Expression of the CYP4F3 Gene

TISSUE-SPECIFIC SPlicing AND ALTERNATIVE PROMOTERS GENERATE HIGH AND LOW Km FORMs OF LEUKOTRIENE B4 OXIDASE

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Cytochrome P450 4F3 (CYP4F3) catalyzes the inactivation of leukotriene B4 by \( \omega \)-oxidation in human neutrophils. To understand the regulation of CYP4F3 expression, we analyzed the CYP4F3 gene and cloned a novel isoform (CYP4F3B) that is expressed in fetal and adult liver, but not in neutrophils. The CYP4F3 gene contains 14 exons and 13 introns. The cDNAs for CYP4F3A (the neutrophil isoform) and CYP4F3B have identical coding regions, except that they contain exons 4 and 3, respectively. Both exons code for amino acids 66–114 but share only 27% identity. When expressed in COS-7 cells, the \( K_m \) of CYP4F3B was determined to be 26-fold higher than the \( K_m \) of CYP4F3A using leukotriene B4 as a substrate. 5′-Rapid amplification of cDNA end studies reveal that the CYP4F3A and CYP4F3B transcripts have 5′-termini derived from different parts of the gene and are initiated from distinct transcription start sites located 519 and 71 base pairs (bp), respectively, from the ATG initiation codon. A consensus TATA box is located 27 bp upstream of the CYP4F3A transcription start site, and a TATA box-like sequence is located 23 bp upstream of the CYP4F3A transcription start site. The data indicate that the tissue-specific expression of functionally distinct CYP4F3 isoforms is regulated by alternative promoter usage and mutually exclusive exon splicing.

Leukotriene B4 (LTB4)\(^1\) is synthesized from arachidonic acid by the sequential action of 5-lipooxygenase and leukotriene A4 hydrolase enzymes in neutrophils, monocytes and macrophages (1). It is a potent chemoattractant for human neutrophils and also has chemotactic activity for monocytes (2–6). LTB4 exerts this activity via a G protein-coupled receptor in target cells (7) and induces a cascade of cellular events that amplify the inflammatory response (8). The generation of LTB4 is implicated in the pathogenesis of inflammatory disorders that involve prominent neutrophil infiltration of tissues (8, 9).

Recently, it has been suggested that LTB4 can also play an intracellular role in regulating transcription by functioning as a ligand for peroxisome proliferator-activated receptor \( \alpha \) (10). The bioactivity of LTB4 is determined by the regulation and kinetic properties of the enzymes that control its synthesis and catalolism. LTB4 is inactivated by \( \omega \)-oxidation of its terminal carbon to yield 20-OH LTB4 and 20-COOH LTB4 (10, 11). The initial \( \omega \)-hydroxylation is catalyzed by CYP4F3 (12–16), which by biochemical analysis has a restricted localization to neutrophils, and to a lesser extent monocytes (12, 17). The CYP4 family of enzymes catalyze the \( \omega \)-hydroxylation of a large number of fatty acids and arachidonic acid derivatives (18). The CYP4F subfamily utilizes LTB4 as a substrate. Two members of this subfamily have been identified in humans and are designated CYP4F3 (16, 19) and CYP4F2 (20). Studies of neutrophil microsomes or recombinant CYP4F3 have determined the specificity of CYP4F3 for LTB4 (15, 21). CYP4F1, -4, -5, and -6 are designations given to rat enzymes (22, 23).

The two human 4F subfamily members differ in their tissue localization and kinetic properties (16, 20). CYP4F3 is expressed in neutrophils and monocytes, whereas CYP4F2 is expressed in liver (20). CYP4F2 has a \( K_m \) for the \( \omega \)-hydroxylation of LTB4 that is ~60-fold higher than CYP4F3 (20). Both enzymes have 520 amino acids and share 93% amino acid sequence identity throughout most of the molecule. However, the sequence diverges between amino acids 66 and 114, where there is only 27% identity. It is not known whether the sequence variation within this 48-amino acid segment accounts for the different \( K_m \) values of the two enzymes.

We have cloned both a novel isoform of CYP4F3 expressed in fetal and adult liver and the CYP4F3 gene. The liver and neutrophil transcripts of CYP4F3 are generated by tissue-specific splicing of exons 3 and 4, respectively, and are initiated from different transcription start sites. Exons 3 and 4 are identical in size and each code for amino acids 66–114 of CYP4F3. Mutually exclusive selection of the exons is correlated with the generation of high \( K_m \) and low \( K_m \) forms of the enzyme; liver CYP4F3 containing exon 3 has a \( K_m \) for LTB4 which is ~26-fold higher than neutrophil CYP4F3 containing exon 4. Analysis of the gene indicates that putative TATA boxes are located upstream of both the liver and neutrophil transcription start sites. These findings suggest that the generation of CYP4F3 isoforms is regulated by a combination of alternative promoter usage and tissue-specific splicing. In addition, we propose a general model for CYP4F family gene organization and expression.

**EXPERIMENTAL PROCEDURES**

Isolation and Analysis of Genomic Clones—A human genomic P1 library (Genome Systems, St. Louis, MO) was screened by PCR using primers N1 and N3 (Table I). The PCR conditions were 94 °C for 1 min,
CYP4F3 Isoform Expression

**Table I**

| Name | Direction | Exon | Sequence (5' to 3') |
|------|-----------|------|--------------------|
| N1   | Sense     | 1–21 | ATGCCACAGCTAGCCTGTCCC |
| N2   | Antisense | 49–32| ATGTCGCCATGCGCCAAA  |
| N3   | Antisense | 78–56| CCCACGACAGCGAGGCCGCC |
| L4   | Antisense | 138–121| GCGCGCTTGTGAAGAGCCCG |
| N5   | Sense     | 165–184| GAACCGGATGTTTCTTGGG |
| L5   | Sense     | 165–184| AAGGCGAAGCCTTTTGGG |
| NL6e3| Antisense | 340–322| AGGCGTAAGCAAGACGCC |
| NL6e4| Antisense | 340–322| GAGCGAAAGCAGGCTT |
| N7   | Antisense | 1205–1188| CAGCAGAGAGAGAGAGCA |
| NL  | Antisense | 1205–1188| ACATCGGCGAGAGAGAC |
| NL9  | Sense     | 1201–1221| TGGCTGACCCACACATTTGG |
| NL10 | Antisense | 1856–1844| GCTGAGGGGGCTCCCAAG |

Sequences of primers referred to in text are shown. Primers are numbered sequentially in order of their cDNA location and are labeled to indicate whether the sequence is specific for CYP4F3 (N) or CYP4F2 (L), or is identical in both CYP4F3 and CYP4F2 (NL).

55 °C for 1 min, and 72 °C for 1.5 min; 35 cycles were followed by 1 cycle with a 10 min extension time. Clones that gave a PCR product of 78 bp on a 1% agarose gel were isolated. Two P1 clones, each with genomic inserts spanning more than 75 kb, were isolated to purity after 3 rounds of screening. A BamHI restriction digest of one clone (P1-A) was shotgun subcloned into pZErO-1 vector (Invitrogen). Positive subclones were identified by Southern blotting using the 32P-labeled coding region of CYP4F3 cDNA as a probe. Five different BamHI fragments were identified, which collectively span the CYP4F3 gene when arranged in a linear order. Plasmid DNA was purified using Qiagen plasmid kits and sequenced at the Massachusetts General Hospital core facility using a combination of forward and reverse primers. Exons and intron-exon junctions were sequenced using primers designed from the cDNA of CYP4F3. Introns and the 3'- and 5'-flanking regions of the gene were sequenced by primer walking. The distances of the large introns were determined by PCR using the Expand Long Template System (Roche Molecular Biochemicals) and primer pairs from flanking exons. 

Human peripheral blood neutrophils were separated from whole blood by fractionation on Mono-Poly resolving medium (ICN) and total RNA was prepared from the cells by the RNA STAT-60 procedure (Tel-Test Inc.). Total RNA from human liver and fetal liver (15–24 weeks) was purchased from CLONTECH. Total RNA was isolated from COS-7 cells using the RNaseasy Mini Kit (Qiagen). First-strand cDNA synthesis was performed using the cDNA Cycle Kit (Invitrogen) with avian myeloblastosis virus reverse transcriptase and random primers. The cDNA was purified by phenol-chloroform extraction and ethanol precipitation. Alternative splice transcripts of CYP4F3 and CYP4F2 were assayed by PCR using forward primers N5 and L5 and reverse primer NL6e3 and NL6e4. The PCR conditions were 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; 30 cycles were followed by 1 cycle with a 10 min extension time. PCR products were analyzed on a 2% agarose gel.

A novel isoform of CYP4F3 containing exon 3 (CYP4F3B) was cloned from fetal liver total RNA following first strand cDNA synthesis with a reverse transcriptase and random primers. The cDNA was purified by phenol-chloroform extraction and ethanol precipitation. Alternative splice transcripts of CYP4F3 and CYP4F2 were assayed by PCR using forward primers N5 and L5 and reverse primer NL6e3 and NL6e4. The PCR conditions were 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min; 30 cycles were followed by 1 cycle with a 10 min extension time. Clones containing exon 3 were identified by PCR screening with primers N5 and NL6e3 and were sequenced. The start sites of transcription were determined by 5'-end labeling of 5'-UTR of CYP4F genes (NL10). The product was sequenced against Western blots containing 1 μg of MBP-CYP4F3(410–520) fusion protein. Peak fractions (2, 3, and 4) were pooled, dialyzed against PBS, and stored in PBS containing 0.05% azide.

**Cell Transfections and Fractionation**—COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The cells were grown to 50% confluence and transfected in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The cells were collected by centrifugation at 100,000 × g for 10 min. The supernatant was centrifuged at 70 °C. The membranes were washed three times in PBS and harvested in disruption buffer (50 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 1 mM EDTA). They were disrupted with a VibraCell probe sonicator (3 × 30 s, 50% output, 5 × 107 cells/ml) and centrifuged at 12,000 × g for 10 min. The supernatant was centrifuged at 100,000 × g for 60 min. The microsomal membrane pellet was resuspended in disruption buffer by brief sonication. All fractionation procedures were at 4 °C. Total protein content was assayed by the Bio-Rad method, and the membranes were stored at 70 °C.

**Western Blot Analysis**—Proteins in cell extracts and microsome preparations were fractionated by SDS-polyacrylamide gel electrophoresis and electrochemically transferred to nitrocellulose Trans-Blot membranes (Bio-Rad). The membranes were blocked for 1 h in PBS containing 3% nonfat dried milk, pH 7.4, and then incubated for 1 h with affinity purified anti-CYP4F3 (410–520) diluted 1:100 in blocking buffer. The membranes were washed three times in PBS and incubated in the appropriate primary antibody, and then incubated with anti-rabbit IgG conjugated with horseradish peroxidase. The membranes were washed three times in PBS and incubated in a substrate solution containing 1 mg/ml of 4-chloro-1-naphthol, 0.1% H2O2, and 0.05% Triton X-100. The blots were washed and dried before being exposed to X-ray film.
for 1 h with peroxidase-conjugated protein A (Roche Molecular Biochemicals) diluted 1:5000 in blocking buffer. Immuno-reactive protein bands were visualized using the enhanced chemiluminescence method (Amersham Pharmacia Biotech).

**LTB**$_4$-ω-Hydroxylase Assay—The conversion of LTB$_4$ to 20-OH-LTB$_4$ was determined as described previously (14). Reaction mixtures containing 20 μg of microsomal protein, [3H]LTB$_4$ (40,000 cpm/nmol), 100 μM NADPH, and 50 mM Tris-HCl, pH 8.0, were incubated in a final volume of 0.1 ml for 20 min at 37 °C. The reaction was terminated by the addition of 4 drops of 2 M citric acid, and the substrate and product bands were visualized using the enhanced chemiluminescence method for 1 h with peroxidase-conjugated protein A (Roche Molecular Biochemicals) diluted 1:5000 in blocking buffer. Immuno-reactive protein bands were visualized using the enhanced chemiluminescence method (Amersham Pharmacia Biotech).

**Chromosomal Localization**—The genomic clone P1-A was used as a probe to map the chromosomal localization of the CYP4F3 gene by fluorescence in situ hybridization to Genome Systems. The clone was labeled with digoxigenin dUTP by nick translation and then hybridized to normal metaphase chromosomes derived from *Phaseolus vulgaris* agglutinin-stimulated lymphocytes from a male donor. The hybridization buffer was 50% formamide, 10% dextran sulfate, and 2 × SSC. Specific hybridization signals were detected by incubating hybridized chromosome slides with fluorescein-conjugated anti-digoxigenin, followed by counterstaining with 4,6-diamidino-2-phenylindole. An anonymous genomic clone known to map to chromosome 19q was used as an internal control in cohybridization experiments with the P1-A probe to confirm the identity of chromosome 19.

**RESULTS**

**Identification of a New Exon in the CYP4F3 Gene**—A map of the CYP4F3 gene comprising 14 exons and 13 introns was constructed by DNA sequencing and PCR analysis of genomic subclones as described under “Experimental Procedures.” The first exon is non-coding, and the ATG translation start codon is located at the beginning of exon 2. All introns conform to the gt-ag rule (24), and a summary of the splice junctions is shown in Table II. These data are in agreement with a recent description of the gene (19), but include a previously unrecognized exon, designated exon 3, which is not incorporated into the characterized cDNA of neutrophil CYP4F3 (16). Examination of the sequence of exons 3 and 4 and the genomic flanking regions (Fig. 1A) revealed several features that suggested exon 3 might have the capacity to participate in alternative splicing reactions with exon 4. 1) The sequences bordering exon 3 conform to recognizable splice junctions. 2) Exon 3 is identical in size to exon 4 (145 bp), and its predicted translation product has 13 amino acids in common with exon 4. 3) Exon 3 has a homolog, which is expressed in the CYP4F2 gene. Analysis of the genomic sequence of CYP4F2 deposited by the Human Genome Project (GenBank accession AC005336) reveals that it contains 14 exons that are directly analogous to those of CYP4F3. A comparison of the similarities in gene sequences in the regions of exons 3 and 4 is shown in Fig. 1A. Examination of the characterized CYP4F2 cDNA from liver (20) indicates that exon 3 from the gene is incorporated in this case whereas exon 4 is excluded. 4) The intron separating exons 3 and 4 (intron 3) of CYP4F3 contains several pyrimidine-rich tracts, which resemble PB in binding sites that regulate alternative splicing in certain pre-mRNAs (25–32). These polypyrimidine tracts are characterized by the inclusion of one or more specific sequence motifs (31). They are functional as splicing repressor signals in pre-mRNA, but their existence can be inferred from the gene sequence. No comparable polypyrimidine tracts were observed in other introns.

To confirm the possibility that exon 3 provides an alternative substrate for splicing we searched for CYP4F3 transcripts that are encoded by a separate gene. The sequences of exons 3 and 4 shown in Fig. 1A were used to develop an RT-PCR assay that distinguishes between all possible isoforms that might be generated by alternative splicing of CYP4F3. Forward primers specific for exon 2 of CYP4F3 and CYP4F2 were used, which had an identical sequence in the genes for these two enzymes. These primers, which had an identical sequence in the genes for CYP4F2 and CYP4F3 but which were specific for either exon 3 (NL6e3) or exon 4 (NL6e4). The four different combinations of primers each generate an isoform-specific PCR product of 158 bp and collectively give a profile of the alternatively spliced transcripts expressed in a given tissue (Fig. 1B).

The RT-PCR assay demonstrated that peripheral blood neutrophils derived from an adult donor exclusively express CYP4F3 containing exon 4 (Fig. 1B, lanes 1–4). This isoform corresponds to the previously characterized cDNA from neutrophils (16). It could not be detected in adult liver (lane 8), but is expressed in fetal liver (lane 12). This observation may derive from hematopoiesis in fetal liver (33). Adult liver expresses CYP4F2 containing exon 3 (lane 5), and this corresponds to the previously characterized cDNA from liver (20). This CYP4F2 isoform could also be detected in fetal liver (lane 6).
but no transcripts of CYP4F2 containing exon 4 were detected in any of the tissues tested (lanes 2, 6, and 10). In contrast, we obtained evidence for a novel isoform of CYP4F3 containing exon 3 in both adult liver (lane 7) and fetal liver (lane 11).

Expression and Kinetic Analysis of a Novel CYP4F3 Isoform (CYP4F3B)—We then cloned a cDNA of the entire coding region of the novel CYP4F3 isoform detected by RT-PCR. The tissue source was fetal liver, which expresses high levels of this transcript, and the cloning conditions are described under “Experimental Procedures.” The complete cDNA sequence of the novel CYP4F3 isoform is not presented here, since it is identi-
cal to the previously characterized cDNA from neutrophils (16) except for the following: 1) residues 199–343 (numbering from ATG initiation codon) are coded for by exon 3 in the CYP4F3 gene, not exon 4; 2) the 5'-UTR is derived from a different part of the CYP4F3 gene (see below). The results confirm that the cDNA is an alternative splice product of the CYP4F3 gene, which shows a distinct tissue-specific pattern of expression.

The CYP4F3 cDNAs derived from neutrophils (containing exon 4) and fetal liver (containing exon 3) were designated CYP4F3A and CYP4F3B, respectively. They were cloned into pcDNA3 (Invitrogen) and expressed in COS-7 cells. CYP4F3A has been purified and characterized previously (19, 21) but was included as a control in expression and kinetic studies to provide a direct experimental comparison with CYP4F3B.

Total RNA was isolated from the COS-7 cells and analyzed by RT-PCR using the same four primer combinations as before (Fig. 2A). Cells transfected with CYP4F3A gave a PCR product of 158 bp with the CYP4F3-specific primer (N5) and exon 4-specific primer (NL6e4) as shown in Fig. 2A (lane 4). All other primer combinations were negative (lanes 1–3). Cells transfected with CYP4F3B gave a PCR product only with the CYP4F3-specific primer (N5) and exon 3-specific primer (NL6e3) as shown in Fig. 2A (lane 7). No PCR products were obtained from non-transfected cells (lanes 9–12).

The expression of CYP4F3 protein was confirmed using affinity-purified anti-CYP4F3 antibody. Microsomal fractions of transfected COS-7 cells were analyzed by Western blotting (Fig. 2B). An immunoreactive band with an apparent molecular mass of 61 kDa was observed in microsomes from cells transfected with CYP4F3A. An immunoreactive band with an identical size was observed in microsomes from cells transfected with CYP4F3B, but this band was not observed in microsomes from non-transfected cells. This confirms that the novel isoform CYP4F3B is expressed in cells as a full-length protein in its predicted subcellular location.

The microsomal fractions of COS-7 cells transfected with CYP4F3A or CYP4F3B were used to determine the \( K_m \) of the two isoforms using LTB$_4$ as a substrate. In a representative experiment (Fig. 2C), the \( K_m \) values in three experiments were 4.0 \( \mu \)M (S.D. \( \pm 0.8 \)) for CYP4F3A, and 105 \( \mu \)M (S.D. \( \pm 13 \)) for CYP4F3B, approximately 26-fold higher than CYP4F3A. No activity was detected if NADPH was removed from the reaction mix, if membrane preparations from non-transfected cells were used for the assay, or if cells were transfected with pcDNA3 vector alone (data not shown).

The CYP4F3A and CYP4F3B enzymes are identical in sequence except for switching of exons 3 and 4. The amino acid
differences between these exons must therefore account for the different kinetic properties. A summary of the alternative splicing pathways of CYP4F3 is shown in Fig. 3. A revised map of the genomic organization, originally suggested to comprise 13 exons and 12 introns (19), is included.

5'-RACE Analysis of CYP4F Transcripts—The sequence for the CYP4F3 gene shares 80% identity with the reported sequence of the CYP4F2 gene over a region that extends into the 5'-flanking region (Fig. 4). The close sequence similarities between CYP4F2 and the two isoforms of CYP4F3 must be taken into account when distinguishing their respective transcription start sites in tissues with overlapping expression. We used a 5'-RACE system, which exploits a series of nested PCR reactions with isoform-specific primers to generate 5'-cDNA ends of determined identity (Fig. 4A).

We first tested the 5'-RACE procedure using neutrophil RNA, which contains a single type of CYP4F'script (CYP4F3A). The sequence of 10 5'-RACE products were identical, and indicated that transcription of CYP4F3A begins 49 bp upstream from the 5'-end of exon 1 (Fig. 4B, start site A). We numbered the gene sequence relative to this start site (+1). The sequence matched the previously reported cDNA sequence of neutrophil CYP4F3 with an additional 17 bp at the 5'-end. These data are in agreement with conclusions derived from S1 nuclease protection analysis of neutrophil RNA (19). The presence of a TATA box-like sequence and a consensus site for the MZF-1 site in CYP4F3 (TGTGGGGA) is not present in CYP4F2 because of two nucleotide differences (TGCAGGGA). The TATA box upstream of start site A in the CYP4F3 gene (CCTTACATCAG) is not observed in CYP4F3 because of two nucleotide differences (TCTACCTCCG). These observations would be consistent with the CYP4F2 gene containing a single promoter region that is not myeloid-specific. A model for CYP4F family gene organization and expression is shown in Fig. 5.

Chromosomal Localization of the CYP4F3 Gene—The chromosomal location of the P1A genomic clone containing the CYP4F3 gene was mapped by fluorescence in situ hybridization (Fig. 6). A total of 80 metaphase cells were analyzed, with 71 exhibiting specific labeling on chromosome 19p. Measurements of 10 specifically hybridized chromosomes demonstrated that the CYP4F3 gene is localized at a position that is 28% of the distance from the centromere to the telomere in an area that corresponds to band 19p13.1. The TATA box upstream of start site B in the CYP4F3 gene (TCTTACCTCCCA) is not observed in CYP4F2 because of two nucleotide differences (TCTACCTCCG). These observations would be consistent with the CYP4F2 gene containing a single promoter region that is not myeloid-specific. A model for CYP4F family gene organization and expression is shown in Fig. 5.
DISCUSSION

Human cytochrome P450 4F3 (CYP4F3) catalyzes the \( \omega \)-hydroxylation of LTB4 and has the potential to act as an inhibitory control point in inflammation. The gene spans approximately 20 kb and comprises 14 exons and 13 introns (Table II), including the previously unrecognized exon 3. Exons 3 and 4 are identical in size but only have 27% amino acid sequence identity (Fig. 1A). We developed an RT-PCR assay, which selectively determined the expression of exons 3 and 4 (Fig. 1B), and demonstrated that the two exons are alternatively spliced in a mutually exclusive fashion to generate distinct CYP4F3 isoforms.

**Fig. 4.** Transcription initiation sites of the CYP4F3 gene. A, single-strand cDNAs were generated from tissue RNA by reverse transcription with primer NL10 and tailed with dCTP using terminal transferase. Each of the three types of CYP4F transcript were uniquely selected for subsequent 5'-RACE analysis by the sequential use of two isoform-specific primers in nested PCR reactions. DNA sequences are in the 5' to 3' direction with respect to the gene orientation. The sequences shown extend from the 5'-end to the ATG translation start codon (underlined). B, the relationship of the 5'-RACE sequences to the gene sequence is shown. Transcription initiation of CYP4F3A in neutrophils from start site A generates a transcript (gray shading) with a 5'-UTR consisting of exon 1, which is spliced to exon 2. Initiation of CYP4F3B in fetal liver from start sites B1 (major) or B2 (minor) generates a transcript (underlined), which excludes exon 1 and extends exon 2 to make a distinct 5'-UTR. The region of exon 2 common to both CYP4F3A and CYP4F3B transcripts begins 1 bp prior to the ATG translation initiation codon (clustered arrowheads). Predicted TATA boxes are labeled A or B to distinguish whether they are located in the region upstream of start site A (putative neutrophil promoter) or start site B (putative liver promoter), respectively. A single start site of CYP4F2 transcription is aligned with start site A in CYP4F3, and nucleotides are numbered relative to this location (+1). Exon sequences are shown in uppercase letters. Intron sequences and 5'-flanking sequences are shown in lowercase letters. Differences in the CYP4F2 gene are shown below the CYP4F3 sequence. 5'-RACE products were sequenced with primer N2.
The involvement of tissue-specific trans-acting factors (circles) is hypothetical. Tissue-specific transcription factors may direct expression in neutrophils (N) or liver (L) by binding to regulatory sites in the promoter regions (colored boxes). Alternative splicing of exons 3 and 4 is tissue-dependent and may require liver- and neutrophil-specific factors represented by S3 and S4, respectively. Polypyrimidine tracts containing splicing repressor motifs are located in intron 3 (black boxes) and may participate by binding PTB. A 25-bp sequence at the junction of exon 4 and intron 4 is duplicated in CYP4F3 (hatched box). The final splicing pattern of exons (thick line) is determined by the combined action of transcription and splicing factors.

Fig. 6. Chromosomal localization of CYP4F3 gene. A, a human chromosome spread was cohybridized with the digoxigenin-labeled P1-A probe, which contains the CYP4F3 gene, and a second probe, which is known to map to chromosome 19q. Specific twin-spot hybridization signals are seen on chromosome 19p (P1-A probe, indicated by arrow) and 19q (control probe, indicated by arrow) after staining with fluoresceinated anti-digoxigenin and counterstaining with 4,6-diamidino-2-phenylindole. B, a human chromosome 19 ideogram showing the location of the CYP4F3 gene in the region of 19p13.1.

Analysis of neutrophil RNA by RT-PCR indicated that myeloid CYP4F3 consists exclusively of the isoform containing exon 4 (CYP4F3A). We identified a novel CYP4F3 cDNA containing exon 3 (CYP4F3B) in adult liver but could not detect CYP4F3A in this tissue. Alternative splicing of CYP4F3 therefore exhibits a distinct tissue specificity. Fetal liver contains both alternative splice forms of CYP4F3 but it is possible that a cell-specific pattern of splicing is maintained. The liver is a site of hematopoiesis in the fetus (33), and although the production of neutrophils may be limited (34, 35), neutrophil-specific proteins can be detected by RT-PCR (35).

The novel CYP4F3 isoform (CYP4F3B) was cloned and expressed in COS-7 cells to enable a comparison of its properties with the previously characterized isoform (CYP4F3A). The Km values for LTB4 (Fig. 2C) were directly correlated with inclusion of exon 4 (low Km) or exon 3 (high Km). The differences in amino acid sequence between the two exons must therefore account for the different kinetic properties of these two isoforms of CYP4F3. The selection of exon 4 in myeloid cells may have importance in maximizing the efficiency of LTB4 inactivation in situations that require fine control of inflammatory reactions.

Exon switching may depend on positive signals to promote selection of exon 3 in liver or negative signals to repress exon 4 selection. Recent work on cell-specific splicing of pre-mRNAs for tropomyosins, c-Src, and GABA_A receptor γ2 suggest that a complex balance of positive and negative regulatory elements is likely to be required (25–32). The negative signals in these pre-mRNAs generally consisted of polypyrimidine tracts with specific sequence motifs, which act as binding sites for PTB. We identified typical polypyrimidine tracts within intron 3 of the CYP4F3 gene. Their distribution is most reminiscent of the rat β-tropomyosin gene, where a cluster of PTB binding sites within one intron suppresses splicing of a skeletal muscle-specific exon immediately downstream. By analogy, PTB may regulate CYP4F3 splicing by repressing exon 4 selection in liver, and some form of derepression may occur in neutrophils. However, polypyrimidine tracts can be located upstream or downstream of a regulated exon, and PTB is a positive regulator of exon splicing in calcitonin pre-mRNA (36). Further analysis is therefore required to determine the functional status of these elements in CYP4F3. There is a duplication of a 5′-splice donor sequence in CYP4F3, which comprises 6 bp at the 3′-end of exon 4 and the following 19 bp of intron 4 (Fig. 1A). It is not clear how this might affect assembly of the spliceosome, but cooperative binding of factors to duplicate binding sites could provide a competitive advantage in the splicing reaction, or could inhibit splicing by hyperstabilizing the spliceosome complex.

The human CYP4F3 and CYP4F2 genes are highly related and have a similar organization. Alternative splicing of CYP4F2 has not been demonstrated, but we suggest a similar designation of 14 exons and 13 introns to the gene. The characterized form of CYP4F2 (20) has a number of properties in common with the novel CYP4F3B isoform described in this study. Both contain exon 3 from their respective genes; they show a similar tissue distribution in liver and fetal liver, but not in neutrophils; and they have a high Km relative to neutrophil CYP4F3A. This is consistent with the interpretation that
the $K_m$ of CYP4F enzymes is determined by the inclusion of exon 3 or 4 into the mature mRNA transcript, and that splicing of these exons occurs in a tissue-specific pattern. Polypyrimidine tracts containing splicing repressor motifs are observed within intron 3 of the CYP4F2 gene, suggesting that splicing might be regulated by a similar mechanism. An alternative splice form of CYP4F2 containing exon 4 has not yet been observed. This exon has 90% homology with exon 4 in the CYP4F3 gene, and its splice junctions are almost identical in sequence (Fig. 1A). However, the splice junction at the 3’-end of exon 4 is not duplicated in CYP4F2.

Neutrophil CYP4F3A and fetal liver CYP4F3B have different 5’-UTRs initiated from distinct transcription start sites (referred to as start sites A and B, respectively, in Fig. 4B). A sequence resembling a TATA box is located 23 bp upstream of start site A, but a stronger consensus TATA box sequence is located 27 bp upstream of start site B. The CYP4F3 gene therefore contains two putative promoter regions. Promoter A (upstream of transcription start site A) may direct myeloid-specific expression. Promoter B (upstream of start site B) may direct expression in liver. There is no evidence for two promoters in the CYP4F2 gene, and its single transcription start site can be aligned exactly with the CYP4F3A start site in neutrophils (start site A). The gene sequences of CYP4F2 and CYP4F3 are 80% identical over a region that extends 168 bp upstream of this start site. However, CYP4F2 is not expressed in neutrophils. A small number of nucleotide changes may therefore have been sufficient to convert the promoter region upstream of start site A to a myeloid-specific form subsequent to the divergence of the two genes. These could include the gain of sites for myeloid factors such as MZF-1, and perhaps the loss of TATA box activity. The consensus TATA box in the CYP4F2 gene is replaced by a weaker TATA-like sequence upstream of start site A in the CYP4F3 gene. Whatever the evolutionary sequence of events, selection has led to greater specificity for LTB4 (CYP4F family), lower $K_m$ (exon 4), and myeloid expression (CYP4F3A).

A hypothetical model for the regulation of CYP4F genes is shown in Fig. 5. The model makes some simple assumptions which are easily testable and provides a basis for future studies. Promoters A and B in CYP4F3 are depicted as independent control elements, but it remains a possibility that they share some regulatory sites. Tissue-specific splicing pathways are shown to be determined by either neutrophil- or liver-specific factors. However, it is possible that regulated splicing occurs in only one of the two tissues while the other exhibits a default pathway determined by the basal splicing factors. Some of the features described in Fig. 5 have similarities in other genes. Mutually exclusive exon splicing is regulated by PTB in the rat α- and β-tropomyosin genes (25–28). The chicken β-tropomyosin gene uses two promoters and mutually exclusive exon splicing to generate three tissue-specific isoforms (37). Alternative promoter usage accounts for tissue-specific patterns of expression of CYP19 (38) and CYP27 (39) but generates an identical enzyme in all cells.

CYP4F3A plays an important role in the control of inflammation by catalyzing the ω-hydroxylation of LTB4 in inflammatory cells with a unique low $K_m$ for the reaction. Bioactivity of CYP4F3A probably depends on the interplay of cell-specific transcription and splicing factors. Characterization of these factors and their target elements in the CYP4F3 gene may help to elucidate the regulatory pathways that determine the extent of an inflammatory response.

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