The Flavin-containing Monooxygenase 2 Gene (FMO2) of Humans, but Not of Other Primates, Encodes a Truncated, Nonfunctional Protein*  

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Flavin-containing monooxygenases (FMOs) are NADPH-dependent flavoenzymes that catalyze the oxidation of heteroatom centers in numerous drugs and xenobiotics. FMO2, or “pulmonary” FMO, one of five forms of the enzyme identified in mammals, is expressed predominantly in lung and differs from other FMOs in that it can catalyze the N-oxidation of certain primary alkylamines. We describe here the isolation and characterization of cDNAs for human FMO2. Analysis of the sequence of the cDNAs and of a section of the corresponding gene revealed that the major FMO2 allele of humans encodes a polypeptide that, compared with the orthologous protein of other mammals, lacks 64 amino acid residues from its C terminus. Heterologous expression of the cDNA revealed that the truncated polypeptide was catalytically inactive. The nonsense mutation that gave rise to the truncated polypeptide, a C → T transition in codon 472, is not present in the FMO2 gene of closely related primates, including gorilla and chimpanzee, and must therefore have arisen in the human lineage after the divergence of the Homo and Pan clades. Possible mechanisms for the fixation of the mutation in the human population and the potential significance of the loss of functional FMO2 in humans are discussed.

The flavin-containing monooxygenases (FMOs) (EC 1.14.13.8) are NADPH-dependent flavoenzymes that catalyze oxidation of soft nucleophilic heteroatom centers in a range of structurally diverse compounds, including drugs, pesticides, and other xenobiotics (1, 2). Functionally, FMOs differ from other monooxygenases in that the active oxygenating species, the C(4a)hydroperoxide derivative of the flavin cofactor, exists stably within the protein in the absence of substrate, thereby enabling the enzyme to oxidize any soft nucleophile able to gain access to the active site. FMO was originally characterized by using a homogeneous enzyme preparation isolated from pig liver (3). Proteins with equivalent catalytic properties and substrate profiles were later isolated from the livers of several other species (4–6). However, contemporaneous studies indicated that rabbit lung contained a form of FMO which, although clearly related to the liver enzyme, possessed distinct physicochemical properties (7) and substrate preferences (8, 9). The purification of this “lung” or “pulmonary” FMO (10–12) confirmed the existence of two distinct FMOs, distinguishable both immunochemically and by substrate preference (10–12), and the subsequent isolation and characterization of the corresponding cDNA clones (13) demonstrated that each was the product of a separate gene.

Following the identification of “liver” and “lung” FMO as discrete enzymes, evidence accumulated indicating the presence in these tissues of additional forms of the enzyme. The existence of multiple mammalian FMOs was subsequently confirmed when new forms were identified, either by direct sequencing of purified proteins (14, 15), or via the isolation and characterization of cDNA clones (16–18). To date, five distinct forms of FMO, designated FMO1–5, each of which is encoded by its own gene and which exhibits approximately 50–60% pairwise amino acid sequence similarity, have been identified in mammals (19). According to the present nomenclature (19) “liver” and “lung” FMO are designated FMO1 and FMO2, respectively, whereas the forms identified subsequently have been designated FMOs 3, 4, and 5. Although detected at other sites, such as the kidney (13, 20, 21), FMO2 is expressed predominantly in pulmonary tissue (10, 11, 13, 20–23) where, in the rabbit, it has been localized to the nonciliated bronchial epithelial (clara) cells and endothelial type I and II cells (24). FMO2 gene expression has been demonstrated to be regulated by sex hormones in experimental animals (25, 26) and putative glucocorticoid responsive elements have been identified in the 5′-flanking region of the rabbit FMO2 gene (27). FMO2 displays catalytic characteristics that distinguish it from other forms of FMO. For instance, although able to oxidize many typical FMO substrates, it is inactive toward certain tertiary amines, such as imipramine and chlorpromazine (9, 10), that are good substrates for other forms of the enzyme. Furthermore, in contrast to other FMOs, FMO2 is capable of mediating the N-oxidation of some primary alkylamines to their oximes, via an N-hydroxylamine intermediate (28, 29).
Human FMO2 Encodes a Truncated, Nonfunctional Protein

We have previously reported the isolation of cDNAs encoding human FMOs 1 (30), 4 (16) (previously designated FMO2), and 3 (31) and have determined that the corresponding genes, plus the genes encoding human FMOs 2 and 5, are all located on the long arm of chromosome 1 (16, 30, 32, 33). In this report we describe the isolation and characterization of cDNA clones encoding human FMO2 and demonstrate that, in common with human FMOs 1 and 3 (31), expression of the corresponding gene is subject to both ontogenic and tissue-specific regulation. Furthermore, we report that, in contrast to apparently all other mammalian species, including closely related primates, in humans the major FMO2 allele encodes a truncated polypeptide which is catalytically inactive.

EXPERIMENTAL PROCEDURES

Oligonucleotide Synthesis—Oligonucleotides were synthesized on a PCR-MATE DNA synthesizer (model 391, Applied Biosystems, Warrington, UK).

Isolation of RNA and Genomic DNA—Human adult and fetal tissue samples were obtained as described previously (31) except for single adult lung and kidney samples, which were obtained from the International Institute for the Advancement of Medicine (Exton, PA). Total RNA was prepared by the guanidinium thiocyanate/LiCl method (34), resuspended in diethylpyrocarbonate-treated water and stored in aliquots at –80 °C. RNA concentration was determined from absorbance at 260 nm. Human genomic DNA was isolated from whole blood by the method of Lahiri and Nurnberger (35) and from solid tissue by use of a commercial DNA isolation kit (Nucleon Biosciences, Coatbridge, Scotland). Gorilla and chimpanzee genomic DNA was isolated by the method of Blin and Stafford (36) from post-mortem tissue samples.

Amplification of a Partial-length cDNA Encoding Rabbit FMO2—Reverse-transcription of rabbit (New Zealand White) lung total RNA (20 μg) was primed with 200 μg of random hexamer oligonucleotides (Pharmacia Biotech, St. Albans, UK) and catalyzed by 200 units of Moloney murine leukemia virus reverse-transcriptase (Life Technologies) in a total volume of 20 μl according to the supplier’s recommendations. The reaction mix was then incubated for 5 min at 75 °C and the volume increased to 100 μl with water. Oligonucleotides 102 (5′-GAGCGACTCATGTTGCTGAGTGGC-3′) and 180 (5′-GATGTAATTTGCTGAGTTTCTGCT-3′), designed from the rabbit FMO2 cDNA sequence (33) and which incorporated PsI restriction endonuclease sites present within the rabbit sequence, were used to prime PCR amplification, by PCR, of 579 bp of coding region. The PCR was performed in a volume of 40 μl containing 15 pmol of each primer, 66.7 mM Tris-HCl (pH 8.4), 1.25 mM MgCl2, 16.7 mM (NH4)2SO4, 0.1% Nonidet P-40, and 0.05% sodium deoxycholate, at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, followed by an additional 5 min at 72 °C and the volume increased to 100 μl with water. Amplification of a Region of the FMO2 Gene—PCR products of the human FMO2 3′- and 5′-RACE-PCRs, using the human FMO2 cDNA-specific primers 498 (5′-CCCTCATCCACACAGC-3′) and 423 (5′-CTCAAGTCCCTATCCACAGCAG-3′), respectively, and the common adapter primer 365 (31), were performed as described previously (31). The products of the seminested 5′-RACE-PCRs were blunted-end with T4 DNA polymerase, phosphorylated with T4 polynucleotide kinase (New England Biolabs, Hitchin, UK), then purified (SpinBind, FMC Bioproducts, Rockland, ME) and inserted into EcoRV-digested pBS.

DNA Sequencing—Plasmid DNA and purified PCR products were sequenced by the dideoxy chain-termination method (39) using DNA sequencing kits (Sequenase II, Amersham International; Cyclist, Stratagene, Cambridge, UK) to give the plasmid pRABLUNG. The identity of the cDNA insert was confirmed by restriction mapping and partial DNA sequencing.

cDNA Library Screening—The insert from pRABLUNG was excised by incubation with PsI, gel-purified, radiolabeled by the oligonucleotide random primer method (37) to a specific radioactivity of approximately 109 cpm/μg (with [α-32P]dATP (800 Ci/mmol, Amersham International, Amersham, UK), and used to screen an adult human lung cDNA library constructed in λgt11 (gift of Dr. K. Reid) plated at a density of 1.5 × 106 plaque-forming units per 20 × 20-cm plate (Nalge Nunc, Hereford, UK). Duplicate filters were prehybridized, hybridized, washed, and subjected to autoradiography as described previously (30). Because of loss of the EcoRI cloning sites during library construction, insert DNAs could not be excised from positive phage clones by restriction digestion and were therefore recovered by PCR as described previously (30).

DNA Polymorphism Analysis—The human FMO2 RNase protection plasmid, pBSFMO2/2/15, was constructed by excising a 985-bp section from within the insert of pBSFMO2/2 (by digestion with HindIII and Styl), followed by blunt-ending of the DNA with T4 DNA polymerase and self-ligation of the larger vector fragment. pBSFMO2/2/15 (10 μg) was linearized by digestion with MscI, after which the reaction mix was treated with proteinase K and SDS (40), and extracted with phenol/chloroform (1:1, v/v). The linearized plasmid was ethanol-precipitated
and resuspended in diethylpyrocarbonate-treated water. In vitro synthesis of radiolabeled antisense RNA, probe purification, and RNAse protection assays were performed as described previously (31, 40, 41). Comparison of the autoradiographic signal derived from the protected species with a standard curve of undigested probe permitted quantification of FMO2 mRNA in terms of molecules/cell (41). Microsomal fraction was obtained by centrifugation of the resulting supernatant at 100,000 × g for 15 min at 4 °C. The microsomal fraction was pelleted, resuspended in 30 ml 0.154 M KCl, 50 mM Tris-HCl (pH 7.4), 0.2 mM phenylmethylsulfonfluryl fluoride, and subjected to two 30-s bursts of sonicatin (Dynatech, model 150) on ice. The cell lysate was centrifuged at 10,000 × g for 15 min at 4 °C. The microsomal fraction was obtained by centrifugation of the resulting supernatant at 100,000 × g for 15 min.

**Southern Blot Hybridization**—Human genomic DNA (20 μg) was incubated overnight at 37 °C with 300 units of EcoRI or HindIII (New England Biolabs), extracted with phenol-chloroform (1:1, v/v), ethanol-precipitated, resuspended through a 1% agarose gel, transferred to a nylon membrane (Hybond, Amersham International). The membrane was prehybridized at 42 °C for 3 h in 50% deionized formamide, 5× SSPE (1× SSPE, 0.18 M NaCl, 10 mM sodium phosphate (pH 7.4), 1 mM EDTA), 5× Denhardt’s reagent, 2% (w/v) SDS, and denatured salmon sperm DNA (100 μg/ml), then hybridized overnight at 42 °C in 50% deionized formamide, 5× SSPE, 2% (w/v) SDS and 10% (w/v) dextran sulfate, containing radiolabeled probe (synthesized as described above for cDNA library screening) at a final concentration of 2–5 ng/ml. After hybridization the membrane was washed once in 2× SSPE/1% (w/v) SDS, (15 min at room temperature), twice in 1× SSPE/1% (w/v) SDS, (15 min each at room temperature), and twice in 0.1× SSPE/1% (w/v) SDS (15 min each, once at room temperature then at 55 °C), then washed in 0.1× SSPE for 75 h at 80 °C with an intensifying screen.

**Northern Blot Hybridization**—RNA samples (15 μg) were electrophoresed through 1.5% agarose gel (43). After transfer to a nylon membrane (BDH, Poole, UK) RNA was immobilized by baking the membrane at 80 °C, followed by UV cross-linking (Stratalinker 1800, Stratagene). The membrane was prehybridized, hybridized, washed, and subjected to autoradiography as described above.

**Construction of Recombinant Baculovirus**—To construct FMO2X472 recombinant baculovirus, pBSFMO2/2 was incubated with XbaI and PstI, and the smaller of the resulting restriction fragments, comprising the 1413-bp ORF, 192 bp of associated 3′ untranslated region, and approximately 50 bp of pBS, was ligated to XbaI/PstI-digested pFastBac (Life Technologies) to give pFastaMO2/2/8. To construct recombinant virus containing FMO2Q472, two oligonucleotides, 5′-GCCTTAGAGATAGGTGCG-3′ and 5′-9AGGCTGTTGCAGGACTTGGCCG-3′, were used to prime the amplification of a 330-bp contiguous region of the FMO2 gene that encompassed codon 472 and the second in-frame stop signal located 192 bp downstream (see Fig. 2). PCR conditions were as described above for FMO2 gene amplification, except that genomic DNA (100 ng) was used as template. A consensus sequence for the human FMO2 cDNA derived from four independent cDNA libraries, each expressed FMO2, was determined by scanning densitometry (BioRad, model GS-870) using a standard curve of authentic rabbit FMO2 (gift of R. Philpot).

**RESULTS**

**The Human FMO2 Gene Contains a Premature In-frame Termination Codon**—A screen of 6 × 10⁸ plaques of a human lung cDNA library with the insert of pRABLUNG, which comprised an 879-bp fragment of rabbit FMO2 cDNA, yielded four positive clones, L1, L2, L3, and L8, containing inserts of 0.6, 2.0, 1.6, and 0.9 kb, respectively. Sequence analysis confirmed that the inserts of all four clones contained sequences that encoded the human ortholog of FMO2. However, two of the clones, L2 and L3, also contained sequences that were not related to FMO2. A search of the GenBank™data base revealed that the latter sequences encode pulmonary surfactant-associated protein and mitochondrial 12 S ribosomal RNA, respectively. In each case, the FMO2 cDNA sequence was found to be joined to the unrelated sequence by an EcoRI restriction site, indicating that both clones were chimeric artifacts formed during library construction.

A consensus sequence for the human FMO2 cDNA derived from these four clones contained a short stretch of 5′-untranslated region and almost 1100 bp of protein-coding region (Fig. 1). The latter region was considerably shorter than the corresponding regions of FMO2 cDNAs of rabbit and guinea pig and did not contain an in-frame stop codon. A cDNA sequence encoding the carboxyl-terminal portion of human FMO2 we carried out 3′-RACE-PCR. Two independent 3′-RACE-PCR products (Fig. 1) were found to have identical sequences, which contained the remainder of the protein-coding region and 45 bp of an apparent 3′-untranslated region. To confirm and extend the sequence of the 5′-untranslated region we performed 5′-RACE-PCR. The products of two independent 5′-RACE-PCRs (Fig. 1) were each found to contain 60 bp of 5′-untranslated region, the sequences of which were identical with each other and with the overlapping regions of the shorter stretches of 5′-untranslated region contained within the clones L3 and L8. A cDNA, FMO2/2 (Fig. 1), containing the entire protein-coding region of FMO2, plus a short stretch of 3′-untranslated region, was obtained by reverse-transcription PCR.
from the partial sequences of the phage clones L1, L2, L3, and L8, the complete sequences of the 5′- and 3′-RACE-PCR products and the complete sequence of the full-length cDNA obtained by reverse-transcription PCR. The sequence contains 60 bp of 5′-untranslated region, followed by an ORF of 1413 bp, a TAG translational termination codon and 237 bp of an apparent premature stop region that lacks a consensus polyadenylation signal. The high degree of sequence identity with FMO2 cDNAs of rabbit (87%) (13) and guinea pig (86%) (46) confirmed the identity of the human clone. However, in contrast to FMO2 proteins of rabbit (13), guinea pig (46) and rhesus macaque (23), each of which contains 535 amino acid residues, the ORF of the human cDNA that we have isolated encodes a polypeptide of only 471 residues, with a calculated molecular mass of 53,639 daltons. Comparison of the human FMO2 cDNA sequence with the corresponding sequences of rabbit, guinea pig, and macaque monkey. Southern blot hybridization of human genomic DNA revealed that the full-length cDNA hybridized to fragments of 7.0, 6.4, 4.7, 3.7, and 3.1 kb in EcoRI-digested DNA (Fig. 4, lane 1), and to fragments of 9.2, 7.2, and 6.9 kb in HindIII-digested DNA (Fig. 4, lane 2). This gives an estimated minimum size for the human FMO2 gene of approximately 23 to 25 kb, similar to that of the human FMO3 gene (47) and suggests that humans contain only one FMO2 gene.

Analysis of Human FMO2 mRNA—The sequence flanking the proposed translational initiation codon of human FMO2 mRNA, GAAGCUUGAG (initiation codon underlined) (Fig. 2), conforms poorly to the consensus sequence, GCC(AG)C-CAUUGG, for efficient initiation of translation of vertebrate mRNAs (48). Of particular note is the lack of a purine at the −3 position (the A of the AUG codon is designated +1), as pyrimidines at this position are rarely associated with functional initiation codons (48, 49). However, the sequence contains a G at the +4 position, which has been shown experimentally (50) to compensate for the otherwise unfavorable presence of a pyrimidine at −3. The remaining 5′-untranslated sequence of the mRNA contained no additional AUG triplets which might serve as alternative translational initiation codons (48, 49). However, the sequence contains a G at the +4 position, which has been shown experimentally (50) to compensate for the otherwise unfavorable presence of a pyrimidine at −3. The remaining 5′-untranslated sequence of the mRNA contained no additional AUG triplets which might serve as alternative translational initiation codons (Fig. 2). The region flanking the initiation codon of the FMO2 mRNA of cynomolgus macaque, determined from the sequence of a 5′-RACE-PCR product (data not shown), also has a poor match to the consensus Kozak sequence, as it differs from the human sequence at only the −4 position, at which an A replaces a G. In contrast, the Kozak regions of mRNAs encoding FMO2s of rabbit, GAGACGAUGG, and guinea pig, GGGCCGAUGG, conform better to the consensus and contain a purine at the important −3 position.
Northern blot hybridization of total RNA isolated from the lungs of three human individuals revealed the presence of a single species of FMO2 mRNA of 5.7 kb (Fig. 5, lanes 1–3), which is similar in size to the mRNAs encoding FMO2 of macaque (5.0 kb) (Fig. 5, lane 4) (23), rabbit (a single major transcript of 4.8 kb and minor transcripts of 6.0, 2.6, and 2.4 kb) (13), guinea pig (6.0 kb) (46), and pig (6.0 kb) (46). The human FMO2 mRNA thus contains a 3′-untranslated region of approximately 4 kb. The much shorter length of 3′-untranslated sequence in the 3′-RACE-PCR products that were obtained (Figs. 1 and 2), together with the absence of a consensus polyadenylation signal, suggests that the reverse-transcription step of the 3′-RACE procedure was primed from an internal adenosine-rich sequence rather than from the poly(A) tail of the mRNA. In support of this, both rabbit and guinea pig FMO2 cDNAs contain adenosine-rich sequences at the corresponding positions within their respective 3′-untranslated regions (13, 46).

The Human FMO2 Gene Is Subject to Developmental and Tissue-specific Regulation—The tissue distribution and developmental expression of the mRNA encoding human FMO2 was investigated by quantitative RNase protection. A representative autoradiogram is shown in Fig. 6 and demonstrates that a discrete protected RNA, of the expected size, was obtained from the FMO2 antisense RNA probe. FMO2 mRNA was expressed relatively abundantly (13–36 molecules/cell) in adult lung samples from four different individuals (Fig. 6B). It was also expressed, but in lower abundance (3 molecules/cell), in one of two adult kidney samples analyzed (Fig. 6A and data not shown), but was not detected in the other kidney sample or in...
either of two adult liver samples, even after prolonged exposure. In fetal tissues, FMO2 mRNA was not detected in samples of liver, kidney or brain (data not shown) but was present in moderate abundance (5–12 molecules/cell) in each of four lung samples (Fig. 6B).

The Nonsense Mutation in the Human FMO2 Gene Occurred after the Pan and Homo Lines Diverged—The FMO2 gene of rhesus monkey encodes a polypeptide of 535 amino acid residues (23), the same length as the orthologous protein of rabbit (13) and guinea pig (46). Analysis of the sequence of cDNA for FMO2 of the cynomolgus monkey, a close relative of the rhesus monkey, revealed that a stop codon was not present at position 472 (data not shown). The mutation that gave rise to the premature stop codon present in the human FMO2 gene must therefore have occurred within the Hominoidea, sometime after this primate superfamily, which includes apes and humans,
diverged from the Cercopithecoida superfamily, which includes the Old World monkeys. To determine at what stage during the evolution of the Hominoidea the mutation occurred, a 235-bp region of the FMO2 gene encompassing codon 472 was amplified by PCR from genomic DNA of gorilla and chimpanzee. Within this region the sequence of the FMO2 gene of these species is 98% identical to that of the corresponding region of the FMO2 gene of humans (data not shown). Both of these nonhuman hominoids were homozygous for a CAG triplet, encoding Gln, at codon 472 of FMO2 (Fig. 3B and data not shown) and contained a translational stop codon at position 536 (data not shown), corresponding to that present in FMO2 of rabbit, guinea pig, and rhesus and cynomolgus monkeys. The mutation that gave rise to the truncated FMO2 of present day humans must therefore have occurred in the human lineage sometime after the divergence, some 4–5 million years ago, of humans from their closest relative, the chimpanzee.

Subsequent analysis, with a PCR-restriction enzyme assay, of individuals of various racial and ethnic backgrounds, including 27 European Caucasians, 18 Orientals (10 Japanese, 8 Chinese), 41 of African descent (20 Africans, 16 African-Americans, 5 UK Afro-Caribbeans), 6 New Guinean Aboriginals, 2 Indians and 2 Maoris, revealed that the allele encoding the truncated FMO2, FMO2X472, occurs at a frequency of essentially 100% in all groups investigated, with the exception of populations of African descent, in which an allele containing a CAG triplet at codon 472 (Q472) is present at a frequency of approximately 4%.2

The Truncated Protein Encoded by the Human Gene Is Catalytically Inactive—FMO2X472, the truncated form encoded by the major FMO2 allele of humans, and FMO2Q472, a “full-length” form containing 535 amino acid residues (see Fig. 2), were produced via heterologous expression of the corresponding cDNAs in insect cells via the baculovirus expression system. Western blotting with antibody against rabbit FMO2 detected proteins of 53 and 57 kDa, respectively, in microsomal membranes isolated from cells infected with virus containing cDNA encoding FMO2X472 (AcFMO2X472) (Fig. 7A, lane 5), or FMO2Q472 (AcFMO2Q472) (Fig. 7A, lane 6). Authentic rabbit FMO2 (Fig. 7A, lane 7) had an estimated molecular mass of 56 kDa. The concentrations of heterologously expressed FMO2X472 and FMO2Q472 were 14 and 19 pmol/mg microsomal membrane protein, respectively. Immunoreactive protein was undetectable in microsomal membranes isolated from noninfected insect cells (Fig. 7A, lane 1) or from cells infected with wild-type virus, AcNPV (Fig. 7A, lane 2), or in the cytosolic fractions of cells infected with AcFMO2X472 (Fig. 7A, lane 3) or AcFMO2Q472 (Fig. 7A, lane 4).

The function of FMO2X472 and FMO2Q472 was investigated by determining their ability to catalyze the S-oxidation of methimazole, an excellent substrate for FMO2s of other mammalian species (Fig. 7B). Microsomal membranes isolated from cells infected with AcFMO2Q472 catalyzed the S-oxidation of methimazole with essentially maximal specific activity of 0.50 nmol of methimazole oxide formed/min/mg microsomal protein ([S] = 2 mM; $K_{ii} = 411 \mu M$) (51) ($k_{cat}^{app} = 27 \min^{-1}$). In the presence of 100 mM Mg$^{2+}$, a known effector of FMO2 (7), this was increased approximately 6-fold to 2.9 nmol/min/mg microsomal protein ($k_{cat}^{app} = 155 \min^{-1}$). In contrast, microsomal membranes isolated from noninfected insect cells, or from cells infected with either AcNPV or AcFMO2X472, failed to catalyze methimazole oxidation. Thus, although the full-length human FMO2 that we have produced can catalyze the S-oxidation of methimazole and respond to Mg$^{2+}$ in similar ways to FMO2s of other mammals, the truncated form of the protein encoded by the major FMO2 allele of humans appears to be catalytically inactive.

**FIG. 7.** Analysis of heterologously expressed human FMO2. A, immunoblot of subcellular fractions of T. ni cells. The microsomal fraction of noninfected cells (200 μg, lane 1), and of cells infected with AcNPV (200 μg, lane 2), AcFMO2X472 (100 μg = 1.4 pmol, lane 5) or AcFMO2Q472 (100 μg = 1.9 pmol, lane 6), and the cytosolic fraction of cells infected with AcFMO2X472 (200 μg, lane 3) or AcFMO2Q472 (200 μg, lane 4), together with 1.0 pmol of a recombinant rabbit FMO2 (lane 7), were analyzed by Western blotting. FMO2s were detected through the use of goat anti-(rabbit FMO2) serum and a rabbit anti-goat IgG-alkaline phosphatase conjugate. The positions and sizes (in kDa) of molecular mass markers are indicated. B, time course of methimazole oxidation-dependent nitro-5-thiobenzoate oxidation. Methimazole oxidation, monitored indirectly as the time-dependent difference in absorbance at 412 nm, was catalyzed by microsomal membrane proteins isolated from noninfected T. ni insect cells (x——x, 420 μg), or from cells infected with AcNPV (———, 300 μg), AcFMO2X472 (———, 555 μg = 8.0 pmol) or AcFMO2Q472, in the absence (▲——▲, 540 μg = 10.3 pmol) or presence (■——■, 540 μg = 10.3 pmol) of 100 mM Mg$^{2+}$. The presence of nonsense mutations within prokaryotic and eukaryotic genes is frequently associated with decreased abundance of the corresponding mRNA, due to an increase in the turnover rate of the mutant transcript (52). This process,

**DISCUSSION**

Our results demonstrate that the major FMO2 allele of humans encodes a polypeptide that, in comparison with the orthologous protein of rabbit (13), guinea pig (46), and rhesus macaque (23), lacks 64 residues from its carboxyl terminus. This is due to the presence in the human gene of an in-frame TAG translational termination triplet at codon 472, 64 codons upstream of a second in-frame termination signal the position of which corresponds exactly with that present in FMO2 of rabbit, guinea pig, and macaque. As the latter species all contain a CAG triplet, encoding glutamine, at codon 472, this is present in the human FMO2 gene as the result of a C to T transition at the first position of codon 472.

The presence of nonsense mutations within prokaryotic and eukaryotic genes is frequently associated with decreased abundance of the corresponding mRNA, due to an increase in the turnover rate of the mutant transcript (52). This process,
termed nonsense-mediated mRNA decay (53), is dependent upon the relative position of the mutation within the protein-coding region; mutations located within the region encoding the amino-terminal two-thirds of a polypeptide accelerate degradation, whereas those within regions encoding sequences closer to the carboxyl terminus may have little or no effect upon mRNA stability (54). Consequently, as the nonsense mutation present in the FMO2 mRNA is located in the 3′-most one-third of the protein-coding region, it would be expected to exert little effect on the stability of the mRNA. In support of this, we found no evidence of degraded FMO2 mRNA in any of the human lung samples analyzed by Northern blot hybridization, and, furthermore, RNase protection analyses demonstrated that FMO2 mRNA was moderately abundant in all samples of human lung examined. Thus, the primary transcript of the human FMO2 gene is apparently correctly processed in pulmonary tissue to produce a stable and abundant mRNA that should be available for potential translation. The conservation throughout mammalian evolution of a long 3′-untranslated region in FMO2 mRNA suggests that it may be important for some aspect of the metabolism or function of the mRNAs, such as stability.

The results of RNase protection assays demonstrate that in humans expression of the FMO2 gene in both the adult and fetus is essentially restricted to the lungs, with the gene being inactive in the liver. The complete absence of hepatic expression of FMO2 has been demonstrated for other species (13, 21, 23) and confirms that FMO2 is essentially a pulmonary-specific FMO. The higher concentration of the FMO2 mRNA present in adult, compared with fetal, human lungs indicates that the gene is also regulated developmentally. Thus, in humans, FMO2, in common with FMO1 and FMO3 (31), is subject to both ontogenic and tissue-specific regulation. The similarities in the size and pattern of expression of FMO2 mRNAs of humans and other species of mammals indicate that the human FMO2 gene has suffered no mutations that affect either the expression of the gene or the processing or stability of the corresponding mRNA.

Heterologous expression studies showed that the loss of 64 amino acid residues from the carboxyl terminus of human FMO2 had no effect on targeting of the protein to the membranes of the endoplasmic reticulum, but abolished its catalytic activity. This is in accord with site-directed mutagenesis studies of rabbit FMO2 (55, 56), which showed that, although the removal of up to 26 amino acid residues from the carboxyl terminus of the enzyme had no effect on catalytic activity, a deletion of 40 or more residues inactivates the enzyme. It is possible that the truncated human FMO2 has some other, unidentified, catalytic activity. However, even if this were so, Williams et al. (57) found that FMO2, although abundant in the lungs of rhesus macaque, was undetectable, by Western blotting, in all but one of 29 human lung samples. The results of our Northern blot hybridization and RNase protection experiments demonstrate that FMO2 mRNA is moderately abundant in human lung. Consequently, the absence of FMO2 protein in this tissue cannot be due to deficient gene transcription or RNA processing, or to instability of the mRNA. The relatively poor conformity of the Kozak region of the human FMO2 mRNA to the consensus sequence may compromise the translational efficiency of the mRNA. However, this seems unlikely as the FMO2 of rhesus macaque, which is abundant in the lungs (23, 57), is encoded by a mRNA that contains a Kozak region that, apart from a single base difference at a noncritical position, is identical to that of the human mRNA. A more likely explanation for the lack of pulmonary expression of FMO2 in man is that, owing to the lack of 64 residues from its carboxyl terminus, the truncated polypeptide is unable to fold correctly and is thus detected by cellular surveillance systems, such as the ubiquitin pathway (58), and rapidly degraded.

The absence of FMO2 in human lung is in marked contrast to the situation in all other species of mammals investigated, in which it represents the major, if not only, form of FMO in adult lung (10, 11, 20–23). In humans, the only member of the FMO family present in adult lung is FMO5 (31, 59). However, as FMO5 has a very restricted substrate range that is quite distinct from that of any other FMO (18, 60), it would be unable to substitute for FMO2. The lung plays a significant role in the metabolism of certain foreign compounds (61, 62). Although the pharmacological or toxicological significance of the absence of FMO2 in human lung remains to be established, it is clear that caution should be exercised when extrapolating pulmonary drug metabolism data from experimental animals to man if an FMO-mediated metabolic pathway is suspected.

Recent reports (63, 64) have indicated the presence in human brain of an FMO that is immunoreactive with, and catalytically inhibited by, antibodies raised against rabbit FMO2. However, as we have demonstrated here, almost all individuals have two FMO2X472 alleles and would thus express a truncated form of the enzyme, which would be nonfunctional and, quite likely, rapidly degraded. The FMO detected by these workers is therefore unlikely to be FMO2. This is supported by the observation that it is able to effectively catalyze the N-oxidation of the non-FMO2 substrate imipramine (63, 64). The precise identity of this FMO is thus unclear.

Our results indicate that the FMO2X472 allele, which encodes a truncated, nonfunctional protein, arose as the result of a point mutation that occurred in the human lineage sometime after the divergence of the Homo and Pan clades took place some 4–5 million years ago and has subsequently spread to attain a frequency of close to 100% in the present day human population. FMO2 thus represents a very unusual case of a gene that has become non-functional in humans but not in other primates. The average time (in generations) for fixation of a neutral mutation is approximately 4N, where N is the effective population size (65). For humans, N has been estimated to have been approximately 10,000 for most of the last 1 million years (66). Assuming a generation time of about 15 years, the average time required for fixation of a neutral mutation in humans is thus 4 × 10,000 × 15 = 6 × 10^7 years. The FMO2X472 allele could therefore have become virtually fixed in the human population merely due to the effects of random genetic drift on a neutral mutation, i.e. one that conferred no, or very little, selective advantage or disadvantage. An alternative, more intriguing, possibility is that the nonsense mutation may have conferred an evolutionary advantage, resulting in a rapid spread of the mutant allele due to a directional selective sweep.

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