

Molecular Cloning and Sequence of the Complementary DNA Encoding Human Mitochondrial Acetoacetyl-Coenzyme A Thiolase and Study of the Variant Enzymes in Cultured Fibroblasts from Patients with 3-Ketothiolase Deficiency

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Abstract

Complementary DNAs encoding the precursor of human hepatic mitochondrial acetoacetyl-CoA thiolase (T2) (EC 2.3.1.9) were cloned and sequenced. The cDNA inserts in these clones were 1,518 bases in length when overlapped, and encoded the 427-amino acid precursor of this enzyme (45,199 mol wt). This amino acid sequence included a 33-residue leader peptide moiety and a 394-amino acid subunit of the mature enzyme (41,385 mol wt). The T2 gene expression in fibroblasts from four patients with 3-ketothiolase deficiency was analyzed by Northern blotting. The T2 mRNA in all four cell lines had the same 1.7 kb as that of the control. However, the amounts of T2 mRNA differed: the content was reduced in two cell lines (cases 1 and 3), whereas it was within a normal range in others (cases 2 and 4). Pulse labeling followed by subcellular fractionation revealed that the T2 proteins in the fibroblasts from these patients are present in the mitochondria. These results suggest that different mechanisms are involved in the enzyme defects in the four patients. (*J. Clin. Invest.* 1990. 86:2086–2092.) Key words: intracellular localization • β -ketothiolase deficiency • Northern blotting • organic acidemia • pulse labeling

Introduction

3-Ketothiolase deficiency (3KTD¹ McKusick 20375) is an inborn error of isoleucine and ketone body catabolism. It is characterized by intermittent ketoacidosis episodes and urinary excretion of organic acids such as 2-methyl-3-hydroxybutyrate, 2-methyl-acetoacetate, and tiglylglycine. Since Daum et al. (1) reported this disorder in 1971, over 10 cases have been documented (2–12). Mammalian tissues have at least four thiolases; mitochondrial 3-ketoacyl-CoA thiolase (T1), acetoacetyl-CoA thiolase (T2), peroxisomal 3-ketoacyl-CoA thiolase (PT), and cytosolic acetoacetyl-CoA thiolase

(CT) (13–15). The etiology of 3KTD has been attributed to a defect in T2 because K⁺ activation of acetoacetyl-CoA thiolase, a characteristic feature of T2 (7), is absent in tissue from patients with this disorder (6, 9, 10, 12). T2 alone seems to be responsible for the cleavage of 2-methylacetoacetyl-CoA, an intermediate of isoleucine catabolism (7, 16). The term “3KTD” is commonly used for T2 deficiency, although deficiencies of PT and CT have also been reported (17, 18).

Previously, we reported that 3KTD in a patient was due to a defect in the T2 biosynthesis (19). Subsequently, we demonstrated using the pulse-chase experiments that, in the fibroblasts from five 3KTD patients (including the first one), the T2 proteins of these patients were synthesized but they differed in stability and in mobility in SDS-PAGE (20, 21), providing evidence for heterogeneity in the molecular defects (21). To better understand 3KTD at the molecular level, we first cloned cDNA for rat T2 (22).

We now report the molecular cloning and nucleotide sequence of cDNA for human T2 and the gene expression of T2 in fibroblasts from four patients with 3KTD.

Methods

Patients. Case 1 is a Japanese boy who presented with episodic ketoacidosis (10) and case 2 is a Spanish girl who repeatedly experienced severe ketoacidosis attacks (9). Case 3 is a Laotian boy who was well developed but had several episodes of ketoacidosis (9). Case 4 is a boy with normal development, despite episodic ketoacidosis. His father is also a patient but without symptoms of this deficiency (7). The diagnosis of 3KTD in the four patients was based on the results of urinary organic acid analyses by gas chromatography-mass spectrometry, and of the enzyme assay of their fibroblasts (7, 9, 10). The four cell lines were well characterized, at the protein level. The amount, biosynthesis, and degradation of T2 protein in the fibroblasts were analyzed by immunoblot and pulse-chase experiments (19–21). These results are listed later in Table II together with the results obtained in the present work.

Screening of the cDNA library. The λ gt11 cDNA library constructed from human hepatic poly(A)RNA with random primers was kindly provided by Dr. M. Mori. The cDNA library was screened with the probe, RT2-6, a full-length cDNA of the rat T2 (22), using the plaque hybridization method (23).

Subcloning and DNA sequencing. Inserts of cDNA clones were subcloned to plasma vectors pTZ (U. S. Biochemical Corp., Cleveland, OH) or pHSG (Takara Shuzo, Kyoto, Japan). Appropriate fragments for sequencing were obtained by treatment with nuclease Ba131 or restriction endonucleases (Takara Shuzo). DNA sequences were analyzed on the double-strand plasmid DNA by the dideoxy chain termination method with the Klenow fragment (Takara Shuzo) or Sequenase (U. S. Biochemical Corp.) (24, 25).

Northern blot analysis. Skin fibroblasts were grown in Eagle's minimum essential medium containing 10% fetal calf serum. The cells were harvested 3–4 d after achieving confluency. Total RNA was pre-

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1. Abbreviations used in this paper: CT, cytosolic acetoacetyl-CoA thiolase; PT, peroxisomal 3-ketoacyl-CoA thiolase; T1, mitochondrial 3-ketoacyl-CoA thiolase; T2, mitochondrial acetoacetyl-CoA thiolase; 3KTD, 3-ketothiolase deficiency.

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pared from fibroblasts by acid guanidium thiocyanate-phenol-chloroform extraction (26). Total RNA was denatured in formamide/formaldehyde and electrophoresed in a 1.0% agarose gel containing formaldehyde (27). RNA was transferred to a sheet of Hybond-N (Amersham Corp., Arlington Heights, IL) by capillary blotting. Prehybridization, hybridization, and washing were carried out using the conditions recommended in the manufacturer's protocol. The insert of HT2-3 (see Fig. 1) was used as a probe. In order to provide standards for the amount of applied RNAs, the same membrane was rehybridized with a β -actin probe (28). Messenger RNA was quantitated by scanning densitometry of the autoradiograms.

Protein analyses. The human enzyme was purified from an post-mortem liver using the procedure previously used for the purification of rat liver enzyme (22). Two peaks of T2 activity on the phosphocellulose column chromatography, isoenzymes A and B (15, 29), were separately purified. The purified proteins were S-carboxymethylated. The amino-terminal amino acid sequence was determined by automated Edman degradation with a protein sequencer (model 470A, Applied Biosystems, Inc., Foster City, CA). Amino acid analysis was performed with an amino acid analyzer (model 6300, Beckman Instruments, Inc., Palo Alto, CA) after hydrolysis of the protein with 5.7 N HCl at 110°C for 24, 48, and 72 h, respectively.

Pulse labeling and subcellular fractionation of fibroblasts. Fibroblasts were pulse-labeled as described (21). Subcellular fractionation of the fibroblasts was done according to the method of Mackall et al. (30), with some modification. Briefly, the harvested cells were suspended in 0.25 M sucrose/20 mM potassium phosphate, pH 7.5, and 1 mM EDTA and digitonin was added to a final concentration of 1 mg/ml. The solution was kept on ice water for 1 min, then was centrifuged for 5 min at 8,000 g. The pellets were rinsed with the same buffer and resuspended in buffer containing 1 mg/ml digitonin. One-third volume of 80 mM Tris HCl, pH 7.5, 0.4% SDS, 0.4% Triton X-100, and 4 mM EDTA was added to the supernatant and the pellet fraction. Immunoprecipitation and fluorography were performed, as described (21).

Results

Isolation of cDNA clones for human T2. The λ gt11 cDNA library was screened using as a probe RT2-6 (22), a full-length cDNA for the rat T2. After screening 4.0×10^5 recombinant plaques, 13 independent positive clones were obtained (Fig. 1). All cDNA inserts were subcloned to plasmid vectors and were sequenced for the entire regions of inserts.

Sequence analysis of the cDNA. The nucleotide sequence of the cDNA for the human T2 is summarized in Fig. 2, together with the deduced amino acid sequence. A 1,281-base open reading frame, starting at the first ATG triplet (positions 1–3), encoded 427 amino acids. The amino-terminal 20-amino acid sequence of the purified enzyme determined by Edman degradation, perfectly matched the sequence (Fig. 2). Hence, the presequence of the thiolase precursor probably consists of 33 amino acid residues. The relative of molecular mass of the precursor and the mature subunit of this enzyme was calculated to be 45,199 and 41,385 D, respectively.

The combined cDNA sequence included 76 bp of the 5'-noncoding region and 161 bp of the 3'-noncoding region. A putative polyadenylation signal and poly(A) tail was not found in the cloned sequence. This may be due to the use of the library constructed with cDNAs synthesized using random primers. The 3'-noncoding regions of rat and human cDNAs were well conserved. In the rat cDNA sequence (22), a typical polyadenylation signal AATAAA was present 15 bp downstream of the site that corresponds to the 3' end of the human cDNA.

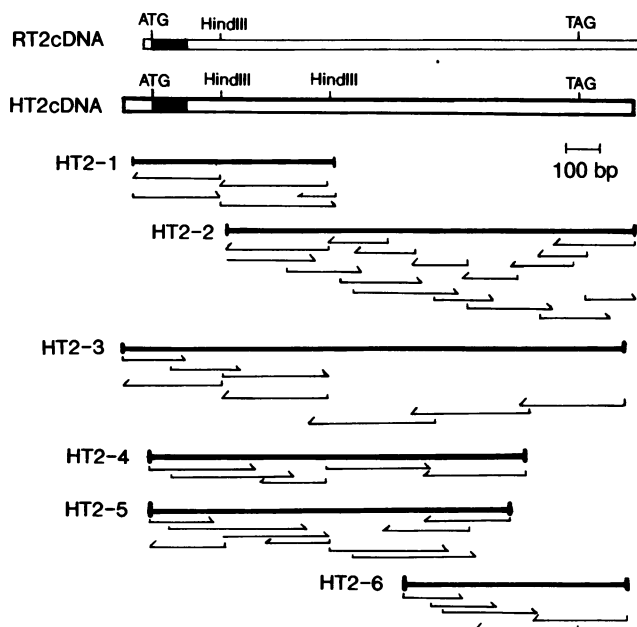


Figure 1. Restriction map and sequencing strategy for the cDNAs of T2. Partial restriction maps of rat (RT2) and human (HT2) cDNAs are shown in the upper scheme. ATGs, TAGs, and solid boxes indicate initiator methionine codons, stop codons, and the coding regions for presequences, respectively. 13 human cDNA clones from HT2-1 to HT2-13 were analyzed, and cDNAs from HT2-1 to HT2-6 are shown. The horizontal arrows under each clone indicate the direction and extent of sequencing.

When the deduced amino acid sequences of human and rat T2 were compared (Fig. 3), these sequences share 84% of identical residues. In contrast, sequence homologies of human T2 to other rat thiolases, T1 and PT (31, 32) are 40 and 33%, respectively. The subunit of the mature enzyme consisted of 394 amino acids in both rat and human T2. The nucleotide sequences of cDNAs for rat and human T2, including the noncoding regions, are 82% identical. In vitro transcription/translation of full-length human cDNA was performed according to the method as described (22). The polypeptide generated was immunoprecipitated with anti-[rat T2]IgG, which cannot cross-react with human T1, PT, and CT (data not shown). Thus, the human cDNA clones shown here encode T2 and the first ATG codon is the true initiation methionine codon. Several base substitutions which led to amino acid changes were found among 13 independent cDNA clones, as shown in Fig. 2: C³²⁰ to A (Ala to Glu) and T⁴⁸³ to A (Tyr to stop codon) in HT2-3, G¹⁰¹⁸ to A (Val to Met), G¹⁰³⁶ to A (Asp to Asn), G¹¹³⁸ to T (Ala to Thr), and A¹²³⁴ to T (Ile to Phe) in HT2-2, and C⁷⁹³ to A (Pro to Thr) in HT2-5. However, there were no substitutions common to more than two cDNA clones. These substitutions may possibly be artifacts occurring during the cDNA synthesis and cloning, but sequence heterogeneity would have to be excluded.

Protein analyses. Two peaks of the thiolase activity on a phosphocellulose column chromatography (termed isoenzymes A and B) were separately purified. The purified isoenzymes A and B were indistinguishable in mobility in SDS-PAGE (data not shown). The amino-terminal sequences of isoenzymes A and B were identical and both matched the amino acid sequence deduced from the cDNA sequence (Fig. 2).


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RT2: 1 MAALA-VLH--GVVRRPLLRLQLQEVRCLEGRSYASKPTLNDVVIVSATRTPIGSFLGSLA
    ** * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
HT2: 1 MAVLAALLRSGARSRSPLLRRLVQEIRYVERSYVSKPTLKEVVIVSATRTPIGSFLGSLA

RT2: 58 SQPATKLGTIAIQGAIEKAGIPKEEVKEVYMGNVIQGGEGQAPTRQATLGAGLPATPCT
    *****
HT2: 61 LLPATKLGSIAIQGAIEKAGIPKEEVKEAYMGNVLQGGEGQAPTRQAVLGAGLPISTPCT

RT2: 118 TVNKVCASGMKAIMMASQSLMCGHQDVMVAGGMESMSNPVYVMSRGATPYGGVKLEDLIV
    * *****
HT2: 121 TINKVCASGMKAIMMASQSLMCGHQDVMVAGGMESMSNPVYVMNRGSTPYGGVKLEDLIV

RT2: 178 KDGLTDVYNKIHMGNCAENTAKKLSISREEQDKYAIGSYTRSKEAWDAGKFANEIMPITI
    *****
HT2: 181 KDGLTDVYNKIHMGSACENTAKKLNIAERNEQDAYAINSYTRSKAAWEAGKFGNEVIPVTV

RT2: 238 SVKGKPDVVVKEDEEYKRVDFSKVPKLKTVFQKENGTVTAANASTLNDGAAAVLMTAEA
    ****
HT2: 241 TVKGQPDVVVKEDEEYKRVDFSKVPKLKTVFQKENGTVTAANASTLNDGAAALVMTADA

RT2: 298 AQRLKVKPLARIAAFADAADVDPIDFPLAPAYAVPKVLKYAGLKKEDIAMWEVNEAFSVVV
    * * * * *
HT2: 301 AKRLNVTPLARIVAFADAAVEPIDFPIAPVYAASMLVDKVLKKEDIAMWEVNEAFSLVV

RT2: 358 LANIKMLEIDPQKVNHHGAVSLGHPIGMSGARIVVHLAHALKQGEFGLASICNGGGGAS
    *****
HT2: 361 LANIKMLEIDPQKVNHHGAVSLGHPIGMSGARIVGHLTHALKQGEYGLASICNGGGGAS

RT2: 418 AVLIEKL
    * * * *
HT2: 421 AMLIQKL

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Figure 3. Comparison of the amino acid sequences of human and rat T2. The standard one-letter amino acid abbreviations are used. RT2 and HT2 represent the sequences of the rat and human enzymes, respectively. Gaps (–) were inserted to achieve maximum homology. Identical residues are indicated by stars. Sequences are numbered, beginning with the initiator methionines. Sequencing data of the rat enzyme are from Fukao et al. (22).

indicated by underlining). Amino acid compositions of the two isoenzymes were also indistinguishable. The composition was also compatible with that calculated from the deduced sequence (Table I).

Expression of the thiolase gene in fibroblasts. Expression of T2 gene in fibroblasts from the controls and from the patients was analyzed by Northern blotting, using the cDNA insert of HT2-3 as a probe (Fig. 4 A). A single band for T2 mRNA was detected in two controls and in all four patients. The size of the T2 mRNAs was 1.7 kb and was indistinguishable. However, in cases 1 and 3, the radioactive signal was very weak and the band was detected only after a long exposure. In order to estimate the amount of RNAs applied, the same membrane was rehybridized with a β -actin probe. The intensities of signals for β -actin band in cases 1 and 3 samples were not significantly smaller than others. It is apparent, therefore, that the amount of T2 mRNA in the fibroblasts from cases 1 and 3 was reduced whereas that for cases 2 and 4 was not.

In order to confirm the reduced quantity of T2 mRNA present in cases 1 and 3, T2 mRNA abundance was quantitated by densitometric scanning of autoradiograms, using 1, 3, and 9 μ g of each of the total RNA samples from four controls and four patients. Typical results are shown in Fig. 4 B. Pa-

tients' RNAs were prepared separately from three fibroblast cultures of different passages and were simultaneously electrophoresed and blotted on a Hybond-N membrane. The ratio of intensity of the band of T2 mRNA to that of β -actin was 0.55 ± 0.32 ($n = 4$), in case of the four controls. The ratio of cases 1, 2, 3, and 4 was 0.085 ± 0.014 , 0.50 ± 0.18 , 0.009 ± 0.013 , and 0.42 ± 0.02 , respectively. These results indicate that the amount of T2 mRNA is lower in cases 1 and 3 than in the controls but is within a normal range in cases 2 and 4.

Subcellular fractionation after pulse labeling of the fibroblasts. Subcellular fractionation was then done to determine whether or not the pulse-labeled T2 of the fibroblasts from the patients were transported into the mitochondria or were degraded in cytosol. The labeled T2 was recovered in the particle fraction, whereas lactate dehydrogenase, a cytosolic enzyme, was present in the cytosol, without exception (Fig. 5). The T2 proteins can therefore be translocated into an organelle, presumably into the mitochondria.

Discussion

The cDNA for human T2 which we cloned was 1,518 bp in length, including a 1,281-base open reading frame encoding

Figure 2. Nucleotide sequence of the cDNA for human T2 and deduced primary structure of the enzyme. Nucleotides are numbered in the direction 5' to 3', beginning at the first residue of the initiator ATG triplet. The nucleotides on the 5'-side from residue 1 are indicated by negative numbers. The deduced amino acid sequence is shown under the nucleotide sequence. The underlined amino acid sequence completely matched the amino-terminal sequence of the purified enzyme, determined by Edman degradation. The stop codon is represented by TER. Base variations found among different clones are as follows: A was found at the positions –22, –6, 33, and 93 in HT2-3, HT2-9, HT2-13, and HT2-1, respectively. These substitutions led to no amino acid changes. Positions 320 and 483 were occupied by A in HT2-3 instead of C and T, respectively. As a result, Ala and Tyr were substituted by Glu and stop codon in HT2-3. HT2-2 contained A at positions 1018 and 1036, T at 1138 and 1234 instead of G, G, G, and A at the respective positions in the sequence shown. These base substitutions caused amino acid changes of Val to Met, Asp to Asn, Ala to Thr, and Ile to Phe in HT2-2. A was found at position 793 in HT2-5 instead of C. As a result, Pro was substituted by Thr in this clone. These sequence data are available from EMBL/GenBank/DBJ under accession number D90228.

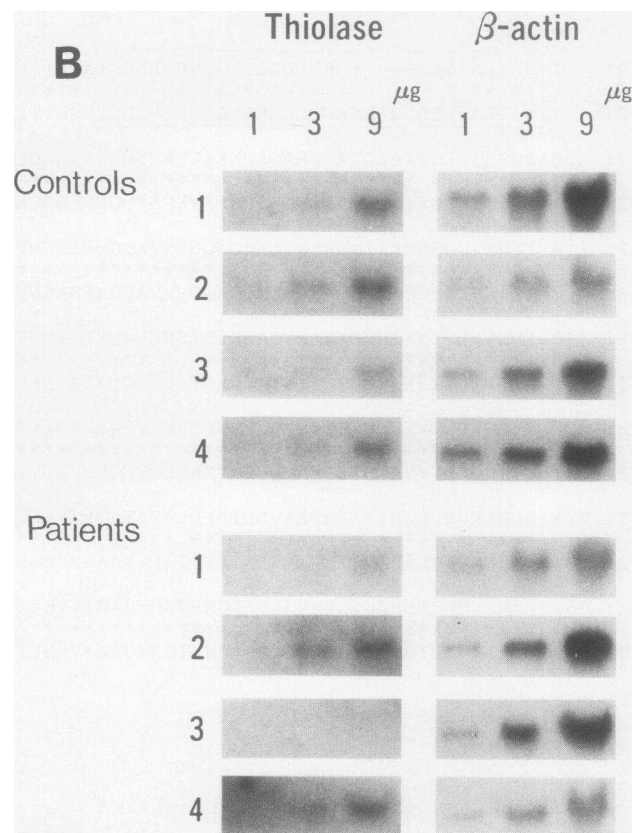
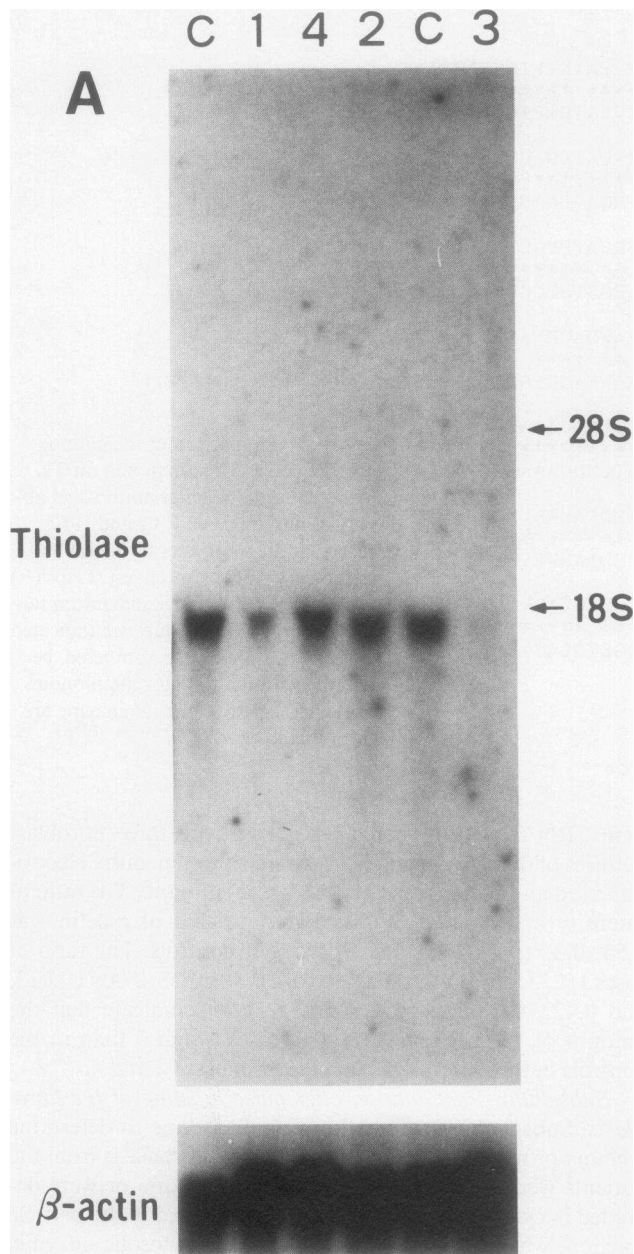


Figure 4. Northern blot analysis of the thiolase mRNA in fibroblasts from controls and patients. (A) 50 μ g of total RNA extracted from fibroblasts were denatured and electrophoresed in a 1.0% agarose-formaldehyde gel. The RNA was transferred to a Hybond-N membrane and hybridized with the 32 P-labeled HT2-3 insert. The same membrane was rehybridized with β -actin probe. The filter was washed to a final stringency of 0.2 \times SSC at 65°C. The cell lines are identified with the case numbers at the top of each lane. (B) RNAs from four controls and four patients were analyzed. Patients' RNAs were prepared separately from three fibroblast cultures of different passages and electrophoresed at the same time followed by blotting on a Hybond-N membrane. The amount of applied RNA is indicated at the top of the figure. The filter was washed to a final stringency of 1 \times SSC, 0.1% SDS at 65°C and exposed to a preflashed X-ray film. A typical blotting pattern of each patient is shown.

the entire precursor for this enzyme. This cDNA will serve as a pertinent tool for further studies on the molecular basis of heterogeneity in 3KTD.

It has been proposed that there are two isoenzymes of T2 since this enzyme was separated into two main peaks on phosphocellulose column chromatography during the process of purification (13, 29). We indeed confirmed the presence of two peaks of T2 activity. The existence of these isoenzymes might cause confusion in the study of 3KTD. However, Huth (33) has proposed that these isoenzymes are produced by post-translational modification such as coenzyme A-mediated transformation, based on the facts that isoenzyme A was spontaneously transformed in vitro to isoenzyme B, and that the rechromatography of coenzyme A-treated isoenzyme B clearly demonstrated the conversion of isoenzyme B to A. Also, we could not distinguish two purified preparations of isoenzymes A and B from rat (22), nor those from human tissue, with regard to several parameters including the amino-

terminal amino acid sequence, amino acid composition, and electrophoretic mobility of the subunits. Only one cDNA clone had a nucleotide substitution causing an amino acid change among the rat 18 cDNA clones, at position 698 (Thr²³³ to Met) (22), but no such substitution was noted in the human cDNA clones. These results support the concept of Huth (33).

We examined by Northern blotting the expression of T2 mRNA in fibroblasts from four patients who have been well characterized at the protein level (19–21). The results are listed in Table II together with a summary of the previous studies done at the protein level. The present results provide further information on the molecular basis of defects in T2 biosynthesis of these patients. T2 mRNAs detected in all cell lines were the same size as those from the controls. However, the amount of the mRNA was reduced in the two cell lines, cases 1 and 3, particularly in the latter. The amount in the other two cell lines, cases 2 and 4, was within a normal range. We previously considered that cases 3 and 4 belong to a same group since the

Table I. Amino Acid Composition of T2

Number of residues per subunit			
Amino acid	Direct analysis		Predicted from the cDNA sequence
	Isoenzyme A	Isoenzyme B	
Asp	33.8	34.2	15
Asn			19
Thr	19.7	20.6	21
Ser	20.7	22.5	24
Glu	35.9	35.4	22
Gln			12
Pro	19.0	18.9	18
Gly	38.7	37.5	38
Ala	48.0	47.9	47
Cys	7.3*	6.2*	5
Val	40.6	40.4	39
Met	13.9	14.3	16
Ile	25.1	25.1	25
Leu	32.8	32.1	31
Tyr	7.2	7.5	9
Phe	7.3	7.4	7
Lys	28.7	29.1	30
His	5.3	5.4	5
Arg	9.9	9.5	9
Trp	ND [‡]	ND [‡]	2

* Determined as S-carboxymethylcysteine.

† Not determined.

pulse-labeled T2 proteins could be detected after a 72-h chase, in either case (21). However, Northern blot analysis revealed that they differed in expression of T2 mRNA.

In case 1, the T2 mRNA was moderately decreased. The pulse-labeled T2 had a slower electrophoretic mobility than did the normal mature T2 subunit. The synthesized T2 was unstable. Fractionation of fibroblasts after pulse labeling revealed that the variant T2 protein is translocated into mitochondria and degraded there. In case 2, the amount of T2 mRNA was within a normal range; however, a smaller amount of normal-sized T2 protein was detected in the mitochondrial

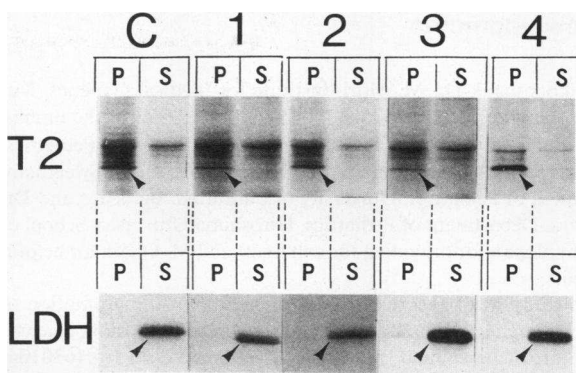


Figure 5. Subcellular fractionation after pulse labeling. The pulse-labeled fibroblasts were fractionated into supernatant (cytosolic fraction, S) and pellets (particle fraction, P), as described in Methods. Each fraction was subjected to immunoprecipitation with anti-mitochondrial acetoacetyl-CoA thiolase (T2) antibody and anti-lactate dehydrogenase (LDH) antibody followed by SDS-PAGE and fluorography. C, control fibroblasts; 1–4, the cell lines of cases 1–4, respectively. Arrowheads indicate the bands for T2 or LDH.

fraction, and was unstable. Hence the mutation of T2 gene must be located in the region coding for the mature T2 protein. In case 3, the amount of T2 mRNA was markedly decreased, and the amount of T2 protein detected was also small. The mobility in SDS-PAGE and stability of the T2 were indistinguishable from those of controls. The cause of an enzyme defect in this patient seems to be a gene mutation affecting the splicing, or inefficient transcription. Middleton et al. (9) reported that some residual T2 activity, ~ 7% of the control, was detected in fibroblasts from this patient. Taken together, the T2 protein in case 3 might be functional. In case 4, the amount of T2 mRNA was within a normal range, and the pulse-labeled T2 was normal in size but unstable. T2 protein was smaller in amount than that of the controls and degraded considerably at a 72-h chase. A point mutation in the protein coding region may be considered as the cause of the enzyme defect in case 4. The mutation sites in the coding region in cases 2 and 4 patients presumably differ because the mutant protein in case 2 degraded more rapidly than did that from case 4. Thus, the molecular basis of the disease appears to be different in all four patients.

Table II. Summary of Heterogeneity in 3KTD

Case	Amount of Northern blot mRNA*	Pulse-chase experiment			Immunoblot	T2 activity
		Pulse		Chase stability		
		CRM [‡]	Size [§]			
			<i>kD</i>			
Control	N	+++++	41	Stable	+++++	+
Case 1	Moderately reduced	+	42	Unstable	—	—
Case 2	N	+	41	Unstable	—	—
Case 3	Markedly reduced	+	41	Stable	+	—
Case 4	N	+	41	Relatively unstable	+	—

Abbreviations: CRM, cross-reactive material; N, Indistinguishable in size or amount from controls. * The size of T2 mRNA was 1.7 kb in control and every case. ‡ The labeled T2 (CRM) was translocated into mitochondria in every case. § Size means an estimation based on the electrophoretic mobility in SDS-PAGE. || T2 activity was measured by K⁺ activation of acetoacetyl-CoA thiolase, a property characteristic of this enzyme (7). Results at the protein level were previously reported (20, 21).

Acknowledgments

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References

1. Daum, R. S., P. H. Lamm, O. A. Mamer, and C. R. Scriver. 1971. A "new" disorder of isoleucine catabolism. *Lancet*. ii:1289-1290.
2. Daum, R. S., C. R. Scriver, O. A. Mamer, E. Devlin, P. Lamm, and H. Goldman. 1973. An inherited disorder of isoleucine catabolism causing accumulation of α -methylacetoacetate and α -methyl- β -hydroxybutyrate, and intermittent metabolic acidosis. *Pediatr. Res.* 7:149-160.
3. Gompertz, D., J. M., Saudubray, C. Charpentier, K. Bartlett, P. A. Goodey, and G. H. Draffan. 1974. A defect in L-isoleucine metabolism associated with α -methyl- β -hydroxybutyric and α -methyl-acetoacetic aciduria: quantitative in vivo and in vitro studies. *Clin. Chim. Acta*. 57:269-281.
4. Hillman, R. E., and J. P. Keating. 1974. Beta-ketothiolase deficiency as a cause of the "ketotic hyperglycinemia syndrome." *Pediatrics*. 53:221-225.
5. Halvorsen, S., O. Stokke, and E. Jellum. 1979. A variant form of 2-methyl-3-hydroxybutyric and 2-methylacetoacetic aciduria. *Acta Paediatr. Scand.* 68:123-128.
6. Robinson, B. H., W. G. Sherwood, J. Taylor, J. W. Balfe, and O. A. Mamer. 1979. Acetoacetyl-CoA thiolase deficiency. A cause of severe ketoacidosis in infancy simulating salicylism. *J. Pediatr.* 95:228-233.
7. Schutgens, R. B. H., B. Middleton, J. F. Blij, W. E. Oorthuys, H. A. Veder, T. Vulsma, and W. H. H. Tegelaers. 1982. Beta-Ketothiolase deficiency in a family confirmed by in vitro enzymatic assays in fibroblasts. *Eur. J. Pediatr.* 139:39-42.
8. Bennett, M. J., J. M. Littlewood, A. MacDonald, R. J. Pollitt, and J. Thompson. 1983. A case report of β -ketothiolase deficiency. *J. Inherited Metab. Dis.* 6:157.
9. Middleton, B., K. Bartlett, A. Romanos, J. Gomez Vazquez, C. Conde, R. A. Cannon, M. Lipson, L. Sweetman, and W. L. Nyhan. 1986. 3-Ketothiolase deficiency. *Eur. J. Pediatr.* 144:586-589.
10. Hiyama, K., N. Sakura, T. Matsumoto, and T. Kuhara. 1986. Deficient beta-ketothiolase activity in leukocytes from a patient with 2-methylacetoacetic aciduria. *Clin. Chim. Acta*. 155:189-194.
11. Hartlage, P., G. Eller, L. Carter, A. Roesel, and F. Hommes. 1986. Mitochondrial acetoacetyl-CoA thiolase deficiency. *Biochem. Med. Metab. Biol.* 36:198-206.
12. Leonard, J. V., B. Middleton, and J. W. T. Seakins. 1987. Acetoacetyl-CoA thiolase deficiency presenting as ketotic hypoglycemia. *Pediatr. Res.* 21:211-213.
13. Middleton, B. 1973. The oxoacetyl-coenzyme A thiolases of animal tissues. *Biochem. J.* 132:717-730.
14. Miyazawa, S., T. Osumi, and T. Hashimoto. 1980. The presence of a new 3-oxoacetyl-CoA thiolase in rat liver peroxisomes. *Eur. J. Biochem.* 103:589-596.
15. Miyazawa, S., S. Furuta, T. Osumi, T. Hashimoto, and N. Ui. 1981. Properties of peroxisomal 3-ketoacetyl-CoA thiolase from rat liver. *J. Biochem.* 90:511-519.
16. Middleton, B., and K. Bartlett. 1983. The synthesis and characterisation of 2-methylacetoacetyl coenzyme A and its use in the identification of the site of the defect in 2-methylacetoacetic and 2-methyl-3-hydroxybutyric aciduria. *Clin. Chim. Acta*. 128:291-305.
17. Schram, A. W., S. Goldfischer, C. W. T. van Roermund, E. M. Brouwer-Kelder, J. Collins, T. Hashimoto, H. S. A. Heymans, H. van den Bosch, R. B. H. Schutgens, J. M. Tager, et al. 1987. Human peroxisomal 3-oxoacetyl-coenzyme A thiolase deficiency. *Proc. Natl. Acad. Sci. USA*. 84:2494-2496.
18. Bennet, M. J., G. P. Hosking, M. F. Smith, R. G. F. Gray, and B. Middleton. 1984. Biochemical investigations on a patient with a defect in cytosolic acetoacetyl-CoA thiolase, associated with mental retardation. *J. Inherited Metab. Dis.* 7:125-128.
19. Yamaguchi, S., T. Orii, N. Sakura, S. Miyazawa, and T. Hashimoto. 1988. Defect in biosynthesis of mitochondrial acetoacetyl-coenzyme A thiolase in cultured fibroblasts from a boy with 3-ketothiolase deficiency. *J. Clin. Invest.* 81:813-817.
20. Yamaguchi, S., T. Orii, N. Sakura, S. Miyazawa, and T. Hashimoto. 1988. Immunohistochemical studies of cultured fibroblasts from a patient with 3-ketothiolase deficiency. *J. Inherited Metab. Dis.* 11:345-347.
21. Nagasawa, H., S. Yamaguchi, T. Orii, R. B. H. Schutgens, L. Sweetman, and T. Hashimoto. 1989. Heterogeneity of defects in mitochondrial acetoacetyl-CoA thiolase biosynthesis in fibroblasts from four patients with 3-ketothiolase deficiency. *Pediatr. Res.* 26:145-149.
22. Fukao, T., K. Kamijo, T. Osumi, Y. Fujiki, S. Yamaguchi, T. Orii, and T. Hashimoto. 1989. Molecular cloning and nucleotide sequence of cDNA encoding the entire precursor of rat mitochondrial acetoacetyl-CoA thiolase. *J. Biochem.* 106:197-204.
23. Huynh, T. V., R. A. Young, and R. W. Davis. 1985. Constructing and screening cDNA libraries in λ gt10 and λ gt11. In *DNA Cloning: A Practical Approach*. D. M. Glover, editor. IRL Press, Ltd., Oxford, England. 49-78.
24. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-termination inhibitors. *Proc. Natl. Acad. Sci. USA*. 74:5463-5467.
25. Hattori, M., and Y. Sakaki. 1986. Dideoxy sequencing method using denatured plasmid templates. *Anal. Biochem.* 152:232-238.
26. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.
27. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular Cloning: A laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 1-545.
28. Nakajima-Iijima S., H. Hamada, P. Reddy, and T. Kakunaga. 1985. Molecular structure of the human cytoplasmic β -actin gene: Interspecies homology of sequences in the introns. *Proc. Natl. Acad. Sci. USA*. 82:6133-6137.
29. Middleton, B. 1978. Enzymatic aspects of ketone body metabolism: the role of mitochondrial acetoacetyl-CoA thiolase isoenzymes. In *Biochemical and Clinical Aspects of Ketone Body Metabolism*. H. D. Söling, and C. D. Seufelt, editors. Georg Thieme Verlag, Stuttgart. 1-10.
30. Mackall, J., M. Meredith, and M. D. Lane. 1979. A mild procedure for the rapid release of cytoplasmic enzymes from cultured animal cells. *Anal. Biochem.* 95:270-274.
31. Arakawa, H., M. Takiguchi, A. Amaya, S. Nagata, H. Hayashi, and M. Mori. 1987. cDNA derived amino acid sequence of rat mitochondrial 3-oxoacetyl-CoA thiolase with no transient presequence: structural relationship with peroxisomal isozyme. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1361-1366.
32. Hijikata, M., N. Ishii, H. Kagamiyama, T. Osumi, and T. Hashimoto. 1987. Structural analysis of cDNA for rat peroxisomal 3-ketoacetyl-CoA thiolase. *J. Biol. Chem.* 262:8151-8158.
33. Huth, W. 1981. The charge heterogeneity of the mitochondrial acetyl-CoA acetyltransferase from rat liver. *Eur. J. Biochem.* 120:557-562.