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D Phaneuf, … , F Lettre, R M Tanguay

Research Article

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Type 1 Hereditary Tyrosinemia

Evidence for Molecular Heterogeneity and Identification of a Causal Mutation in a French Canadian Patient

Daniel Phaneuf,* Marie Lambert,‡ Rachel Laframboise,*§ Grant Mitchell,‡‖ Francine Lettre,* and Robert M. Tanguay*
*Ontogénèse et Génétique Moléculaire, §Département de Pédiatrie, Centre de Recherche du Centre Hospitalier Université Laval, Ste-Foy, Québec, Canada G1V 4G2; ‡Service de Génétique Médicale, Hôpital Sainte-Justine, Montréal, Québec, Canada; and ‖Canadian Centers of Excellence in Human Genetic Diseases, Montreal, Québec, Canada

Abstract

Type 1 hereditary tyrosinemia (HT1) is a metabolic disorder caused by a deficiency of fumarylacetoacetate hydrolase (FAH). Using a full-length FAH cDNA and specific antibodies, we investigated liver specimens from seven unrelated HT1 patients (six of French Canadian and one of Scandinavian origin). The expression of FAH in livers of these individuals was analyzed at several molecular levels including mRNA, immunoreactive material (IRM), and enzymatic activity. Four phenotypic variants were differentiated by these assays: (i) presence of FAH mRNA without any IRM or enzymatic activity, (ii) decreased FAH mRNA, IRM, and enzymatic activity, (iii) moderately decreased FAH mRNA and IRM with severely reduced enzymatic activity, and (iv) undetectable FAH mRNA, IRM, and enzymatic activity. These various molecular phenotypes suggest that this disorder may be caused by a variety of FAH mutations. Interestingly, we found no apparent relationship between the clinical and the molecular phenotypes, except that patients with absent IRM and enzymatic activity tend to have higher levels of serum alpha-fetoprotein and an earlier clinical onset. To further analyze the molecular basis of HT1, the FAH cDNA of a patient designated as variant A was amplified and sequenced. An A-to-T transversion, which substitutes asparagine[16] with isoleucine (N16I), was identified. This patient was heterozygous as shown by direct sequencing of the amplified region and hybridization with allele-specific oligonucleotide probes. The N16I allele originates from the father and the second allele appears not to be expressed in the liver of the proband. CV-1 cells transfected with the mutant cDNA produced FAH mRNA, but no protein or hydrolytic activity, as predicted by the "A" phenotype of the patient. This is the first demonstration of heterogeneity in the expression of FAH at the levels of protein, mRNA, and enzymatic activity in the livers of HT1 patients and is the first identification of a causal mutation in this disease. (*J. Clin. Invest.* 1992. 90:1185–1192.) Key words: liver disease • amino acid metabolism, inborn errors • fumarylacetoacetate hydrolase • tyrosine • missense mutation

Introduction

Type 1 hereditary tyrosinemia (HT1;[1] McKusick No. 276700) is an autosomal recessive aminoacidopathy caused by a deficiency of fumarylacetoacetate hydrolase (FAH; EC 3.7.1.2), the last enzyme of tyrosine catabolism (1). The deficiency of FAH leads to the accumulation of succinylacetone, which can be detected in serum, urine, and amniotic fluid and is used for the diagnosis of HT1 (1–4). The disease has been reported worldwide with a particularly high incidence in the French Canadian (5) and Scandinavian populations (6). The clinical spectrum of the disease is extremely wide and ranges from death due to hepatic failure within the first months of life to chronic complications such as hepatocellular carcinoma (7), renal tubular dysfunction, renal failure (8), and episodes of peripheral neuropathy during childhood and early adulthood (9). The mechanisms underlying these variable clinical outcomes have not been elucidated. We have previously reported the purification of human and rat FAH and the production of antibodies to analyze FAH expression in tissues of normal and HT1 individuals (10, 11). Our initial results (11) and those of others (12) suggested that patients with an early onset and severe symptoms lacked FAH immunoreactive material (IRM), whereas patients with a more chronic course had detectable amounts of enzyme. The antibodies were also used to purify rat FAH mRNA (13) and to isolate full-length cDNA clones for both human and rat FAH mRNAs (14, 15). Introduction of the human FAH cDNA into CV-1 monkey kidney cells resulted in the synthesis of a fully functional enzyme, indicating that the encoded sequence contained all the genetic information necessary for the hydrolytic activity (14). We mapped the structural gene for human FAH to the long arm of chromosome 15 in the region q23–25 (14) and identified restriction site polymorphisms at the FAH locus in normal humans (16–19).

We have taken two approaches to further enhance our understanding of the molecular basis of the clinical variability of the disease. First, we studied FAH expression in a group of seven clinically and biochemically characterized cases of HT1. Northern and Western blotting and FAH assays were performed on liver samples obtained from these patients at the time of transplantation. We have identified four distinct molecular phenotypes in the livers of HT1 patients, suggesting that different mutations at the FAH locus may be involved in the pathogenesis of this metabolic disorder. There was no correlation between the molecular phenotypes and the clinical sever-

Address correspondence to Dr. Robert M. Tanguay, Ontogénèse et Génétique Moléculaire, Centre de Recherche du CHUL, 2705 Boulevard Laurier, Sainte-Foy, Quebec, Canada, G1V 4G2.

1. *Abbreviations used in this paper:* FAH, fumarylacetoacetate hydrolase; HT1, type 1 hereditary tyrosinemia; IRM, immunoreactive material; PCR, polymerase chain reaction.

ity of the disease with the possible exception of blood levels of alpha-fetoprotein. We further investigated the molecular basis of this heterogeneity using the polymerase chain reaction to amplify the FAH cDNA from a tyrosinemia patient who had a normal level of FAH mRNA but not detectable FAH protein. Sequence analysis of this cDNA identified an A[47] to T transversion that predicted an asparagine to isoleucine substitution at codon 16 (N16I). Amplification and sequencing of this specific region on genomic DNA suggested that the patient is a genetic compound. Transfection assays in CV-1 monkey kidney cells using a mutant FAH cDNA containing N16I confirmed that this mutation was responsible for the lack of detectable FAH IRM and enzymatic activity.

Methods

cDNA probes and antibodies. We used HA2 (14), a 1477-bp human FAH cDNA containing the complete coding sequence, and rabbit anti-FAH antisera purified by affinity chromatography (11). Antisera were used at a 1:1,000 dilution. The 830-bp rat albumin cDNA probe (20) was a gift from Dr. Luc Bélanger, Hôtel-Dieu de Québec.

Isolation of RNA and Northern blot analysis. Total cellular RNAs were isolated from livers of normal and HT1 patients using the guanidine thiocyanate procedure of Chirgwin et al. (21). RNA was fractionated by electrophoresis in formaldehyde-containing agarose gels (1.2%) and transferred to nylon membranes (Hybond N; Amersham Corp., Arlington Heights, IL) as described by Khandjian (22). The FAH cDNA insert was labeled with $[\alpha$-^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear, Du Pont, Boston, MA) to a specific activity of 10^9 dpm/μg using the random primer method (Multiprime DNA Labelling System; Amersham Corp.). Prehybridization was done for 4 h at 42°C in 50% deionized formamide, 1 M NaCl, 1% SDS, 0.1% sodium pyrophosphate, 10× Denhardt's (0.2% Ficoll, 0.2% polyvinylpyrrolidone, and 0.2% BSA), 50 mM Tris-Cl, pH 7.5, 100 μg/ml sonicated herring sperm DNA. Hybridization was carried out in the same solution overnight at 42°C with the addition of the ^{32}P-labeled probe. Membranes were washed in 2× SSC, 0.1% SDS at room temperature for 15 min, and then for 30 min each time at 65°C in 1× SSC, 0.1% SDS; in 0.5× SSC, 0.1% SDS; and 0.1× SSC, 0.1% SDS. Wet membranes were exposed to Kodak X-Omat AR films at −70°C with intensifying screens. All Northern blots were reprobed with a ^{32}P-labeled rat albumin cDNA to provide an internal standard for mRNA quality. The amounts of specific mRNAs on the blots were determined by densitometric scanning of the autoradiogram (Research Analysis System, GL-1000 program; Amersham Corp., Arlington Heights, IL).

Determination of FAH activity and Western blot analysis. Hepatic tissues were homogenized in 10 vol (wt/vol) of cold 0.01 M potassium phosphate buffer (pH 7.3) and centrifuged at 12,000 *g* for 30 min at 4°C. Protein concentrations in the supernatants were assayed by the method of Bradford (23). FAH was assayed spectrophotometrically as the change in absorption at 330 nm at 37°C using fumarylacetoacetate as substrate (24). Proteins were separated on SDS polyacrylamide gels, transferred to nitrocellulose (Biotrace; Gelman Sciences, Montreal, Canada) and blotted with affinity-purified anti–human FAH antibodies as described (11). The secondary antibody was a ^{125}I-labeled goat anti–rabbit IgG. Nitrocellulose filters were dried and exposed to Kodak X-Omat AR films at −70°C with intensifying screens. FAH IRM was quantified by scanning of the autoradiogram and also by direct counting of the reactive bands in a gamma counter.

Patients. Material from all patients was obtained at Hôpital Ste-Justine, Montreal except for patient 1 who was from Norway and has been previously described (patient N.A.; reference 25). Liver specimens from these patients were obtained at the time of transplantation. Patients 2–7 were from French Canadian families. Genealogical analysis of the French Canadian pedigrees, performed to detect first- or second-degree relationships within or among the families, showed no evidence of consanguinity. For the families of patients 2, 3, 5, and 6, which originated from a small region in northern Quebec, this analysis was carried to the level of third-degree consanguinity and was also negative. Patients 2–7 were diagnosed during the first month of life by the Quebec Newborn Screening Program (26). They were all treated with mild to moderate protein restriction except for patient 6 who has consumed a normal diet since 6 yr of age. Patients 2–6 were in stable metabolic condition at the time of the evaluation. Patient 7 experienced a neurological crisis a few days after his evaluation. Some pertinent clinical and laboratory data are presented in Table I. All patients were found to have detectable succinylacetone levels. Other data on patients 2–6 are the following: (*i*) in all, plasma alanine aminotransferase and aspartate aminotransferase values were less than two times the upper limit of normal, and prothrombin and partial thromboplastin times were less than 1.5 times the upper limit of normal; (*ii*) one patient (No. 2) had portal hypertension; (*iii*) only patient 6 had glycosuria and/or generalized aminoaciduria, which we used to screen for renal tubular dysfunc-

Table I. Clinical, Biochemical, and Molecular Data of Type 1 Hereditary Tyrosinemia Patients

Case*	Sex	Age	Blood tyrosine	Blood succinylacetone	Serum α-fetoprotein	Liver histology‡	Hepatocellular carcinoma	Neurological crises§	FAH mRNA‖	FAH IRM‖	Enzymatic activity‖	Variant
		yr	μmol/liter	μmol/liter	μg/liter			No.				
1	F	21	270–406	NAV	17	3	Yes	NAV	++	++	−	C
2	M	7-3/12	93	14.8	70	3	No	2	+	+	+	B
3	M	7-6/12	104	26.8	60	2	No	20	+	+	+	B
4	F	2-8/12	65	9.0	1,380	3	No	>4	+	−	−	A
5	M	2	37	25	177	1	No	4	−	−	−	D
6	F	16-8/12	60	ND¶	8	3	No	21	+	+	+	B
7	M	1-1/12	40	5.6	1,400	3	No	7	++	−	−	A
Normal	NAP	NAP	45–101	ND	<10	0	NAP	NAP	++	++	++	NAP

NAP: not applicable; NAV: not available; ND: not detected.
* Values from patient 1 are published (25). For patients 2–7 the age and biochemical values given were obtained at the time of evaluation for transplantation. Hepatic transplantation and renal biopsies were performed from 3 mo to 2 yr later. ‡ Histological changes in the excised liver were graded as follows: 0, normal; 1, fibrosis; 2, micronodular cirrhosis; 3, macronodular cirrhosis. § Neurological crises were defined as the presence of paralysis or painful dysesthesia with or without hypertonic posturing during hospitalization (9). ‖ −: 0–5%, +: 5–50%, ++: 50–100% (of control value). ¶ Neonatal blood succinylacetone screening was positive but levels fell below the detection limit for filter paper spots (5 μmol/liter) during therapy.

tion. Patient 1 had normal serum transaminase values (25). The control adult liver was from a 50-yr-old woman who died of a myocardial infarction.

cDNA amplification and sequence analysis. Polyadenylated RNAs from the liver of a control subject and patient 7 were reverse-transcribed into cDNA with an oligo (dT) primer, followed by amplification of the FAH cDNAs using specific oligonucleotides primers (27). The sequence of oligonucleotides TAN18 (5'-GGGGATCCTGCCGG-GTGCTCTTCAGC-3') and TAN19(5'-GGGAATTCTGTCACTGA-ATGGCGGAC-3') was derived from the normal FAH cDNA sequence. These oligonucleotides bear a BamHI (TAN 18) or an EcoRI (TAN 19) site, at their 5' end, and were used as primers to amplify a 1,397-bp DNA fragment. This fragment contains a 1,381-bp section of the FAH cDNA, spanning nucleotides −18–1,363 and covering the complete translated region. The amplified DNA was purified from agarose gels and cloned into the EcoRI and BamHI sites of plasmid pGEM-7Zf. Sequencing was performed on several isolated recombinant clones by the dideoxy chain-termination method of Sanger et al. (28) using synthetic oligodeoxynucleotides as primers and phage T7 DNA polymerase (Pharmacia Fine Chemicals, Piscataway, NJ). The entire coding sequence of the amplified FAH cDNA from patient 7 was also sequenced directly using lambda exonuclease (Bethesda Research Laboratories, Gaithersburg, MD) as described by Higuchi and Ochman (29).

PCR amplification of genomic DNA and allele-specific oligonucleotides hybridization. Genomic DNA was isolated from peripheral blood lymphocyte nuclei by the procedure of Bell et al. (30). Human DNA of normal and HT1 livers was isolated by the procedure of Marmur (31). A 100-ng portion of genomic DNA was amplified with *Thermus aquaticus* DNA polymerase (Amplitaq; Perkin Elmer-Cetus, Norwalk, CT) as described by Saiki (32). The oligonucleotides TAN 18 and TAN 38 (5'-CCCCGCGCCCGGACGCCAAAGCCCC-3') were used as primers in the polymerase chain reaction PCR assay to amplify a 141-bp DNA fragment that completely covered exon 1 of the FAH gene. Amplified fragments were directly sequenced as described above. For allele-specific oligonucleotides hybridization, one fifth of the amplified DNA was denatured with NaOH at a final concentration of 0.4 M and applied to a nylon filter membrane under vacuum with a manifold (Hybri-Dot; Bethesda Research Laboratories). The oligonucleotides probes TAN41 (5'-CATCCACAACCTGCCCT-3') and TAN42 (5'-CATCCACATCCTGCCCT-3') were end labeled with [γ-^{32}]ATP (6,000 Ci/mmol; New England Nuclear, Du Pont) by using T4-polynucleotide kinase as described by Sambrook et al. (33). Duplicate filters were prehybridized at 50°C for 30 min in 5× SSPE (1× SSPE is 0.15 M NaCl, 10 mM NaH$_2$PO$_4$, 1 mM EDTA, pH 7.4), 5× Denhardt's solution and 0.5% SDS. Hybridization was carried out in the same solution at 50°C for 1 h with the addition of 2 × 10^6 cpm/ml of probe. Mem-

branes were rinsed twice in 2× SSPE, 0.1% SDS at room temperature, and then washed for 10 min each time at 50°C in 2× SSPE, 0.1% SDS; in 1× SSPE, 0.1% SDS; and were followed by a final wash for 15 min at 50°C in 0.5× SSPE, 0.1% SDS. Wet membranes were exposed to Kodak X-Omat AR films for 12 h at −70°C with intensifying screens.

Expression analysis. The specific A → T point mutation at base 47 in the mutant cDNA was introduced into the pSV2FAH recombinant plasmid, which expressed a functional FAH polypeptide (14). Oligonucleotides TAN35(5'-GGGGTACCGAATTCCGTGCCGGGTGCTC-TTCAGC-3') and TAN10 (5'-GTCCAAGAGCTTGCAGGC-3') were used as primers to amplify a 625-bp DNA fragment containing a 609-bp section of the mutant FAH cDNA. Amplified fragments were digested with restriction endonucleases KpnI and EcoNI (Pharmacia Fine Chemicals), and the 247-bp restriction cassette was inserted in the KpnI-EcoNI–digested pSV2FAH vector. The integrity of the whole FAH cDNA from this construction, named pSV2MUT-Ile16, was verified by DNA sequencing as described above. Normal and mutant recombinant plasmids were transfected into CV-1 monkey kidney cells using the calcium phosphate precipitation method of Gorman (34). Protein extracts from transfected cells were analyzed by Western blot as previously described (14). Northern blot analysis was performed on total RNA from transfected cells as described above.

Results

Southern blot analysis carried out on different restriction enzyme digests of genomic DNA from seven tyrosinemia patients did not show evidence of large gene deletion or rearrangement in the FAH gene (data not shown), suggesting that small mutation(s) may be responsible for HT1. We next determined if there was any significant alteration in the amount of the FAH mRNA in livers of these tyrosinemia patients by Northern blot hybridization. This analysis revealed the presence in control liver of an mRNA species that migrated at ∼ 1.6 kb (Fig. 1 *A*, lane *C*). FAH mRNA of normal size was present in varying amount in the livers of certain patients (Fig. 1 *A*, lanes *1–4*, *6–7*). In patient 5 FAH mRNA was undetectable.

Western blot analysis of liver proteins from the same patients using FAH antibodies revealed a variable amount of 43-kD FAH IRM in four patients (Fig. 1 *B*, lanes *1, 2, 3, 6*). No FAH IRM was detected in the liver of the other tyrosinemia patients (Fig. 1 *B*, lanes *4, 5, 7*) even after overexposure of the autoradiogram as shown in Fig. 1 *B*. The results of enzymatic assay of FAH are summarized in Table II. The control liver had
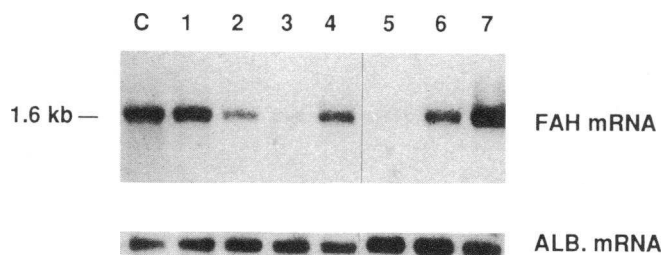
A NORTHERN BLOT

Figure 1. Expression analysis of FAH at different molecular levels in livers of seven unrelated HT1 patients. (*A*) Northern blot analysis of liver mRNA. 20 μg of total liver RNA from control and HT1 individuals was probed with the ^{32}P-labeled human FAH cDNA insert as described in Methods. Normal adult liver RNA (lane *C*), HT1 patients' liver RNA (lanes *1–7*). The 1.6-kb FAH mRNA band is indicated (*FAH mRNA*). Lower panel, hybridization of the same filter with a rat albumin cDNA insert (*ALB. mRNA*). (*B*) Western blot analysis of proteins from liver extracts of the same individuals. 40 μg of liver proteins was loaded in each lane. The immunoblot was autoradiographed and overexposed. The 43-kD FAH immunoreactive band is indicated (*FAH IRM*).

Table II. Fumarylacetoacetate Hydrolase Enzymatic Activity
of Liver Homogenates from Normal and Type I
Hereditary Tyrosinemia Patients

Case	Activity*	n	Percentage of activity in relation to the control
C	1.11±0.16	18	100
1	0.05±0.01	2	4.5
2	0.40±0.17	4	36
3	0.22±0.02	3	20
4	Not detected	4	0
5	Not detected	3	0
6	0.14±0.02	4	13
7	Not detected	2	0

* Mean±standard deviation. Expressed in $\mu mol \cdot h^{-1} \cdot mg\ protein^{-1}$. Hydrolase activity was measured at 37°C as a decrease in the optical density (330 nm) of the substrate fumarylacetoacetate. n, number of determinations.

an activity of 1.11±0.16 μmol/h per mg protein^{-1}, which is within the normal range that we established in seven other control livers (0.94±0.23 μmol/h per mg protein^{-1}; D. Phaneuf, unpublished data). FAH hydrolytic activity in patient livers was deficient in all cases and ranged from an undetectable level in patients 4, 5, 7 to 0.40 μmol/h per mg protein^{-1} in patient 2 (range, 0–36% of normal). Based on the levels of FAH mRNA, immunoreactive protein, and enzymatic activity, we classified HT1 patients into four different molecular variants (A–D), which are summarized in Table I.

Next, we analyzed the FAH mRNA from patient 7 (variant A) by reverse-transcription of cDNA, amplification, and sequencing (Fig. 2, A and C). Four separate reverse transcription and PCR amplification experiments were performed using hepatic FAH mRNA of patient 7. Sixteen independent cDNAs were subcloned and sequenced. All 16 clones contained an A to T transversion at position 47 from the first ATG that results in the replacement of asparagine[16] with isoleucine (N16I) (Fig. 3, arrow). Direct sequencing of amplified material from the patient's FAH cDNA confirmed that the A to T substitution at position 47 was the only nucleotide alteration present in the entire amplified FAH cDNA. No evidence of the presence of a normal A residue was seen on direct sequencing.

To determine if the patient was homozygous for N16I, a 141-bp DNA fragment (which includes the first coding exon of the FAH gene; R. M. Tanguay, unpublished data) was amplified from genomic DNA and sequenced. The presence of two bands comigrating in lanes A and T at nucleotide position 47 (Fig. 4 B, proband) clearly indicates that patient 7 is a genetic compound for N16I and another allele with normal sequence in this region. Sequence analysis of the amplified 141-bp DNA fragment from the parents revealed that the N16I allele originates from the father (Fig. 4 B). These results were confirmed by dot blot hybridization experiments using allele-specific oligonucleotide probes corresponding to either the normal or mutant sequences (Fig. 4 C). Genomic DNA from 37 other tyrosinemia patients (25 of French Canadian origin and 12 from other countries) was similarly amplified and analyzed with allele-specific oligonucleotide probes. The N16I allele was not found in any of these individuals (data not shown).

Transfection and expression analyses were next performed to determine if the N16I substitution could in itself be responsible for the deficiency of FAH in patient 7. Recombinant plasmids pSV2FAH, pSV2MUT-Ile16, and pSV2CAT (35), as a negative control, were transfected into CV-1 monkey kidney cells, which do not normally express FAH. Northern blot analysis of total RNA from transfected and untransfected CV-1 cells showed that FAH mRNA was present at similar levels in cells transfected with vectors containing either normal or mutant FAH cDNA, whereas such a message was undetectable after transfection with pSV2CAT or in untransfected CV-1 cells (blank) (Fig. 5 A). The corresponding Western blot analysis of protein extracts from these cells showed that cells transfected with the normal cDNA (pSV2FAH) expressed immunoreactive material as well as FAH activity whereas cells transfected with either the N16I-containing mutant cDNA or negative controls (pSV2CAT and blank) did not express detectable immunoreactive material (Fig. 5 B) or FAH hydrolytic activity (data not shown).

Discussion

Analysis of livers from seven patients with proven hereditary tyrosinemia type 1 was performed by measuring FAH mRNA levels, the presence of an immunoreactive protein, and FAH enzymatic activity. Our results indicate substantial molecular and biochemical heterogeneity in HT1. Four variants, summarized in Table I, can be distinguished. The livers of patients 4 and 7 (variant A) contained FAH mRNA of normal size but
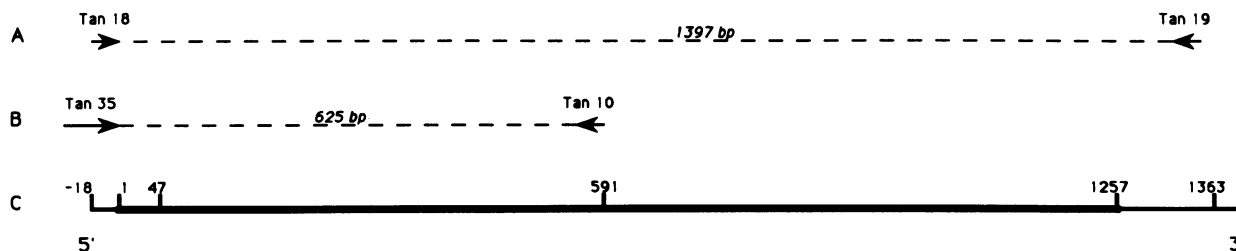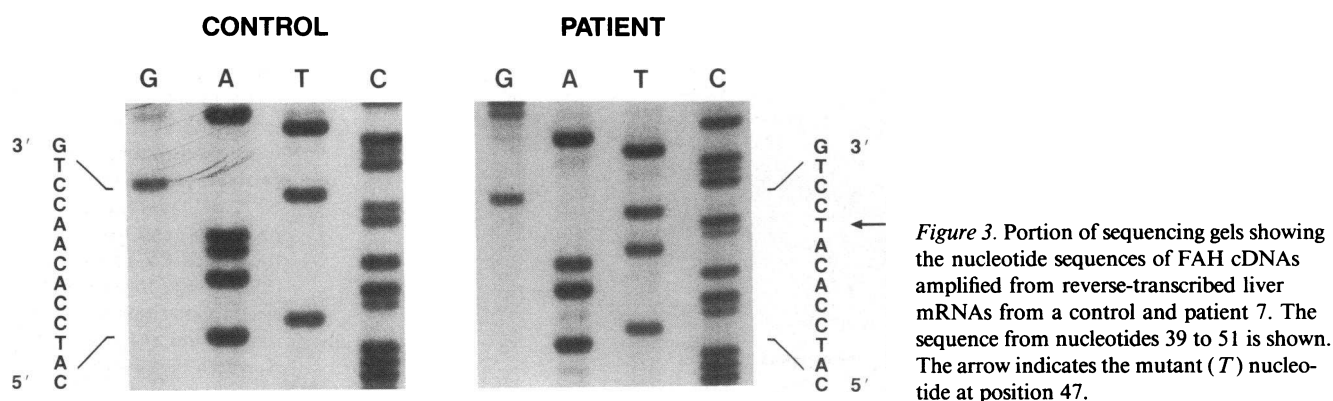

Figure 2. Diagram of cDNA fragments from patient 7 amplified by PCR and used for sequence analysis and construction of pSV2MUT-Ile16. (A) 1,397-bp DNA fragment containing a 1,381-bp section of FAH cDNA (bp −18–1,363) amplified from reverse-transcribed mRNAs from the liver of patient 7 with the specific primers TAN 18 and TAN 19. (B) 625-bp DNA fragment containing a 609-bp section of FAH cDNA (bp −18–591) amplified from a mutant FAH cDNA with primers TAN 35 and TAN 10. (C) Schematic representation of the FAH cDNA (5' → 3') including the entire coding region from nucleotides 1–1,257.

CONTROL   **PATIENT**

Figure 3. Portion of sequencing gels showing the nucleotide sequences of FAH cDNAs amplified from reverse-transcribed liver mRNAs from a control and patient 7. The sequence from nucleotides 39 to 51 is shown. The arrow indicates the mutant (*T*) nucleotide at position 47.

no detectable IRM or enzymatic activity. The livers of patients 2, 3, and 6 (variant B) contained a reduced amount of normal-sized FAH mRNA with reduced levels of immunodetectable protein, which could be correlated with FAH hydrolytic activity. In the case of patient 1 (variant C), the slight reduction of FAH mRNA and IRM contrast with the markedly diminished enzymatic activity. Interestingly, this patient is from Norway (all other patients are of French Canadian origin). A similar biochemical phenotype has not been seen in over 30 Quebec patients that we have examined to date. Finally, no FAH mRNA or enzyme could be detected in the liver of patient 5 (variant D). Although the findings of separate molecular and biochemical variants is reproducible and consistent with the concept of several underlying mutations, we cannot formally conclude that they represent distinct mutations at present. Mutational analysis to address this question directly is underway.

A Genomic Amplification
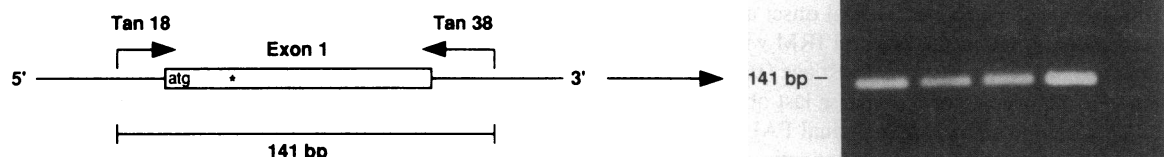
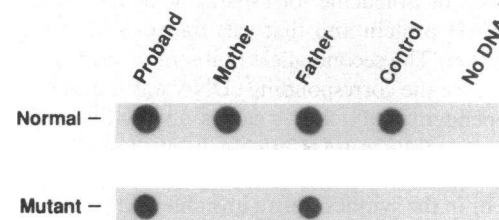## B Sequence Analysis
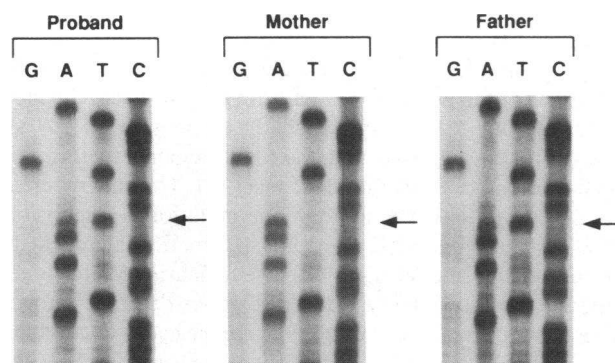
C Dot Blot Hybridization

Figure 4. Amplification and analysis of a 141-bp genomic DNA fragment containing exon 1 of the FAH gene from the proband, his mother, his father, and a control. (*A*) Schematic representation of the strategy used to amplify the 141-bp DNA fragment by PCR with primers TAN 18 and TAN 38 and analysis of amplified materials on a 2% agarose gel stained with ethidium bromide. The asterisk inside the exon 1 box designates nucleotide 47. (*B*) Direct DNA sequence analysis of amplified alleles. Arrows indicate nucleotide 47. (*C*) Dot-blot hybridization analysis. One fifth of amplified materials was denatured, applied in duplicate on nylon filters, and hybridized separately to either allele-specific oligonucleotide probes for the normal or the mutant sequences as described in Methods. Autoradiography was performed.
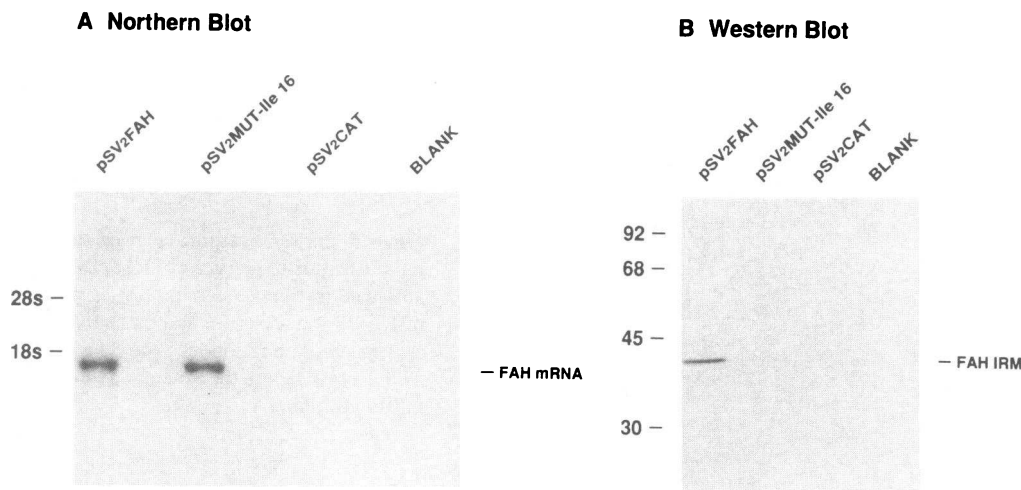
A Northern Blot

B Western Blot

Figure 5. Expression analysis of FAH in transfected CV-1 cells. (*A*) Northern blot analysis of total RNA. 20 μg of total RNA from CV-1 cells transfected with recombinant plasmids pSV2FAH, pSV2MUT-ile16, and pSV2CAT, and from untransfected CV-1 cells (blank) were fractionated by electrophoresis on a 1% agarose gel and analyzed by Northern blot with the ^{32}P-labeled human FAH cDNA insert as described in Methods. The Northern blot was autoradiographed. The positions of 28S and 18S ribosomal subunits are indicated to the left of the panel. (*B*) Corresponding Western blot analysis of protein extracts. 40 μg of protein extracts was analyzed as described in Methods. Molecular mass (expressed in kD) is indicated to the left side of the panel.

In general, we found no clear relationship between the clinical, routine laboratory, and histologic results of the patients and the levels of FAH mRNA, protein, or enzymatic activity. This apparent clinical-molecular discordance is consistent with reports of variable clinical severity among affected siblings (7). It is nonetheless interesting that patients 4, 5, and 7, who did not have any FAH activity, had the highest levels of serum alpha-fetoprotein, a marker of hepatocyte injury and proliferation. It is also interesting to compare our earlier report (11) that a group of HT1 patients with early clinical onset and severe symptoms had no detectable hepatic FAH IRM with the observation that patients who reached the oldest age (patients 1, 2, 3, and 6) all had some FAH activity. These last observations are consistent with the concept that residual FAH IRM and enzyme activity may have favorable prognostic significance in tyrosinemia, although the current data do not allow stronger conclusions to be drawn. Another group has recently failed to find a relationship between clinical and biochemical phenotypes (36).

To further investigate the basis of this heterogeneity, the molecular defect of one tyrosinemia patient designated as variant A (patient 7) was examined. This analysis revealed that this patient was heterozygous for a missense mutation that results in the substitution of isoleucine for asparagine at residue 16 (N16I) of the FAH protein and that was transmitted to the patient by his father. The second allele is absent or present in very low levels, since the corresponding cDNA was found neither in 16 independent FAH cDNA clones nor by direct sequence analysis of amplified FAH mRNA from the proband. This could be caused by another mutation resulting in a defect of transcription or in the synthesis of an unstable FAH mRNA. Further studies will be necessary to determine the exact molecular nature of the defect responsible for the lack of detectable FAH mRNA from this allele. A similar case has recently been described in a patient with Pompe disease (37).

The level of mutated FAH mRNA expressed in transfected CV-1 cells is similar to that found in cells transfected with the normal FAH cDNA. However, neither immunoreactive material nor enzymatic activity were detected in CV-1 cells transfected with the mutant FAH cDNA. These results are compatible with the molecular phenotype found in the patient's liver and confirms that the N16I substitution is responsible for the enzyme deficiency.

The N16I substitution may affect the stability of the FAH protein. It is interesting to note that Asn^{16} is conserved in human, rat, and mouse FAH (14, 15, 38). In addition, N16I results in the substitution of a polar with a nonpolar residue, which may alter the regional hydrophobicity pattern and the secondary structure of the protein as predicted by the Chou-Fasman algorithm (39). Walker et al. (40) described a missense mutation in argininosuccinic acid lyase, affecting a highly conserved region of the argininosuccinate lyase protein and which could be responsible for a decreased stability of the enzyme. A similar mutant allele was also reported at the phenylalanine hydroxylase (phenylketonuria) locus in a Chinese patient (41) and in most alleles of ornithine δ-amino-transferase (42). N16I is not a common mutation at the FAH locus, since it is not observed in 37 additional HT1 patients (25 of whom are French Canadians). Moreover, N16I was not detected in amplified genomic DNA from another patient with a variant A phenotype (FAH mRNA$^+$, IRM$^-$, EA$^-$, see patient 4). This demonstrates that the same molecular phenotype may be caused by different genetic lesions.

The highest reported incidence of HT1 is found in French Canadians of the northeastern part of Quebec, where the incidence at birth is 1:1,846 and where the carrier rate is 1:20 (5). It was initially suggested that the high incidence within this population was due to a founder effect (43). The data reported here demonstrate that more than one mutation is responsible for HT1 among French Canadians. Genetic heterogeneity within French Canadians has been well documented for other hereditary diseases. The existence of seven different mutant alleles has been reported at the phenylalanine hydroxylase (phenylketonuria) locus (44, 45) and four at the cystic fibrosis (46) locus. Two different mutations at the β-globin locus (β-thalassemia) (47) and in hexosaminidase A (Tay-Sachs disease) (48) have been identified within this population. At least two alleles cause hyperchylomicronemia in French Canadians (49). In Finland, another region with genetic isolation, population stratification, and characteristic autosomal recessive diseases, at

least three mutant alleles of ornithine δ-amino-transferase cause gyrate atrophy of the choroid and retina, indicating a more complex situation than initially expected (50).

The molecular lesion(s) causing tyrosinemia in the majority of French Canadian patients remain unknown. It remains possible that a prevailing allele or alleles exist, as is the case in the above examples (44–50). Although the different variants that we have observed may be due to epigenetic effects upon the FAH mRNA and protein, they may also reflect substantial mutational heterogeneity among French Canadian patients with HT1.

Acknowledgments

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