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Cloning and Expression of the cDNA Encoding Human Fumarylacetoacetate Hydrolase, the Enzyme Deficient in Hereditary Tyrosinemia: Assignment of the Gene to Chromosome 15

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Summary

Type 1 hereditary tyrosinemia (HT) is an autosomal recessive disease characterized by a deficiency of the enzyme fumarylacetoacetate hydrolase (FAH;E.C.3.7.1.2). We have isolated human FAH cDNA clones by screening a liver cDNA expression library using specific antibodies and plaque hybridization with a rat FAH cDNA probe. A 1,477-bp cDNA was sequenced and shown to code for FAH by an in vitro transcription-translation assay and sequence homology with tryptic fragments of purified FAH. Transient expression of this FAH cDNA in transfected CV-1 mammalian cells resulted in the synthesis of an immunoreactive protein comigrating with purified human liver FAH on SDS-PAGE and having enzymatic activity as shown by the hydrolysis of the natural substrate fumarylacetoacetate. This indicates that the single polypeptide chain encoded by the FAH gene contains all the genetic information required for functional activity, suggesting that the dimer found in vivo is a homodimer. The human FAH cDNA was used as a probe to determine the gene's chromosomal localization using somatic cell hybrids and in situ hybridization. The human FAH gene maps to the long arm of chromosome 15 in the region q23-q25.

Introduction

Type 1 hereditary tyrosinemia (HT; McKusick no. 27670) is an autosomal recessive inborn error of tyrosine metabolism (Kvittingen 1986; Goldsmith and Laberge 1989). The disease has been reported worldwide but with a variable frequency. In the province of Quebec, the incidence of HT in the general population is one per 10,000 births, but in the region of Saguenay-Lac-St-Jean it rises to 1:1846, indicating a carrier rate of 1:20 (De Braekeleer and Larochelle 1990). Clinically, hereditary tyrosinemia is characterized by acute or chronic hepatic and renal dysfunctions resulting in vitamin D–resistant rickets, hepatic cirrhosis, liver failure, and development of hepatocarcinoma in adolescence. Neurological crises have also been reported to occur in over 40% of patients (Mitchell et al. 1990). Dietary restriction of tyrosine and phenylalanine can improve the condition of patients but does not reverse the damage to the liver and kidneys. In some cases, liver transplantation has been reported (Starzl et al. 1985; Flatmark et al. 1986); however, these patients still show biochemical abnormalities likely due to kidney and possibly other tissue dysfunctions (Tuckman et al. 1987; Kvittingen et al. 1986*b*). Excretion of succinylacetone and its metabolites in the urine of HT patients led Lindblad et al. (1977) to suggest that the primary defect of the disease was a decreased activity of the enzyme fumarylacetoacetate hydrolase (FAH; E.C.3.7.1.2), the last enzyme involved in the catabolism of tyrosine. Using antibodies against purified rat and human FAH, we previously showed that two clinical forms of the disease could be differentiated in an immunoblot assay (Tanguay et al. 1984; Tanguay et

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al. 1990). The acute form is characterized by an absence of FAH immunoreactive material (IRM) in liver and kidneys of patients. Using an antibody against beef liver FAH, Berger et al. (1987) also reported similar findings in their studies of Dutch HT patients. The chronic form is characterized by a lowered amount of FAH IRM which is correlated with the amount measured in the enzymatic assay (Tanguay et al. 1990).

In addition to hepatocytes, a low FAH enzymatic activity has been measured in lymphocytes, fibroblasts, amniocytes, chorionic villi, and erythrocytes. These cell types have been used for prenatal and neonatal diagnosis and heterozygote detection (Kvittingen et al. 1983, 1985, 1986*a*; Holme et al. 1985). However, the reliability of heterozygote detection based on such enzymatic assays is not absolute. Genetic variants showing little FAH activity but without the disease have been reported, and this can limit the usefulness of such tests for carrier detection (Kvittingen et al. 1983). Furthermore, the primary molecular defect is still unknown. It could result from total or partial deletion of the gene, frameshift mutations, or multiple other molecular defects affecting the expression of the gene. A study of the FAH gene was undertaken to determine the molecular basis of the disease in both of its clinical forms and to develop a DNA-based carrier detection test. We have reported elsewhere the purification of rat mRNA coding for FAH (Nicole et al. 1986) and the isolation and sequence of partial cDNA clones coding for rat and human FAH (Phaneuf et al. 1989). Here we report the cloning and sequence of full-length human FAH cDNA and its expression in transfected CV-1 cells. The FAH structural gene was assigned to chromosome 15 by Southern blot analysis of human-rodent somatic cell hybrid panels. In situ hybridization confirms the localization of the FAH gene locus within the region 15q23-q25.

Material and Methods

Isolation of cDNA Clones

A human liver λgt11 cDNA library (Kwok et al. 1985) provided by Dr. S. L. C. Woo (Howard Hughes Medical Institute, Houston) was screened by plaque hybridization with a [^{32}P]-labeled rat FAH cDNA insert (Y. Labelle, unpublished data) at 68°C as described elsewhere (Mason and Williams 1985). This library was also screened with affinity-purified rabbit anti-human FAH antibodies (Tanguay et al. 1990) and [^{125}I]-labeled goat anti-rabbit IgG according to the method of Huynh et al. (1985). The longest cDNA inserts (named HA2 and HK) were subcloned into pGEM-7Zf.

In Vitro Transcription, Translation, and Immunoprecipitation

Prior to transcription, the plasmids containing the human FAH cDNA inserts were linearized with *Sma*I or *Xho*I (in vitro transcriptions with T7 or SP6 RNA polymerases, respectively), deproteinized, and precipitated with ethanol. Transcription reactions were carried out as described elsewhere (Nicole and Tanguay 1987). The synthetic RNAs were translated in micrococcal nuclease-treated rabbit reticulocyte lysates (Amersham) in the presence of [^{35}S]methionine for 60 min at 37°C. The translation products were analyzed by SDS-PAGE (Thomas and Kornberg 1975) prior to or after immunoprecipitation with an affinity-purified rabbit anti-human FAH antibody and *Staphylococcus aureus* cell suspension (Pansorbin, Calbiochem, San Diego, CA) as described elsewhere (Kelley and Schlesinger 1982). Gels were stained with Coomassie brilliant blue, dried and autoradiographed on Kodak X-Omat AR film.

Construction of Plasmids for In Vitro Transcription-Translation

To construct a clone without the first ATG, the HA2 FAH cDNA insert was cut with *Alu*I, removing nucleotides − 18 to + 206, and the complete and truncated inserts subcloned into a Bluescript transcription vector. To validate the constructions, recombinant plasmids were sequenced at their 5′ and 3′ ends. The inserts were transcribed and translated in vitro as described above.

Sequencing and Extension of the FAH cDNA

Sequencing was performed on both strands by the dideoxy chain-termination method (Sanger et al. 1977) using synthetic oligodeoxynucleotides as primers and phage T7 DNA polymerase (Pharmacia). Extension of the 5′ end of FAH cDNA was performed by reverse transcription of human liver mRNA using a gene-specific oligodeoxynucleotide followed by PCR amplification as described by Frohman (1990). PCR-amplified products were cloned into pGEM-7Zf and sequenced. (The nucleotide sequence data reported in this paper are deposited in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under the accession number M55150.)

Expression of the FAH cDNA in CV-1 Cells

To construct pSV2FAH, the cDNA insert from phFAH HA2 was inserted in place of the *Hin*dIII-*Hpa*I fragment of the pSV2CAT vector containing the chloramphenicol acetyltransferase coding sequence (Gorman et al. 1982). DNA transfections into CV-1 cells were performed by the calcium phosphate precipitation method (Ausubel et al. 1987). Cells were harvested by centrifugation and sonicated in 10 mM phosphate buffer, pH 7.3. Protein concentrations of cell extracts were assayed according to the method of Bradford (1976). The enzymatic activity of FAH was measured using fumarylacetoacetate as the substrate as described elsewhere (Tanguay et al. 1990). Proteins from cell extracts were also separated on SDS polyacrylamide gels, transferred to nitrocellulose (Biotrace, Gelman Sciences, Montreal) and blotted with affinity-purified rabbit anti-human FAH antibodies as described elsewhere (Towbin et al. 1979). The secondary antibody used was a [^{125}I]-labeled goat anti-rabbit IgG.

Analysis of Somatic Cell Hybrids

The panel of rodent-human somatic cell hybrids and its characterization have been described elsewhere (Griffin et al. 1987). The hybrids designated GM were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ); those designated A3 were from P. Pearson (University of Leiden). The 1.5-kb insert derived from recombinant plasmid phFAH HA2 was labeled with [^{32}P]-dCTP (3,000 Ci/mmol) to a specific activity of 10^9 dpm/μg by the method of Feinberg and Vogelstein (1983). DNA was isolated from hybrid cells, digested with *Hin*dIII, fractionated by electrophoresis on 0.8% agarose gel, and transferred to a nylon membrane (Schleicher and Schuell) as described elsewhere (Griffin et al. 1987). Filters were prehybridized in 6 × SSC, 10 × Denhardt's, 1% SDS, and 100 μg boiled salmon sperm DNA/ml for 16 h at 42°C. Hybridization was carried out at 47°C overnight in the same solution containing 50% deionized formamide and 5% dextran sulfate. The membranes were washed twice in 2 × SSC, 0.1% SDS for 15 min at room temperature followed by two 30-min washes at 65°C in 0.1 × SSC, 0.1% SDS and a 5-min final wash in 0.1 × SSC at room temperature.

In Situ Hybridization

Human metaphase chromosomes were obtained from phytohemagglutinin-stimulated peripheral blood lymphocytes from a normal male as described elsewhere (Mattei et al. 1985). 5-Bromo-deoxyuridine (30 μg) was added for the last 7 h of culture to ensure high-quality chromosomal banding. Slides were treated with RNase and DNA denatured with alkali prior to hybridization. The FAH cDNA probe was nick-translated in the presence of [^{3}H]-dCTP (50 Ci/mmol) and [^{3}H]-dTTP (80.5 Ci/mmol) to a specific activity of 2.6 × 10^7 dpm/μg. The probe was hybridized to metaphase preparations at final concentrations of 0.01–0.5 μg/ml hybridization solution at 42°C for 16–18 h as described elsewhere (Berge-Lefranc et al. 1985; Mattei et al. 1985). Slides were then rinsed in 50% formamide, 2 × SSC at 39°C, followed by successive washes in 2 × SSC at 39°C and room temperature before dehydration in ethanol and air-drying. Hybridized slides were coated with nuclear track emulsion (Kodak NTB2) and exposed at 4°C for 7–19 d. Chromosomes were stained with buffered Giemsa solution, and the metaphase chromosomes were photographed. R-banding was then performed using a slight modification of the fluorochrome-photolysis-Giemsa (FPG) method of Perry and Wolf (1974), and metaphase chromosomes were rephotographed before analysis.

Peptide Sequencing

FAH from rat and human liver was purified to homogeneity as described previously (Tanguay et al. 1990). Tryptic peptides were obtained by high-performance liquid chromatography, and the sequence analysis was performed by M. Van der Rest at the Shriners Hospital for Crippled Children, Montreal.

Results

Isolation of Human FAH cDNA Clones

A human liver λgt11 cDNA library was initially screened with a rat FAH cDNA probe (Y. Labelle, unpublished data). Nine positive clones were isolated from 10^5 recombinant phages. The same library was also screened with rabbit anti-human FAH antibodies, and four other clones were isolated from a total of 4.0 × 10^4 recombinant phages. The cDNA inserts of the clones (800–1,500 bp) isolated with the two techniques hybridized with each other. To confirm the identity of the isolated human clones, the two longest cDNA inserts (named phFAH HA2 and phFAH HK) were subcloned into a pGEM transcription vector, transcribed with T7 or SP6 RNA polymerases and

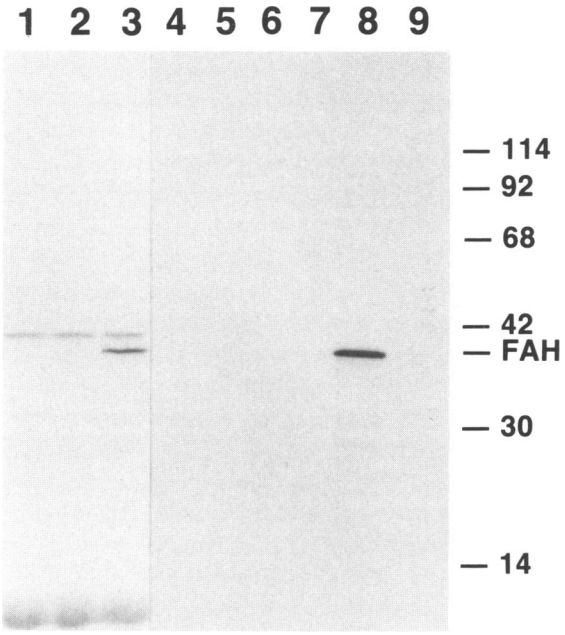

Figure 1 Translation products of SP6 and T7 transcripts of human FAH clone. In vitro–transcribed sense and antisense RNAs of the cDNA insert of phFAH HA2 were translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. The translation products were immunoprecipitated with affinity-purified rabbit anti-human FAH antibodies as described in Material and Methods. Translation products were analyzed on a 12% polyacrylamide gel. Lanes 1–3, autoradiogram of translation products from blank (lane 1), antisense RNA (lane 2), and sense RNA (lane 3). Lanes 4–9, immunoprecipitation of translation products with immune (lanes 4, 6, and 8) and nonimmune (lanes 5, 7, and 9) sera: blank (lanes 4 and 5), antisense RNA (lanes 6 and 7), and sense RNA (lanes 8 and 9). Molecular masses (in kilodaltons) of protein markers including human FAH are shown on the right side of the panel.

the synthetic RNAs were translated in a reticulocyte lysate. SDS gel analysis of the translation products of the sense-synthetic mRNA generated from the cDNA insert of phFAH HA2 showed a polypeptide of the size expected for the human FAH monomer (fig. 1, lane 3). This translation product was specifically immunoprecipitated with an affinity-purified rabbit anti-human FAH antibody (fig. 1, lane 8) but not with the preimmune serum (fig. 1, lane 9). Antisense mRNA could not direct the translation of this specific product (fig. 1, lanes 2 and 6). Similar results were obtained with the second phFAH HK clone (data not shown).

Sequence and 5′ Extension of the Human FAH cDNA

The cDNA insert in phFAH HA2 was sequenced in both directions. The complete sequence was 1,477 bp long, including 68 bp of poly (A) tail (fig. 2). There is a single continuous open reading frame of 1,257 bp coding for 419 amino acids starting from the first ATG at position 1 and ending at the TGA at position 1258. As shown in figure 2, homologies between four sequenced oligopeptide fragments from purified rat FAH and the deduced amino acid sequence of the human FAH cDNA at positions 48–57, 85–90, 213–218, and 383–392 provide evidence for the authenticity of the clone. The observation that the in vitro translation product of the synthetic mRNA corresponding to the cDNA insert of phFAH HA2 comigrates on SDS gels with the human FAH monomer suggests that the ATG at position 1 is the translation initiation codon (also see below). Since there were only 18 bp before the first ATG in phFAH HA2, 5′ extension of the cDNA was performed. Liver mRNAs from two individuals were reverse transcribed using an FAH gene–specific oligodeoxynucleotide primer followed by PCR amplification. Sequence analysis of PCR-amplified products from eight cDNA clones (four from each individual) revealed that they all overlap with the cDNA insert of phFAH HA2. This analysis allowed us to add 38 bp at the 5′ end of the FAH cDNA (fig. 2). No other ATG initiation codon was found in that sequence.

Expression of FAH cDNA in CV-1 Cells

To establish whether the cloned FAH cDNA contained all the genetic information necessary for expression of FAH enzymatic activity, the phFAH HA2 insert was subcloned in a pSV2 expression vector and transfected into CV-1 cells which do not normally express FAH. Proteins from transfected and untransfected cells and from human liver were separated on SDS polyacrylamide gels and immunoblotted with affinity-purified anti-human FAH antibodies. As shown in figure 3, cells transfected with pSV2FAH (lane 3) had an immunoreactive band which comigrated with human liver FAH (lane 1). No immunoreactive bands were seen in untransfected CV-1 cells (lane 2) or in cells transfected with the original vector pSV2CAT (data not shown). Parallel examination of cells by immunofluorescence with the anti-FAH antibody indicated that ~15% of cells transfected with pSV2FAH expressed FAH (data not shown). To determine if the expressed protein was biologically active, the hydrolytic activity of FAH against its natural substrate fumarylacetoacetate was measured in these cell extracts. FAH activity was present in the extract from pSV2FAH-transfected CV-1 cells but not in extracts from untransfected cells (table 1). The activity of FAH expressed in transfected CV-1 cells was comparable

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                                                         -56 AGTCCTGCTCTCCGCACGCCACCTTAGGCCCGCAGCCGTGCCGGGTGCTCTTCAGC

   1 ATG TCC TTC ATC CCG GTG GCC GAG GAT TCC GAC TTC CCC ATC CAC AAC CTG CCC TAC GGC GTC TTC TCG ACC AGA GGC GAC CCA AGA CCG
   1 Met Ser Phe Ile Pro Val Ala Glu Asp Ser Asp Phe Pro Ile His Asn Leu Pro Tyr Gly Val Phe Ser Thr Arg Gly Asp Pro Arg Pro

  91 AGG ATA GGT GTG GCC ATT GGC GAC CAG ATC CTG GAC CTC AGC ATC ATC AAG CAC CTC TTT ACT GGT CCT GTC CTC TCC AAA CAC CAG GAT
  31 Arg Ile Gly Val Ala Ile Gly Asp Gln Ile Leu Asp Leu Ser Ile Ile Lys His Leu Phe Thr Gly Pro Val Leu Ser Lys His Gln Asp

 181 GTC TTC AAT CAG CCT ACA CTC AAC AGC TTC ATG GGC CTG GGT CAG GCT GCC TGG AAG GAG GCG AGA GTG TTC TTG CAG AAC TTG CTG TCT
  61 Val Phe Asn Gln Pro Thr Leu Asn Ser Phe Met Gly Leu Gly Gln Ala Ala Trp Lys Glu Ala Arg Val Phe Leu Gln Asn Leu Leu Ser

 271 GTG AGC CAA GCC AGG CTC AGA GAT GAC ACC GAA CTT CGG AAG TGT GCA TTC ATC TCC CAG GCT TCT GCC ACG ATG CAC CTT CCA GCC ACC
  91 Val Ser Gln Ala Arg Leu Arg Asp Asp Thr Glu Leu Arg Lys Cys Ala Phe Ile Ser Gln Ala Ser Ala Thr Met His Leu Pro Ala Thr

 361 ATA GGA GAC TAC ACA GAC TTC TAT TCC TCT CGG CAG CAT GCT ACC AAC GTC GGA ATC ATG TTC AGG GAC AAG GAG AAT GCG TTG ATG CCA
 121 Ile Gly Asp Tyr Thr Asp Phe Tyr Ser Ser Arg Gln His Ala Thr Asn Val Gly Ile Met Phe Arg Asp Lys Glu Asn Ala Leu Met Pro

 451 AAT TGG CTG CAC TTA CCA GTG GGC TAC CAT GGC CGT GCC TCC TCT GTC GTG GTG TCT GGC ACC CCA ATC CGA AGG CCC ATG GGA CAG ATG
 151 Asn Trp Leu His Leu Pro Val Gly Tyr His Gly Arg Ala Ser Ser Val Val Val Ser Gly Thr Pro Ile Arg Arg Pro Met Gly Gln Met

 541 AAA CCT GAT GAC TCT AAG CCT CCC GTA TAT GGT GCC TGC AAG CTC TTG GAC ATG GAG CTG GAA ATG GCT TTT TTT GTA GGC CCT GGA AAC
 181 Lys Pro Asp Asp Ser Lys Pro Pro Val Tyr Gly Ala Cys Lys Leu Leu Asp Met Glu Leu Glu Met Ala Phe Phe Val Gly Pro Gly Asn

 631 AGA TTG GGA GAG CCG ATC CCC ATT TCC AAG GCC CAT GAG CAC ATT TTT GGA ATG GTC CTT ATG AAC GAC TGG AGT GCA CGA GAC ATT CAG
 211 Arg Leu Gly Glu Pro Ile Pro Ile Ser Lys Ala His Glu His Ile Phe Gly Met Val Leu Met Asn Asp Trp Ser Ala Arg Asp Ile Gln

 721 AAG TGG GAG TAT GTC CCT CTC GGG CCA TTC CTT GGG AAG AGT TTT GGG ACC ACT GTC TCT CCG TGG GTG GTG CCC ATG GAT GCT CTC ATG
 241 Lys Trp Glu Tyr Val Pro Leu Gly Pro Phe Leu Gly Lys Ser Phe Gly Thr Thr Val Ser Pro Trp Val Val Pro Met Asp Ala Leu Met

 811 CCC TTT GCT GTG CCC AAC CCG AAG CAG GAC CCC AGG CCC CTG CCG TAT CTG TGC CAT GAC GAG CCC TAC ACA TTT GAC ATC AAC CTC TCT
 271 Pro Phe Ala Val Pro Asn Pro Lys Gln Asp Pro Arg Pro Leu Pro Tyr Leu Cys His Asp Glu Pro Tyr Thr Phe Asp Ile Asn Leu Ser

 901 GTT AAC CTG AAA GGA GAA GGA ATG AGC CAG GCG GCT ACC ATA TGC AAG TCC AAT TTT AAG TAC ATG TAC TGG ACG ATG CTG CAG CAG CTC
 301 Val Asn Leu Lys Gly Glu Gly Met Ser Gln Ala Ala Thr Ile Cys Lys Ser Asn Phe Lys Tyr Met Tyr Trp Thr Met Leu Gln Gln Leu

 991 ACT CAC CAC TCT GTC AAC GGC TGC AAC CTG CGG CCG GGG GAC CTC CTG GCT TCT GGG ACC ATC AGC GGG CCG GAG CCA GAA AAC TTC GGC
 331 Thr His His Ser Val Asn Gly Cys Asn Leu Arg Pro Gly Asp Leu Leu Ala Ser Gly Thr Ile Ser Gly Pro Glu Pro Glu Asn Phe Gly

1081 TCC ATG TTG GAA CTG TCG TGG AAG GGA ACG AAG CCC ATA GAC CTG GGG AAT GGT CAG ACC AGG AAG TTT CTG CTG GAC GGG GAT GAA GTC
 361 Ser Met Leu Glu Leu Ser Trp Lys Gly Thr Lys Pro Ile Asp Leu Gly Asn Gly Gln Thr Arg Lys Phe Leu Leu Asp Gly Asp Glu Val

1171 ATC ATA ACA GGG TAC TGC CAG GGG GAT GGT TAC CGC ATC GGC TTT GGC CAG TGT GCT GGA AAA GTG CTG CCT GCT CTC CTG CCA TCA TGA
 391 Ile Ile Thr Gly Tyr Cys Gln Gly Asp Gly Tyr Arg Ile Gly Phe Gly Gln Cys Ala Gly Lys Val Leu Pro Ala Leu Leu Pro Ser  *

1261 GATTTTCTCTGCTCTTCTGGAAACAAAGGGCTCAAGCACCCCTTTCAACCCTGTGACTGGGGTCCTCCCTCGGGCTGTAGGCCTGGTCCGCCATTCAGTGACAAATAAAGCCATTGTG

1379 CTCTGAGGCCTGC(A)68
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Figure 2 Sequence of human FAH cDNA. The 38-bp (− 19 to − 56) added by 5′ extension of the FAH cDNA are underlined. Regions showing homologies with sequenced tryptic fragments from purified rat FAH are underlined. The polyadenylation signal (AATAAA) is in bold characters.

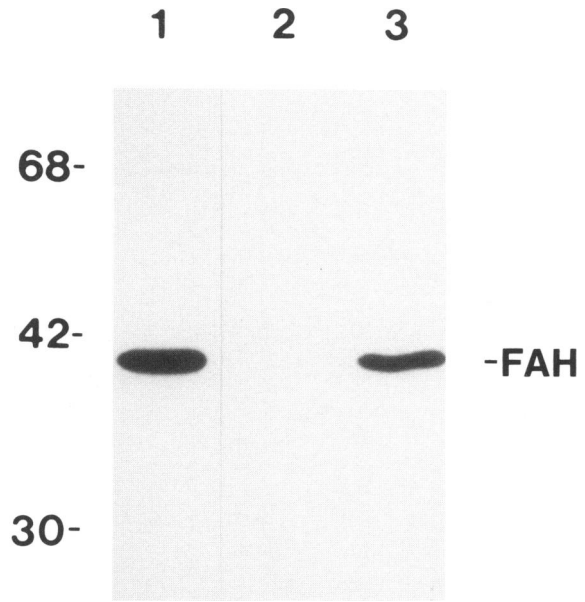

Figure 3 Expression of human FAH cDNA in CV-1 cells. Proteins were fractionated by electrophoresis on an SDS polyacrylamide gel and analyzed by immunoblotting with affinity-purified anti-human FAH antibodies. Gels were loaded with 25 μg of proteins from human liver (lane 1), untransfected (lane 2), and pSV2FAH-transfected CV-1 cells (lane 3). Molecular masses of protein markers are indicated on the left side of the panel.

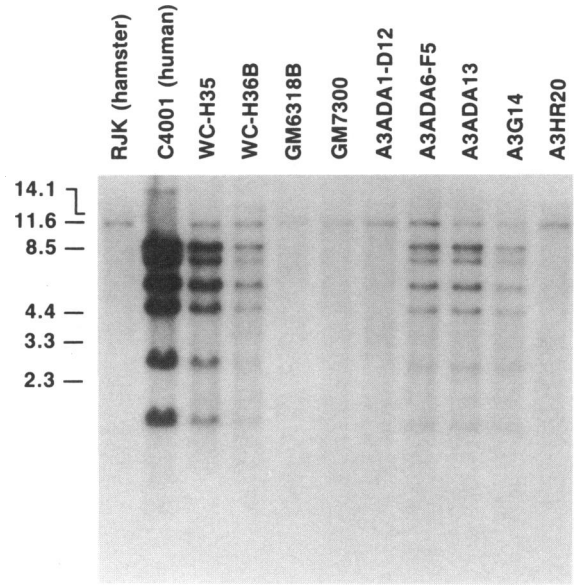

Figure 4 Southern blot hybridization of *Hin*dIII-digested DNA from hybrid cell lines. The cell line hybrid DNA analyzed is indicated on the top of each lane. Cell lines RJK (hamster) and C4001 (human) are negative and positive controls respectively. The cell line A3G14 is missing 2p23-p24. The full length phFAH HA2 insert was used as the probe. The sized markers (in kilobases) are indicated on the left side of the panel.

to that of the liver enzyme, indicating that the FAH cDNA clone contains all the information to produce a functional enzyme.

Chromosomal Localization of FAH

To determine the localization of the FAH gene, Southern blot analysis of a panel of rodent-human somatic cell hybrids was performed. The full-length FAH cDNA probe was hybridized to *Hin*dIII-digested genomic DNA from 17 rodent-human somatic cell hybrids. As shown in figure 4, the probe hybridized to six *Hin*dIII fragments of 8.4, 6.6, 5.1, 4.4, 2.8, and 1.6 kb in human genomic DNA. Under the hybridization conditions used, only one cross-hybridizing fragment of 11.6 kb likely corresponding to the rodent FAH homologous gene was detected (fig. 4, lane marked *hamster*). The chromosomal localization was determined by scoring the presence or the absence of the human bands on the Southern blot. The results of this analysis reveal that chromosome 15 is the only one having 0% discordancy (data not shown). The regional localization of the FAH gene to human chromosome 15 was further determined by in situ hybridization using [^{3}H]-labeled human FAH cDNA as probe. Analysis of 103 metaphase cells hybridized with this probe revealed 267 silver grains associated with chromosomes. This distribution of silver grains is shown in figure 5. Of the total number of silver grains, 15% (40/267) were localized on chromosome 15. No other chromosome showed specific hybridization. Figure 6*a* shows three examples of Giemsa-stained chromosomes with silver grains on chromosome 15. Subsequent R-banding of the same chromosomes reveals that the grains are localized in region q23-q25 (fig. 6*b*). As shown on the ideogram

Table 1

FAH Enzymatic Activity in CV-1 Cells and Human Liver Homogenates

Sample	Activity (nmol·s^{-1}·mg protein^{-1})
CV-1	.1
CV-1(pSV2FAH)	2.7
Human liver	9.3

NOTE.—Hydrolase activity was measured at 37°C as a decrease in the optical density (330 mm) of the substrate fumarylacetoacetate.

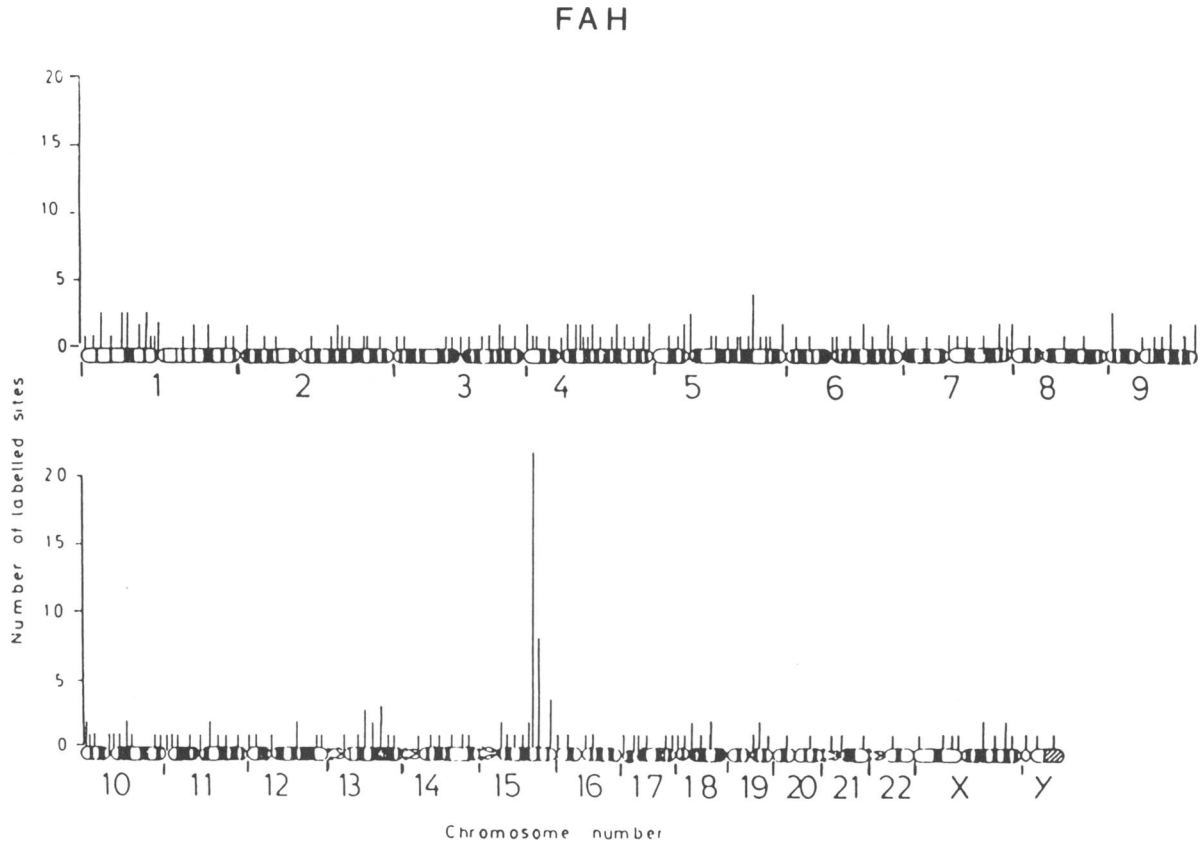

Figure 5 In situ hybridization of ^{3}H-labeled human phFAH-HA2 cDNA probe to normal human metaphase chromosomes. The distribution of labeled sites on the chromosomes of 103 metaphases is shown. Specific labeling was observed on the long arm of chromosome 15.

of figure 7, 77% of the silver grains scored on the long arm of chromosome 15 are located on bands q23-q25. This data confirms the mapping of the gene encoding FAH to chromosome 15q23-q25.

Determination of Translational Start of FAH cDNA

While this manuscript was being finalized, Agsteribbe et al. (1990) reported the nucleotide sequence of a cDNA clone putatively encoding human FAH. Their sequence is identical to the one reported here from position + 82 to the terminal TGA (nt + 1260). However our clone has an extended open reading frame at the 5′ end from + 81 to another ATG initiation codon at + 1. This region has been sequenced in three different individuals, none of which had the upstream stop codon described by Agsteribbe et al. (1990). We had some indications that the first methionine codon reported in our study (+ 1) was the one utilized in vivo, since a sequenced tryptic peptide of 10 amino acids from purified rat FAH was encoded in the region between the first and second ATG codons (see fig. 2). The first ATG codon described here is also in a favorable position conforming to Kozak's scanning initiation model (Kozak 1986) with a purine in position − 3.

To further clarify the issue of the true translation start site, we constructed a truncated version of the full-length cDNA clone (HA2) in which the region containing the first ATG was removed (region − 18 to + 206). The complete and truncated inserts were subcloned into the Bluescript transcription vector and transcribed in vitro with T7 RNA polymerase, and the sense synthetic RNAs were translated in a reticulocyte lysate. The translation product from the transcript of our full-length HA2 cDNA insert comigrates with purified human liver FAH monomer on SDS polyacrylamide gel (fig. 8, lane 2). On the other hand, analysis of the translation products of the truncated insert containing only the second ATG reveals a polypeptide

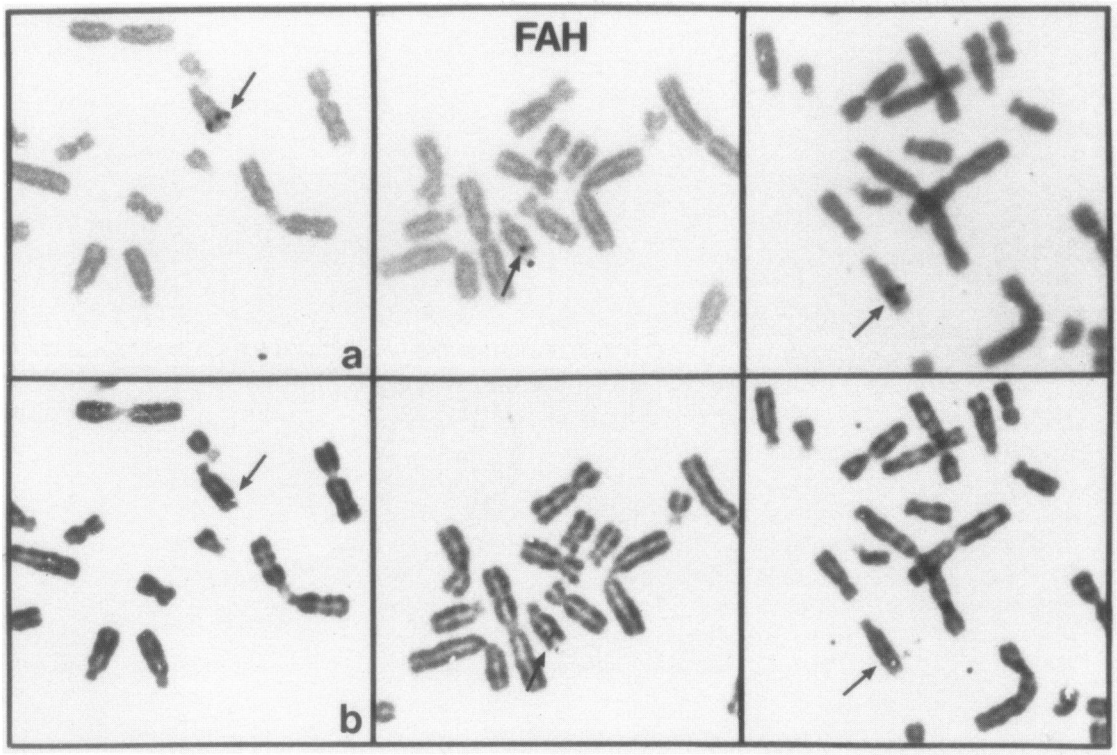

Figure 6 Examples of representative partial human metaphases after in situ hybridization with FAH cDNA probe. *a,* Giemsa-stained chromosomes with silver grains. Arrows indicate clusters of silver grains associated with chromosome 15, *b,* Same metaphases after subsequent R-banding.

that migrates faster than human liver FAH (fig. 8, lane 3). The other lower-molecular-weight polypeptides seen (fig. 8, lane 3) may correspond to other in-frame ATG start sites. These data further confirm that the first methionine codon reported here (+ 1) is the translation initiation site utilized in vivo.

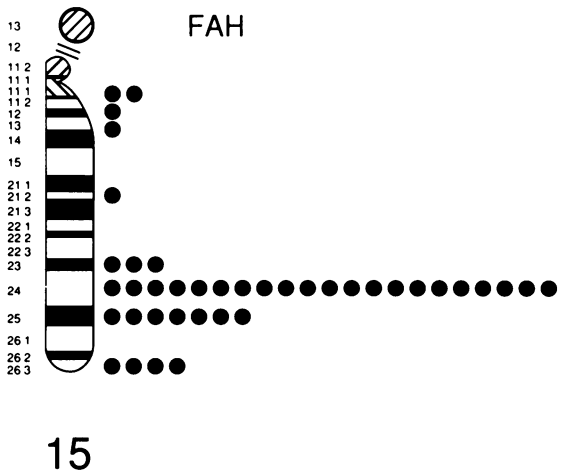

Figure 7 Distribution of labeled sites on chromosome 15. This ideogram shows a clustering of grains on bands q23-q25.

Discussion

The present work describes the isolation and sequencing of human FAH cDNA clones using plaque hybridization screening with a rat FAH cDNA insert. We have shown elsewhere that antibodies directed against rat liver FAH crossreacted with the human enzyme (Tanguay et al. 1984; Tanguay et al. 1990), indicating that the human and rat enzymes exhibited a certain degree of structural and/or sequence homology. The human cDNA clones isolated with the rat FAH cDNA probe hybridized with those isolated by immunological screening with a rabbit anti-human FAH antibody. The longest cDNA clone, phFAH HA2, was further characterized by sequencing. The authenticity of this clone was confirmed in several ways. First, in vitro-transcribed RNA from the cDNA insert of phFAH HA2 was translated into a polypep-

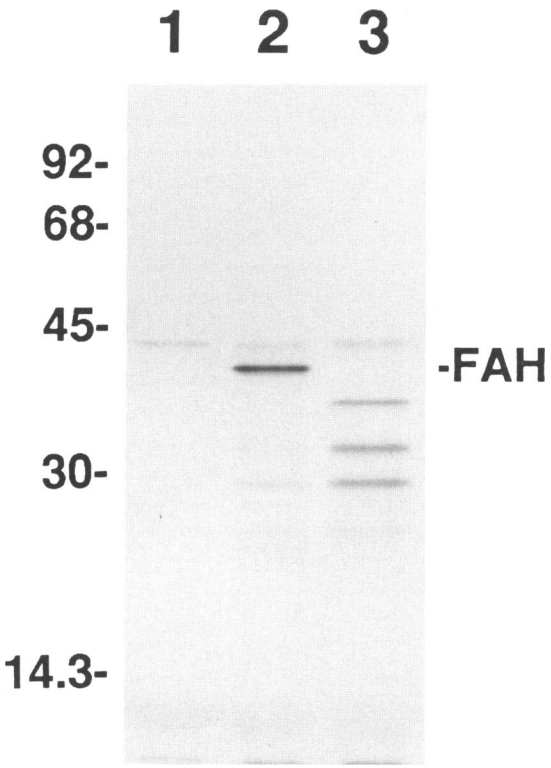

Figure 8 Translation products of T7 transcripts of full-length FAH clone (HA2) and FAH clone deleted from nucleotides −18 to +206. In vitro–transcribed sense RNAs of the cDNA inserts were translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Translation products were analyzed on a 12% polyacrylamide gel. The figure shows an autoradiogram of translation products from a blank (lane 1), an HA2 clone (lane 2), and a deleted FAH clone (lane 3). Molecular masses (in kilodaltons) of protein markers including human FAH are shown on both sides of the panel.

tide which comigrated with the human FAH monomer on SDS gels and was immunoprecipitated by affinity-purified anti-human FAH antibodies. Second, sequence analysis of the human FAH cDNA clone showed the presence of four regions coding for sequenced tryptic fragments from purified rat FAH. Comparison of the coding region of rat and human FAH cDNAs shows a 94% homology at the amino acid level (Y. Labelle, unpublished data). Since one of our sequenced peptides was found to be in the region 5′ to the ATG codon putatively described as the first codon by Agsteribbe et al. (1990), we compared the translation products from our full-length cDNA and the one obtained from a truncated version containing only the second ATG described by Agsteribbe et al. The construction containing the first ATG described here yielded a translation product comigrating with purified human liver FAH while that of the truncated version containing only the second ATG gave a polypeptide shorter than the liver monomer. Third, after transfection with our FAH cDNA clone, CV-1 cells expressed an immunoreactive band comigrating with human liver FAH. Moreover, these pSV2 FAH-transfected cells expressed enzymatic activity as shown by the hydrolysis of the natural substrate, fumarylacetoacetate. This finding is of particular interest, as FAH is known to be a dimer of about 80 kDA in its native condition (Mahuran et al. 1977). The present study indicates that the single polypeptide chain encoded by the FAH gene contains all the necessary genetic information for functional enzymatic activity. This is consistent with the suggestion of Mahuran et al. (1977) that the enzyme is a homodimer.

Southern blot analysis of human genomic DNA with FAH cDNA probes indicates that the FAH gene is present as a single copy per haploid genome (data not shown). Analysis of DNA from rodent-human somatic cell hybrids shows that the FAH gene is localized on chromosome 15. This chromosomal assignment is confirmed by in situ hybridization, which shows a regional localization to bands q23-q25.

Very little is known about the molecular basis of the deficiencies in the various clinical forms of hereditary tyrosinemia. We recently suggested that both clinical forms of the disease, acute and chronic, have different molecular bases (Tanguay et al. 1990). The availability of human FAH cDNA clones now opens the possibility of precisely defining the mutations in the various forms of type 1 hereditary tyrosinemia. Expression of the FAH human enzyme by gene transfer in CV-1 cells also offers the possibility of analyzing suspected mutant cDNA clones from FAH mRNA of tyrosinemia patients. The identification of the mutations responsible for type 1 hereditary tyrosinemia will have important implications for the diagnosis of the disease and should lead to the development of genomic-based tests for the identification of carriers.

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