	Cit	Asp	ATP
Control:								
0–3 mos	31	490	2.7	18	6,500			
	±4	±150	±0.9	±6	±2,600			
Adult	43	660	9.2	35	9,200	0.05	0.03	0.09
	±14	±240	±2.1	±3	±2,300	±0.01	±0.01	±0.01
Type II:								
AP no. 17	85	1,300	1.5	27	8,100	0.04	0.02	0.07
AP no. 18	100	720	1.3	42	13,000	0.06	0.05	0.09
AP no. 21	41	660	1.1	37	10,300	0.03	0.03	0.10
AP no. 24	51	940	0.9	49	13,500	0.05	0.04	0.09
AP no. 27	47	1,240	1.0	45	8,300	0.03	0.03	0.10
AP no. 29	25	840	1.0	30	4,200	0.02	0.02	0.09
AP no. 30	26	240	1.8	28	8,850	0.03	0.06	n.d.
NP no. 6	39	1,280	1.0	21	4,300	0.04	0.03	0.09
Type III:								
NP no. 4	16	520	<0.01	21	15,100	*	*	*
NP no. 7	26	880	<0.01	35	9,500	*	*	*

NOTE: CPS, OTC, ASS, and ASase denote carbamylphosphate synthetase, ornithine transcarbamylase, argininosuccinate synthetase, and argininosuccinase, respectively; Cit and Asp, citrulline and aspartate, respectively. AP and NP denote adult, and neonatal or infantile citrullinemic patients, respectively. n.d.; not determined.

* Activity was undetectable even under the condition of increased concentration of substrate.

In type III citrullinemia, the ASS activity in the liver (table 1) and the cultured skin fibroblasts [7] was undetectable even under assay conditions using more than 20 times the ordinary concentration of substrates. Only a very small amount of the immune crossreactive material could be detected in the liver of patient NP no. 7 by means of a sensitive enzyme immunoassay with Fab'-peroxidase conjugates [29] for ASS (Imamura et al., manuscript in preparation).

Translational Activity of mRNA for ASS

Following quantification of mRNA activity with the cell-free translation system, Sase et al. [8] showed that in two cases of type II citrullinemia (AP nos. 17 and 18), the ASS mRNA levels were comparable to those of controls, although the actual enzyme quantity was reduced.

To investigate the pathogenesis of type III citrullinemia, we examined the translation activity of mRNA coding for ASS in total RNA extracted from the liver. We also determined and confirmed the ASS mRNA activity in type II citrullinemia using poly(A)$^{+}$RNA from AP no. 18. At the same time, the translation activity for aldolase B was measured as an internal standard to compare with that for ASS. Because aldolase B activities were not changed in the citrullinemic liver and were almost the same as those in control liver, as presented later, it seems that aldolase B is well suited for an internal standard to measure ASS mRNA content.

As given in table 2, we confirmed in type II (AP no. 18) that hepatic content of the enzyme was about 10% of the control value (table 1), whereas the translatable mRNA level for ASS was similar to that of control liver as reported [8]. In type III (NP no. 7), ASS-related protein isolated by immunoprecipitation with anti-rat ASS immunoglobulin was not visualized by fluorography after SDS-polyacrylamide gel electrophoresis, although aldolase B-related protein and TCA-insoluble total protein were synthesized in significantly large quantities by the cell-free translation system. Therefore, the livers of patients with type III citrullinemia contained neither detectable enzymatic nor translational activities for ASS, differing from type II citrullinemia.

TABLE 2

THE TRANSLATION ACTIVITY OF mRNA CODING FOR ASS AND ALDOLASE IN TOTAL OR POLY(A)$^{+}$RNA EXTRACTED FROM THE LIVER OF CONTROL AND TYPES II AND III CITRULLINEMIA

	INCORPORATION OF [^{35}S]METHIONINE		
	Total protein	ASS (dpm)	Aldolase
Control	340×10^4	283	2,845
Type II (AP no. 18)	122×10^4	81	943
Type III (NP no. 7)	287×10^4	≒0	3,227

Note: The amount of RNA in the translation mixture (50 μl system) was 40 μg of total RNA from control, 0.8 μg of poly(A)$^{+}$RNA from AP no. 18 and 16.5 μg of total RNA from NP no. 7, respectively. The reaction was carried out at 25°C for 90 min.

Determination of ASS mRNA Content by Dot-Blot Hybridization

To further understand the molecular basis of the pathogenesis in type II and III citrullinemia, we used the dot and Northern blot hybridization with the specific cDNA. Dot-blot hybridization assay was used to determine the amount of ASS mRNA in the liver from the control and each citrullinemic patient (fig. 1, table 3). Each cDNA insert from pAS1 [9, 10] for ASS mRNA and pHABL120-3 for aldolase B mRNA as an internal standard were used as a hybridization probe. The amount of ^{32}P-labeled cDNA hybridized to the filter-bound RNA increased linearly with up to 4 μg of total RNA under the conditions described in MATERIALS AND METHODS (fig. 1).

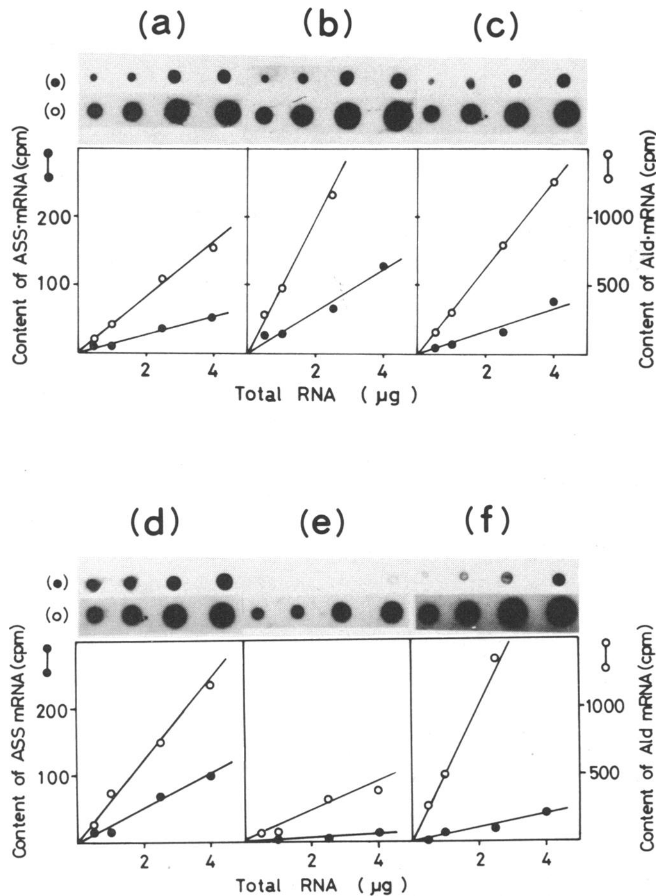

FIG. 1.—Dot-blot hybridization of pAS 1 (●) and pHABL 120-3 (○) with total RNA from the liver of control and citrullinemic patients. Increasing amounts of total RNA were applied to nitrocellulose filters. Filters were hybridized with each nick-translated cDNA from pAS 1 and from pHABL 120-3. Filters were exposed for autoradiography, and spots were cut out for scintillation counting. The hepatic total RNA from control: (*a*) and (*d*); AP: no. 18 (*b*) and no. 27 (*c*); and NP: no. 4 (*e*) and no. 7 (*f*) was used for the quantification, respectively. *Each upper part* shows the autoradiographic spots employed for the analysis.

TABLE 3

COMPARISON OF ASS mRNA LEVELS IN THE LIVER OF CONTROL AND TYPES II AND III CITRULLINEMIA

	DOT BLOTTING (cpm/μg TOTAL RNA)		RATIO ASS/Ald	ENZYME ACTIVITY (mU/mg PROTEIN)		RATIO ASS/Ald
	ASS	Aldolase	(A)	ASS	Aldolase	(B)
Control (no. = 7)	29.6 ±8.4	383 ±93	0.077 ±0.012	10.8 ±2.0	25.9 ±5.6	0.40 ±0.11
Type II:						
AP no. 17	66.3	772	0.086	1.5	35	0.041
AP no. 18	34.5	538	0.065	1.0	18	0.056
AP no. 21	18.5	250	0.074	1.1	24	0.046
AP no. 24	8.3	136	0.061	0.9	24	0.038
AP no. 27	15.5	237	0.065	0.95	30	0.032
AP no. 29	13.2	317	0.042	1.0	24	0.042
AP no. 30	17.6	374	0.047	1.8	34	0.053
NP no. 6	18.4	298	0.062	0.95	24	0.040
Type III:						
NP no. 4	1.8	110	0.016	<0.01	...	...
NP no. 7	9.2	473	0.019	<0.01	...	...

The results from the dot-blot hybridization are listed in table 3. Hepatic ASS activities in citrullinemia were significantly lower than those of control, but aldolase B activities were almost the same. This means that, in the type II liver, the ratio (B) of activity in ASS to aldolase B was lower than that of control. Quantifying the amount of ASS mRNA revealed that the ratio (A) of radioactivity obtained by the labeled ASS cDNA probe to that by the aldolase B cDNA probe was within the control range for type II. Therefore, these results indicate that the liver of patients with type II citrullinemia contained an almost normal amount of mRNA coding for ASS, judged by the dot-blot hybridization test with cDNA for ASS using aldolase B as an internal standard and comparable translatable activity of mRNA for ASS with a cell-free protein synthesis system.

In the case of type III citrullinemia, we detected a smaller amount of RNA molecule hybridized for ASS cDNA (table 3); however, there was no translational activity (table 2).

Northern Blot Hybridization of ASS mRNA

The total or poly(A)$^+$RNA from control and type II citrullinemia patients was examined by electrophoresis on agarose gel (1%) followed by Northern blot hybridization, as shown in figure 2. A major species of RNA was observed with all samples and was calculated to be about 1.7 kb in size, based on the migration of ribosomal RNA as markers. The values obtained in this study are similar to those of one major mRNA species (1.67 kb) as reported by Su et al. [9, 30]. Since various amounts of RNA were applied for analysis in figure 2 and

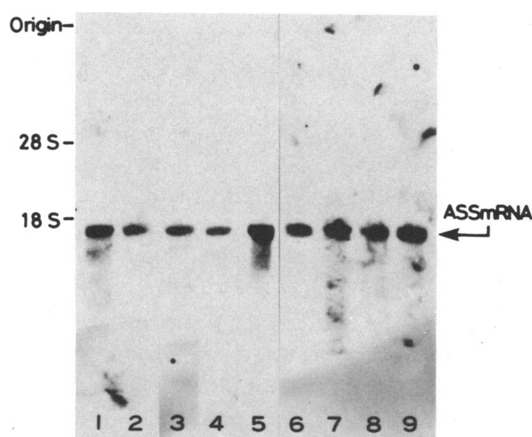

FIG. 2.—Autoradiograph of a Northern blot hybridization of total or poly(A)$^+$RNA extracted from the liver of control and citrullinemic patients. The RNA (40 μl reaction mixture) from four control and five patients was denatured with glyoxal and applied to a 1% agarose gel for electrophoresis. After transfer, the filters were hybridized with nick-translated pAS 1 probe. *Lanes* are displayed for *1:* 18 μg of poly(A)$^+$RNA from control; *2:* 3 μg of poly(A)$^+$RNA from AP no. 18; *3:* 5 μg of poly(A)$^+$RNA from AP no. 27; *4:* 10 μg of total RNA from AP no. 17; *5:* 20 μg of poly(A)$^+$RNA from AP no. 21; *6:* 50 μg of total RNA from AP no. 30; and *7–9:* 50 μg of total RNA from control.

since there is considerable doubt whether each RNA is completely transferred to the nitrocellulose filter, the relative intensity of the bands should not be interpreted quantitatively. The detection of hybridizable mRNA suggests that large structural gene deletions have not occurred. Furthermore, these findings are supported by preliminary S1 nuclease mapping studies (Kobayashi et al., unpublished data, 1985).

DISCUSSION

Comparisons between mRNA levels with their corresponding protein levels have been performed for other hereditary diseases. In thalassemia, a low level of or a complete lack of the globin polypeptide was found to be caused by nonsense mutation, frameshift mutation, and mutations in an intervening sequence or a 5′-flanking sequence [31–33]. Investigations of hereditary diseases in experimental animals, such as rat analbuminemia [34] and ornithine transcarbamylase deficiency in mice [35, 36], revealed a decrease in mRNA content in the liver, which, in the former case, was shown to be due to a defect in the maturation of mRNA caused by mutation in an intervening sequence.

Recently, we analyzed abnormal ASSs of eight neonates and infants with citrullinemia in Japan [7] and found a third type (type III) of enzyme abnormalities in addition to the qualitative type (type I) and the quantitative type (type II), a classification for adult patients [4–6]. The majority of Caucasian patients suffer from severe symptoms in the neonatal period. Su et al. [30] reported that only two of 11 cell lines of cultured skin fibroblasts from citrullinemia patients contained a detectable enzyme antigen and that all citrullinemia cell lines contained hybridizable mRNA, but slight size heterogeneity was noted. Furthermore, structural abnormalities of mRNA were detectable by S1 nuclease analysis [37]. They suggested that these stable mRNAs either are not translated or they encode a product which is rapidly degraded or is immunologically nonreactive.

In the liver of one case of type III (NP no. 7), we detected a smaller amount of RNA molecule hybridized with ASS cDNA and no translatable activity with a cell-free system. We would speculate from these results that the primary defect of type III citrullinemia may be located in the intervening sequence or splicing process.

Because the cultured skin fibroblasts studied by Su et al. showed almost no ASS activities [30, 37], it is considered that none of the 11 cell lines are applicable to type II in which the ASS abnormality was found only in the liver. Two of those cell lines shown to be kinetic mutants seem to correspond to type I in which kinetically abnormal ASSs have been found in all tissues. Our type III contained a very small amount of ASS mRNA, whereas the cell lines of Su et al. [30, 37] contain stable ASS mRNA with structural abnormalities, suggesting that these cell lines do not belong to our type III either. The molecular nature of ASS deficiency in type III should be the subject of continuing investigation by Northern blot hybridization, S1 nuclease mapping, and sequencing analysis of cloned cDNA for ASS from patients. However, the amount of the liver specimens from type III was too small to perform the above analyses.

On the contrary, the analysis of mRNA for type II citrullinemia was consistent with the hypothesis [8] that no major deletion in the structural gene has occurred in these individuals. Hybridizable ASS mRNA was detected in the liver from all type II patients studied and was of near-normal size in all instances. These findings indicate that the gene for ASS is transcribed and produces stable mRNA in these patients at the same level as in the control.

Because ASS protein was defective in the liver of type II citrullinemia, it was interesting to find the translatable and cDNA-hybridizable ASS mRNA comparable to the controls. Some investigators have also demonstrated the existence of stable mRNA with a low level or a complete lack of enzyme protein in some hereditary diseases or mutant cells [30, 37–41]. There are two possible explanations for these results. One possibility is that there is a rapid degradation of the enzyme protein, and the other is that there is a lack of synthesis of the enzyme protein in vivo.

The enzyme protein that shows normal kinetic and immunochemical properties, however, may have a structural instability caused by a mutation in the structural gene. Wiginton et al. [39] found that the enzyme in the cell line from adenosine deaminase deficiency patients was more thermolabile than the normal enzyme. However, instabilities caused by structural gene mutations are unlikely in the case of type II citrullinemia, although it cannot be ruled out completely now. It has previously been demonstrated that ASS in other organs or cells, derived from the same structural gene as the hepatic enzyme, are as active as the control [5–7]. We cannot rule out the possibility that the ASS mRNA content for the cultured skin fibroblasts and/or kidney is augmented, thus accounting for normal enzyme levels in these loci from type II patients. An approach to test this possibility would be to determine if the ASS mRNA contents for the loci from type II are the same as the control. Akaboshi et al. [42] reported a case of quantitative type (type II) citrullinemia in which the hepatic ASS was not thermolabile as compared with the controls. Although the normal enzyme protein is synthesized, other factors that inactivate or degrade the enzyme protein may be present or enhanced. Some stabilizing factors for enzyme protein might be reduced in the liver of type II citrullinemia. The latter mechanism may be a more likely possibility, as shown by Takada et al. [43] and Saheki et al. [44].

Although the ASS mRNA extracted from the liver of type II citrullinemia demonstrates its coding activity in an in vitro cell-free translation system, it might not be completely active in vivo due to a defect in some RNAs and/or proteins necessary for organ-specific expression. As for this possibility, no negating or supporting evidence is yet available for type II citrullinemia, although some examples for post-transcriptional or translational regulation have been reported [45–47]. Glynias et al. [40] suggested that a mutant mRNA may have missing or altered recognition sites for translational initiation factors that are not required when translating RNA in reticulocyte lysates.

Finally, it might be considered that small RNA species, like an anti-mRNA, hybridized with ASS mRNA in vivo, but lost during RNA extraction, increase specifically in the liver of type II citrullinemia, and, consequently, ASS protein

synthesis is inhibited or blocked in vivo by RNA-RNA hybridization. Simons and Kleckner [48] reported that translation of the "transposase" mRNA of transposon Tn10 is inhibited by direct pairing with a small RNA of 180 bases in length. Pestka et al. [49] also described that the anti-mRNA (RNA complementary to the β-galactosidase mRNA) blocked highly specifically β-galactosidase synthesis at a translational level within the *E. coli* cell. It might be possible that anti-mRNA transcripts have physiological roles in cells as suggested by Mizuno et al. [50].

Our results cannot completely discriminate between rapid degradation and/or lack of synthesis in vivo. Sequencing analysis of the ASS protein or of cDNA prepared from patients with type II citrullinemia is now being studied in our laboratory and will be helpful to clarify the hepatic ASS deficiency in type II citrullinemia. At the present, there is no explanation for the large number of type II patients found only in Japan. One study that should be performed in the near future is genotyping of the patients to establish whether or not these different types have a common origin.

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