Biochemistry. In the article “Molecular cloning of the flavin-containing monooxygenase (form II) cDNA from adult human liver” by N. Lomri, Q. Gu, and J. R. Cashman, which appeared in number 5, March, 1992, of Proc. Natl. Acad. Sci. USA (89, 1685–1689), the authors ask that the following be noted. Subsequent work with clones derived from their original cDNA of the human liver flavin-containing monooxygenase (form II), which is presently referred to as human liver flavin-containing monooxygenase form 3 (or HLMO 3), has led them to resequence the gene: They found a number of sequencing errors of the original clone. Fig. 1 is the corrected sequence and translated open reading frame for HLMO 3. By comparison with the previously published sequence, corrections to errors that involved a change in the predicted identity of amino acid residues can be found at nt 879, 1105, 1107; the region spanning 1198–1215; the region spanning 1251–1260; the region spanning 1330–1333; nt 1345, 1346, and 1361; the region spanning 1381–1395; and nt 1432, 1433, and 1434. Additional changes in the nucleotide sequence not affecting amino acid identity are at nt 330, 1104, 1353, 1380, and 1431.

![Fig. 1](image_url)
Molecular cloning of the flavin-containing monoxygenase (form II) cDNA from adult human liver

(mammalian amine/sulfide oxygenase/Ziegler's enzyme)

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ABSTRACT Complementary DNA (cDNA) clones encoding the adult human liver flavin-containing monoxygenase (FMO; dimethylaniline N-oxidase, EC 1.14.13.8) were isolated from Agt10 and Agt11 libraries. The cDNA libraries were screened with three synthetic 36-mer oligonucleotide probes derived from the nucleic acid sequence of the pig liver FMO cDNA. The deduced amino acid sequence for the adult human liver FMO was quite distinct from the pig liver FMO, and adult human liver FMO was designated form II (HLFMO II). The full-length cDNA sequence of HLFMO II (2119 base pairs (bp)) had an open reading frame of 1599 nucleotides, which encoded a 533-amino acid protein of M, 59,179, a 5'-noncoding region of 136 nucleotides and a 3'-noncoding region of 369 nucleotides excluding the poly(A) tail. The deduced amino acid sequence of HLFMO II had 80% similarity with the rabbit liver FMO I1 but only a 52%, 55%, and 53% amino acid similarity with the rabbit liver (form I), the pig liver (form I), and fetal human liver (form I) FMOs, respectively. RNA analysis of adult human liver RNA showed that there was one HLFMO II mRNA species. Analysis of genomic DNA indicated that HLFMO II was the product of a single gene. These results indicated that the deduced amino acid sequence for HLFMO II contained highly conserved residues and suggested that FMO enzymes were closely related and, undoubtedly, derived from the same ancestral gene.

The mammalian flavin-containing monoxygenase (FMO, dimethylaniline N-oxidase, EC 1.14.13.8) is a widely distributed enzyme that catalyzes the NADPH-dependent oxygenation of a wide variety of nucleophilic nitrogen-, sulfur-, and phosphorous-containing drugs, chemicals, and xenobiotics (1, 2). To date, essentially all of the investigations examining hepatic FMO have been performed with animal tissues, possibly because of the instability of human liver FMO preparations. In contrast to adult human liver cytochrome P-450, almost nothing is known about the structure of adult human liver FMO. A few studies with human liver microsomes have demonstrated FMO-like enzyme activity (3–5) and immunoreactivity with the antibody against pig liver FMO (4, 6). Dimethylaniline N-oxidation was observed in adult (5) and fetal (7) human liver microsome preparations. In contrast to dimethylaniline N-oxidation, which was observed in both kidney and liver tissues, impromine N-oxidation was only observed in microsome preparations from human kidney but not from human liver (4). The conclusion from these studies was that FMO was present in human liver tissue, albeit with low specific activity and possibly as multiple enzyme forms.

In animals, FMO is present at least one pulmonary form (8, 9) and as two or more hepatic forms (e.g., forms I and II) (10, 11). In rabbit liver, form I and form II are 54% identical to one another, but the amino acid sequence similarity between hog liver FMO and rabbit liver FMO form I is ~90% (12). Although studies are limited, forms I and II FMO apparently differ in many important properties including substrate specificity (10), enzyme stability (12), and other physical properties. For example, form I FMO activity is stimulated by primary aliphatic alkylamines, and form I FMO catalyzes the N-oxidation of secondary and tertiary amines (1). In contrast, form II FMO N-oxygenates primary aliphatic alkylamines as well as secondary and tertiary amines (10). Some aliphatic tertiary amines, such as chlorpromazine, are preferentially N-oxygenated by form II FMO (10).

Rabbit liver FMO form I (11), like pig liver FMO (13) and rabbit lung FMO (14), is NH2-terminal blocked with an acetyl group. Form II FMO in rabbit liver is not modified at its NH2 terminus. Rabbit liver FMO form II (RLFMO II) possesses a proteolytically active center near amino acid 280 and appears to be readily degraded in microsome preparations (12). It is possible that NH2-terminal acetylation and/or other posttranslational modifications, such as glycosylation, may contribute to the relative susceptibility of FMO to proteolytic degradation. Undoubtedly, there are other factors that help to determine the catalytic and structural properties of form II FMO. In this study, we report the primary structure of adult human liver FMO form II (HLFMO II) deduced from the cDNA data.† RNA and Southern blot analyses indicated that HLFMO II was the product of a single gene. In the present study we also analyzed the deduced amino acid sequence and demonstrated that HLFMO II is 52–80% similar with other hepatic and pulmonary FMOs. These data reinforce our hypothesis that another FMO isoform (form II) exists and is the major form in adult human liver.

MATERIALS AND METHODS

The adult human liver Agt11 cDNA library was obtained from Clontech, and the adult human liver Agt10 library was constructed by standard procedures (15). Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, avian myeloblastosis virus reverse transcriptase, S1 nuclease, Pol.1, and Pol.1k were purchased from Boehringer Mannheim. Bluescript II vectors, Escherichia coli strains, and kits for deletions were from Stratagene. Oligo(dT)-cellulose T7 was from Sigma, nitrocellulose filters (BA-85) were from Schleicher & Schuell, nylon filters (Hybond N+) and radiochemicals were from Amersham, X-OMat AR films were from Kodak, and Sequenase kits were purchased from United States Biochemical. Human tissue was from the Liver.

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Abbreviations: FMO, flavin-containing monoxygenase; HLFMO II, adult human liver FMO form II; RLFMO II, rabbit liver FMO form II; PLFMO, pig liver FMO; RLFMO form I, rabbit liver FMO; RLuFMO, rabbit lung FMO.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M83772).
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Isolation of Poly(A)+ RNA and Genomic DNA. Human adult liver was obtained and RNA was extracted by the acid guanidium thiocyanate/phenol/chloroform method (16). Poly(A)+ RNAs were purified by chromatography with oligo-(d-T)-cellulose T7 (17). The genomic DNA was isolated according to a standard protocol (18).

Screening of Human Liver CDNA Libraries. The cDNA libraries were screened by an in situ hybridization technique (19) at a density of 100,000 plaque-forming units per 132-mm-diameter plate. Approximately 2 × 10⁸ plagues were examined. The probe used was a mixture of three oligonucleotides complementary to the pig liver FMO (PLFMO) cDNA (20). The three 36-mer oligonucleotide probes were prepared by the University of California at San Francisco Biomolecular Resource Center with an Applied Biosystems model 380B DNA synthesizer using phosphoramidite chemistry. PLFMO1, a 36-mer of sequence 5'-ATCGCTTCTCTCAAACGAGTGGGCTCAGCCCCCTC-3', is complementary to the pig liver cDNA nucleotide sequence 127–162; PLFMO2, a 36-mer of sequence 5'-CTCATCAAGGGAAGCAGGATGATCCAGT-3', is complementary to the pig liver cDNA nucleotide sequence 104–139; and PLFMO3, a 36-mer of sequence 5'-GAATGTTCGGTCCACCAGTGATGATAGCTTC-3', is complementary to the pig liver cDNA nucleotide sequence 1590–1545. Purified oligonucleotides were 5'-end-labeled with [γ-32P]ATP and T4 polynucleotide kinase. Filters from the first screening were prewashed in 50 mM Tris-HCl (pH 8.0)/1 M NaCl/1 mM EDTA/0.1% SDS for 2 hr at 42°C. Prehybridization was done with 6× standard saline citrate (SSC)/0.5% N-lauroylsarcosine/10× Denhardt's solution/denatured salmon sperm DNA at 50 μg/ml for 4 hr at 45°C. The 32P-labeled probes (10⁶ cpm per filter) were added to the same hybridization buffer without N-lauroylsarcosine, and hybridization occurred for 16 hr at 45°C. The filters were washed five times with 2× SSC/0.1% SDS for 10 min at room temperature, twice for 1 hr at 55°C, dried, and exposed to Kodak X-Omat AR5 films at −80°C for 24 hr with intensifying screens. The positive clones were further purified by additional screenings under the same hybridization conditions.

Preparation of Phage DNA, Subcloning, and Restriction Endonuclease Mapping. Phage stocks of the positive clones were prepared, and the cDNA EcoRI inserts were isolated. The various EcoRI fragments were subcloned into Bluescript II vectors with competent E. coli XL1-Blue and NM 522 cells. A restriction endonuclease map was obtained by digestion with appropriate restriction enzymes.

Nucleotide Sequence Analysis. The nucleotide fragments generated by treatment with EcoRI and other restriction endonucleases were subcloned into Bluescript (KS+/-) and SK+/- vectors with competent E. coli NM 522 and XL1-Blue cells. For sequencing, the directed deletion strategy (21) was used to obtain convenient sizes. Both strands of the double-stranded DNA clone were prepared and completely sequenced by the dyeoxynucleotide chain-termination method (22). Nucleotide sequences were compiled by the socrates program (University of California, San Francisco) using the Eugene system (Baylor College, Houston).

RNA Blot Analysis. Formamide-denatured poly(A)+ RNAs were fractionated by electrophoresis on a 1% agarose gel containing formaldehyde, transferred to nylon membranes, prehybridized for 4 hr at 42°C, and hybridized with the nick-translated 5'-[α-32P]dATP cDNA insert (23) for 16 hr at 42°C. The membrane was washed under strong stringency conditions with five changes of 1× SSC/0.1% SDS for 5 min each at room temperature and then washed twice with 0.1× SSC/0.1% SDS for 1 hr at 65°C. The blots were dried and autoradiographed at −80°C by using an intensifying screen.

RESULTS

From two adult human cDNA libraries, 2 × 10⁶ phage plagues were screened with a mixture of three 36-mer synthetic oligonucleotide probes derived from the cDNA sequences of PLFMO (20) as described in Materials and Methods. A total of five clones were isolated and purified to homogeneity and mapped by restriction endonuclease digestion. The largest clone, which contained ~2200 base pairs (bp) (Fig. 1), was subcloned into Bluescript vectors (KS+/- and SK+/-), and both strands were entirely sequenced by the dyeoxynucleotide chain-termination method (22). The sequencing strategy is depicted in Fig. 1. The complete nucleotide sequence of the HLFMO II cDNA was determined (Fig. 2). The sequence contained a 5'-untranslated region of 136 bp, followed by an open reading frame of 1599 bp, encoding a 533 amino acid protein of Mr 39,179, a termination codon, and a 3'-untranslated region of 384 bp. The sequence ACCATGG (bp 134–140) contained the initiating codon ATG and corresponded to the sequence found optimal for initiation of transcription by eukaryotic ribosomes (27).

Two consensus polyadenylation signals were also found: ATTAAA (bp 1805) and AATAAA (bp 2083), which were situated 15 nucleotides upstream from the poly(A) tail (28).

The amino acid sequence of HLFMO II deduced from the cDNA clone is shown in Fig. 3. Comparison of HLFMO II


Southern Blot Analysis. Samples of restriction endonuclease-digested genomic DNA were fractionated by electrophoresis on a 0.8% agarose gel, transferred to a nylon membrane, and prehybridized in 50% (vol/vol) formaldehyde/5× SSC/10× Denhardt's solution/salmon sperm DNA at 50 μg/ml/20 mM Na₂HPO₄, pH 7, for 4 hr at 42°C. The 32P-labeled probe (10⁶ cpm/ml) was added to the same prehybridization buffer, and hybridization occurred for 16 hr at 42°C. The filter was washed five times with 2× SSC/0.1% SDS for 10 min at room temperature and twice with 0.1× SSC/0.1% SDS for 1 hr at 65°C, dried, and then visualized by autoradiography.

Protein Structure Analysis. The amino acid sequences of HLFMO II, RLFMO II (12), human fetal liver form I (24), PLFMO (20), RLFMO form I (25), and rabbit lung FMO were analyzed for hydropathy (26) by using PROTEIN WORKS. Computer programs used to carry out these studies were provided by the University of California at San Francisco Computer Graphics Facility.

FIG. 1. Restriction endonuclease map and sequencing strategy of the HLFMO II cDNA insert. Only the restriction sites used for subcloning are shown. The 1599-nucleotide open reading frame (ORF) is indicated in the box. The 5' and 3' ends of the HLFMO II cDNA are shown. The sequencing strategy is indicated by horizontal arrows. The cDNA fragments were subcloned in Bluescript (KS+/-, SK+/-), and ordered deletions were produced using Exonuclease III/mung bean nuclease. The deleted (○) and the restriction endonuclease (○) fragments were sequenced by the dyeoxynucleotide chain-termination method (22). Fragment B indicates the cDNA fragment used as a probe for hybridization analyses (Fig. 5).
with human fetal liver FMO form I (24), PLFMO (20), rabbit liver FMO form I (25), and rabbit lung FMO (25) showed only a modest degree of primary sequence identity (e.g., 53–57%). HLFFMO II contained a putative FAD-binding do-

main at amino acid residues 9–14 (e.g., GAGVSG) and a putative NADPH binding domain at residues 191–196 (e.g., GLGNSG). These cofactor-binding regions were highly conserved among all of the mammalian FMO enzymes known as
well as the FMO bacterial equivalent, cyclohexanone monoxygenase (29). The hydropathy profiles in Fig. 4 showed several regions of apparent hydrophobicity that were highly conserved. These corresponded to the FAD, the NADP⁺-binding sites, and the COOH termini of the respective proteins as well as some internal regions. The productive regions may be involved in substrate binding, membrane association, or posttranslational processing. In contrast to other mammalian hepatic FMO forms (see Fig. 3), HLFMO II has only a single putative consensus N-glycosylation site (Asn-Xaa-Ser/Thr) at residues 61–63. It was notable that HLFMO II did not contain the putative N-glycosylation sites at residues 269–271, 276–278, and 120–123, 315–317 that were present in form II and in form I FMOs, respectively.

Adult human liver mRNA was analyzed by using the cDNA clone shown in Fig. 1. This cDNA was radiolabeled, and the 32P probe was hybridized to poly(A)⁺ RNA as described. The radiolabeled probe detected one mRNA species (2300 bp) in human liver (data not shown). The genomic DNA extracted from adult human liver was treated with restriction endonucleases EcoRI (E), Pst I (P), BamHI (B), and Xho I (X) (Fig. 5). The samples were fractionated on a 0.7% agarose gel, transferred to a nylon membrane, and probed with HLFMO II EcoRI B fragment (Fig. 1). As shown in Fig. 5, this probe hybridized to a single band with each sample. The apparent sizes of these bands ranged from 800 bases (EcoRI) to 4000 bases (Pst I, BamHI, and Xho I).

When genomic Southern blot analyses were conducted under reduced-stringency hybridization conditions with the three EcoRI fragments (A, B, and C, Fig. 1), additional bands were observed, which indicated that the HLFMO II-encoding gene was probably part of a multigene family (data not shown).
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The hydropathic character analysis showed that the amino acid sequence suggests another approach for the isolation and purification of native HLFMO II based on the similarity to animal form II FMO enzymes.

In summary, we have isolated a full-length cDNA encoding adult human liver FMO, and we have deduced its primary structure. The availability of a HLFMO II clone will now allow us to examine in detail the structure–function relationships of this important enzyme. In addition, analysis of the deduced amino acid sequence suggests another approach for the isolation and purification of native HLFMO II based on the similarity to animal form II FMO enzymes.

DISCUSSION

In this report we describe the identification of another FMO-encoding gene in adult human liver, and we designate the corresponding protein as HLFMO II. The complete HLFMO II cDNA was cloned and sequenced. Nucleotide sequence analysis showed the presence of two polyadenylation signals at the 3′-noncoding region of the cDNA. Accordingly, we anticipated that two HLFMO II transcripts would be present. However, Northern (RNA) analysis revealed the presence of only one transcript with a size of 2300 bp. The size of the transcript indicated that only the polyadenylation signal at bp 2085 was used. Analysis of genomic DNA suggested that HLFMO II was encoded by a single gene.

Based on comparisons of the deduced amino acid sequences of known FMOs, HLFMO II is markedly more hydrophobic than other reported FMOs. HLFMO II contained the highly conserved FAD- and NADP+-binding domains near deduced amino acid positions 9–14 and 191–196, respectively. In contrast to other hepatic FMOs, which contained multiple putative glycosylation sites, HLFMO II has only one consensus sequence at amino acid position 61–63. The conserved nature of this glycosylation site suggests that this region may play an important structural and/or functional role in catalysis. The absence of other conserved glycosylation sites may also affect HLFMO II stability or activity.

In summary, we have isolated a full-length cDNA encoding adult human liver FMO, and we have deduced its primary structure. The availability of a HLFMO II clone will now allow us to examine in detail the structure–function relationships of this important enzyme. In addition, analysis of the deduced amino acid sequence suggests another approach for the isolation and purification of native HLFMO II based on the similarity to animal form II FMO enzymes.

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