Expression and alternative splicing of the cytochrome P-450 CYP2A7

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In order to investigate the relative levels of expression of human cytochrome P-450 (P-450) CYP2A genes and determine how this relates to polymorphism in coumarin hydroxylase activity, cDNA clones for members of the CYP2A gene family were isolated. These clones were CYP2A6, CYP2A7 and an alternatively spliced version of CYP2A7 (CYP2A7AS). The latter clone was missing exon 2, but contained a 10 bp segment of intron 1. Translation of CYP2A7AS resulted in an in-frame deletion of 51 amino acids. The expression of these cDNAs in COS-7 cells showed that both CYP2A6 and CYP2A7 generated a protein of molecular mass 49 kDa, whereas the protein product of CYP2A7AS was about 44 kDa. Only the CYP2A6 had coumarin hydroxylase activity. The relative level of CYP2A7 and CYP2A7AS mRNA was investigated by reverse transcription followed by PCR (RT-PCR) using human liver RNAs and an RNA sample from a human skin fibroblast cell line. In one of five liver RNAs studied, the aberrantly spliced CYP2A7 mRNA was 3–4-fold more abundant than the normal mRNA. The other samples contained very low levels of this mRNA species. Interestingly, CYP2A7AS mRNA was the major CYP2A7 mRNA detected in the fibroblast cell line. In this case only a protein band of 44 kDa was observed by Western-blot analysis. The relative level of mRNA encoding CYP2A6 and CYP2A7 was established in seven human liver samples by RT-PCR and found to range between 1:0.5 and 1:3. These data strengthen the previous findings that alternative splicing is an important factor in determining the levels of many human P-450s and that this may be subject to tissue-specific effects. Whether in this case the protein product has some function remains to be determined.

INTRODUCTION

In mammals, cytochrome P-450 (P-450) enzymes play a pivotal role in the biotransformation of endogenous compounds involved in intermediary metabolism and of xenobiotic substances such as drugs, environmental toxins and carcinogens [1,2]. It is recognized that P-450s are not only inducible by foreign chemicals, but also subject to hormonal regulation and genetic polymorphism. As a consequence, hepatic P-450 levels in man are subject to marked individual differences. The importance of these differences is exemplified by studies on the genetic polymorphism at the CYP2D6 locus. Two distinct phenotypes, namely 'extensive metabolizer' (EM) and 'poor metabolizer' (PM), have been described [3]. The ability of individuals with the EM phenotype to metabolize some chemicals is 10–20 times higher than that of individuals with the PM phenotype [4]. In some cases the distribution of phenotypes is changed in disease populations [5] and can also be a critical determinant in inter-individual differences in toxic responses to clinical drugs.

In order to be able to determine the role of P-450s in adverse drug reactions and chemical toxicity, the basis for individuality in gene expression needs to be established. In this regard the genes of the human CYP2A subfamily have not been extensively investigated. Two cDNAs, designated CYP2A6 and CYP2A7, have been isolated. Both genes are expressed in human liver and share 96% nucleotide sequence identity and 94% identity at the amino acid level [6,7]. CYP2A6 has been found to be responsible for the metabolism of coumarin [7,8] as well as the carcinogen aflatoxin B1 [9,10]. The functions of CYP2A7 are currently unknown.

The level of expression of CYP2A6 is highly variable within the population. Coumarin hydroxylase activity varies greatly as well (up to 144-fold). This is consistent with the difference in hepatic CYP2A6 expression [11,12]. Northern-blot analysis with CYP2A6 as a probe identifies two mRNAs of 2.3 and 2.8 kb in human liver [8], and immunoblotting of liver microsomes also reveals two or three bands with molecular masses of 49, 51 and 55 kDa [7,8,12]. These results suggest that more than two CYP2A genes are expressed in this tissue.

In order to study the factors involved in the inter-individual variability in the expression levels of human CYP2A genes, we have studied the expression of CYP2A genes in human liver. We report the identification of three mRNA species encoding CYP2A6, CYP2A7 and an alternatively spliced form of CYP2A7, designated CYP2A7AS. The identification of this latter mRNA exemplifies previous reports indicating that alternative splicing is an important factor in determining cytochrome P-450 levels in man.

EXPERIMENTAL

Cells

A Simian-virus-40-transformed monkey kidney fibroblast cell line (COS-7) was maintained under standard cell-culture conditions in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% foetal-calf serum, glutamine (2 mM), and antibiotics (penicillin and streptomycin); the human skin fibroblast cell line was kindly donated by Dr. S. Keyse.
Preparation of radioactive DNA probes

A 0.78 kb cDNA fragment (from exon 1 to exon 5) of the CYP2A7 gene was radioactively labelled with \([\alpha-^{32}P]dCTP\) (3000 Ci/mmol) by random primer extension [14].

Isolation of CYP2A genomic clone

A human acute-lymphocytic-leukaemia DNA library in an Epstein–Barr-virus-based cosmid vector cos202 (a gift from Dr. D. Kioussis, The Middlesex Hospital Medical School, London, U.K.) was screened using a 0.7 kb cDNA fragment of CYP2A6 cDNA as a probe. Hybridization and washing of replica filters were performed as described by Kioussis et al. [15]. Analyses by restriction-enzyme digestion were carried out using standard techniques [16].

Southern-blot hybridization analysis

DNA was separated on 1%-agarose gels and then transferred to a Hybond-N nylon membrane using the conditions recommended by the manufacturer (Amersham International). The membrane was hybridized with the cDNA probe spanning exons 1 to 5 of CYP2A7 at 65 °C overnight, followed by washing the membrane in a final salt concentration of 0.1×SSC/0.1% SDS at 65 °C (1×SSC is 0.15 M NaCl/0.015 M sodium citrate).

DNA sequencing

The dideoxy chain-termination method with \([\alpha-^{35}S]dATP\) (400 Ci/mmol) was used to sequence plasmid DNA. The DNA was prepared by alkaline lysis. RNAase A was added to final concentration of 50–100 μg/ml and incubated at 37 °C overnight. The degraded RNA was removed by precipitating plasmid DNA with 20% poly(ethylene glycol) 6000/2.5 M NaCl (3:5, v/v). Sequences were compiled and analysed by using Gene Jockey software (published and distributed by BIOSOFT).

RNA preparation from human liver and tissue-culture cells

Human livers were obtained from kidney-transplant donors. Livers were stored at −70 °C within 1 h of removal. Information about the patient case histories has been described previously [8,9]. Total cellular RNA was prepared by the guanidinium isothiocyanate method [16] or a single-step method [17] from human liver and from cultured human skin fibroblast cells. The designation of the RNA samples is the same as that described in the literature. RNA concentration and purity were estimated spectrophotometrically.

Reverse transcription and PCR (RT-PCR)

Before reverse transcription, RNA was tested for integrity by ethidium bromide staining following separation on a denaturing formaldehyde gel. The reagents were mixed in 1 × PCR buffer (Promega), as described by Innis et al. [18], in a final volume of 20 μl. These were dNTPs (1 mM), MgCl\(_2\) (4 mM), 1 unit/μl of RNasin, 0.1 μg of oligo(dT)\(_{\text{12-18}}\) 1–5 μl of total RNA sample (10 μg) and 200 units of Moloney-murine-leukaemia-virus reverse transcriptase. A negative control without RNA was carried out in all the RT-PCR reactions, and no product was found in these negative controls. The mixture was incubated for 15 min at 23 °C, 60 min at 43 °C and then transferred to a water bath at 95 °C for 6 min. After heat treatment the reaction mixture was quickly chilled on ice. An 80 μl volume of 1×PCR buffer, containing 10–50 pmol of each primer, MgCl\(_2\) (1 mM) and 2.5 units of Taq polymerase (Promega) was then added. Mineral oil (100 μl) was then layered on top of the solution. To amplify the full-length CYP2A cDNAs, oligonucleotide A (upstream primer):

\[5'-\text{CATGCTGGCCTAGGCTGTT-3'}\]

and oligonucleotide B (downstream primer):

\[5'-\text{GCCTTAAGGCTCCCCCATCTTTATACC-3'}\]

containing an additional EcoRI site were used. To amplify a cDNA fragment spanning exons 1–5, oligonucleotide A and oligonucleotide C:

\[5'-\text{GAAGTCTCCAGCCTGCAGC-3'}\]

were used. The thermal cycle was: (1) denaturing; 1 min at 94 °C; (2) annealing; 1 min at 58 °C; and (3) primer extension; 1 min or 2 min at 72 °C. After 30–35 cycles, the reaction mixture was incubated for 8 min at 72 °C. The PCR product was purified by phenol/chloroform (1:1, v/v) and chloroform extractions. After ethanol precipitation the amplified product using oligonucleotides A and C was resuspended in 50 μl of TE buffer (10 mM Tris/1 mM EDTA, pH 8.0). Samples (10 μl) were digested for 4 h at 37 °C with 20 units of PstI.

Isolation of CYP2A7 cDNAs

Full-length CYP2A7 and CYP2A7AS cDNAs were obtained by RT-PCR using oligonucleotides A and B. The amplified products were first blunted using Klenow DNA polymerase, digested with EcoRI and then ligated into the EcoRI–Smal sites of pUC19 and transformed into Escherichia coli DH5a.

Construction of expression plasmids

The full-length CYP2A6 cDNA was described previously by Miles et al. [8]. In order to clone the CYP2A6, CYP2A7 and CYP2A7AS into the mammalian expression vector pCMV [19], the subcloned cDNAs were digested with EcoRI, blunted, and then cut with HindIII. The fragments were then ligated into the Smal–HindIII sites of pCMVx to form the expression plasmids of pCMVx-CYP2A6, pCMVx-CYP2A7 and pCMVx-CYP2A7AS.

Transient transfection

COS-7 cells were seeded at a density of 3 × 10\(^4\) cells/75 cm\(^2\) flask and incubated overnight at 37 °C. Cells were then transfected using the DEAE-dextran method [20]. Briefly, 5 μg of plasmid DNA in 100 mM NaCl/10 mM Tris/HCl, pH 7.5, was diluted to 2.0 ml with PBS (Ca\(^{2+}\)- and Mg\(^{2+}\)-free; Gibco) containing 0.5 mg/ml of DEAE-dextran (Pharmacia; molecular mass 500 KDa). Then the mixture was added to the PBS-washed cells and incubated at 37 °C for 30 min with occasional shaking. Culture medium (10 ml), supplemented with 80 μM chloroquine, was then added to the flask and the cells incubated for 2.5 h at 37 °C. The transfection mixture was aspirated off and replaced for 2.5 min with medium (10 ml) containing 10% dimethyl sulfoxide (DMSO), followed by washing once with fresh
medium; then fresh medium (15 ml) was added and the cells were cultured for 48 h before use.

**Assay of coumarin 7-hydroxylase activity in transfected COS-7 cells**

The transfected cells were washed with PBS and then re-fed with 5 ml of fresh serum-free medium, 25 l of 10 mM coumarin in DMSO and 10 l of [3-14C]coumarin (13.25 mCi in 0.5 ml of DMSO) were added to the flask and the cells were then cultured for 6 h at 37 °C. After incubation the medium was collected and an equal volume of ice-cold methanol was added. 7-Hydroxycoumarin was then assayed in the medium by h.p.l.c. analysis [21].

**Western-blot analysis**

The transfected COS cells and cultured skin fibroblast cells were harvested by trypsin treatment and resuspended in 0.5 ml of 10 mM sodium phosphate buffer, pH 8.0, containing 2 mM MgCl₂, 2 mM dithiothreitol and 1 mM EDTA. Samples were lysed by sonication using an MSE Soniprep (two 5 s bursts at an amplitude of 12 µm with the sample kept on ice). The lysed samples were centrifuged at 11 000 g for 10 min to prepare crude supernatant and pellet fractions for Western-blot analysis. Proteins were separated by SDS/PAGE [22], transferred to nitrocellulose membrane and probed with anti-(rat CYP2A) antiserum using the method of Lewis et al. [23]. For human liver microsomal preparation [8], 10 µg of protein was loaded per track, whereas for transfected COS cells and skin fibroblast cells, 100 µg of crude supernatant and pellet fractions were used.

**RESULTS**

**Determination of CYP2A mRNA levels**

Analysis of the human CYP2A6 and CYP2A7 cDNA sequences [7] shows the presence of a PstI restriction site at bp 143 (+1 indicates the start of the open reading frame) of CYP2A6, which is absent in CYP2A7. This restriction site was used to establish the relative mRNA levels encoded by these two genes. Hepatic mRNA was reverse-transcribed, and CYP2A6 and CYP2A7 were amplified by PCR using oligonucleotides A and C as primers. The expected 750 bp fragments of CYP2A6 and CYP2A7 were observed (Figure 1b; the tracks indicated by a ‘U’). After digestion with PstI (20 units and 4 h incubation at 37 °C), CYP2A6 cDNA was, as expected, cut into two fragments of 607 bp and 143 bp, whereas CYP2A7 cDNA was unaffected by this procedure. During subsequent electrophoresis the 143 bp fragment migrated out of the gel and was not revealed (Figure 1b). In a separate experiment, 0.4 mg of pBluescript DNA was added into the reaction mixture as an internal control (Figure 1a) to verify a complete digestion. In order to ensure that the analysis was reproducible, the RT-PCR and PstI digestion for samples L8, L6 and L4 were carried out twice. The results revealed that the ratio of CYP2A7 to CYP2A6 in these three RNA samples was reproducible (results not shown). Both the CYP2A6 and CYP2A7 cDNA were expressed in all liver samples. The ratio of the expression level of CYP2A7 to that of CYP2A6 was subject to some variation, ranging from slightly lower than 1:1 in sample L6, to about 3:4:1 in sample L4. The relative expression of CYP2A7 to CYP2A6 in one RNA sample (L6) was determined over a range of PCR cycles. The results showed that the expression level of CYP2A7 to that of CYP2A6 was constant with the number of PCR cycles (Figure 1c).

![Figure 1](https://example.com/f1.png)

**Figure 1** Relative amounts of CYP2A6 and CYP2A7 mRNA in six human livers

The amplified DNA was cut with 20 units of PstI and longer incubation (4 h). (a) Agarose-gel electrophoresis of PstI digested RT-PCR products. A 0.4 µg portion of plasmid pBluescript DNA (2.94 kb) was added to the reaction mixture to verify complete digestion. Lanes M, 1 kb ladder marker; lanes 1–3, digested pBluescript DNA (2.94 kb) with RT-PCR products from RNA samples L8, L9 and L11 respectively; 4, uncut pBluescript DNA. (b) Southern-blot analysis of PstI digested RT-PCR products. The fragment of 607 bp represents the digested CYP2A6, and that of 750 bp is CYP2A7. (c) The ratio of the expression of the CYP2A7 relative to CYP2A6 over a range of PCR cycles with RNA sample L6; lanes U, uncut PCR product; lanes P, PstI-digested product. The designation of the samples is the same as that described in the literature [8,9].

**The identification of an alternatively spliced CYP2A7 mRNA**

As part of the analysis of CYP2A6 and CYP2A7 mRNAs, RT-PCR was carried out on the entire coding region of the CYP2A genes using a human total liver RNA sample (L8) and oligonucleotide primers; A (complementary to the 5’ end, bp 1–22) and B (complementary to the 3’ end, bp 1583–1564). In addition to the expected fragment of 1.6 kb, a weak fragment of about 1.45 kb was observed. The PCR products were subcloned into the vector pUC19. PstI digestion was used to screen the colonies, as this site is present at bp 143 (+1 indicates the start of the open reading frame) of CYP2A6, but absent in CYP2A7. Three colonies with different PstI digestion patterns were isolated and sequenced. The results indicated that the colonies with a 1.6 kb insert contained a CYP2A6 or a CYP2A7 cDNA. The colony with a 1.45 kb insert appeared to contain an aberrantly spliced version of CYP2A7 (CYP2A7AS).

To determine the basis for the alternative splicing, we isolated
of CYP2A7AS mRNA would result in an in-frame deletion of 51 amino acids to generate a protein product of molecular mass 44 kDa. The sequence of the 10 bp segment adds three amino acid residues at position 60 and links to the amino acid at position 114 in exon 3 (Figure 2b). All the intron/exon junctions conformed to the normal GT/AG consensus splicing recognition site. However, the intron 1 contained an additional 5'-splice site, G/gcagg (exon sequences are designated by upper-case letters and intron sequences by lower-case letters), which resulted in CYP2A7AS (Figure 3). This alternative 5'-splice site does not conform to the GT/AG consensus sequence; however, it is similar to a ‘non-conforming’ 5' splice site sequence found in a few genes [24,25].

Expression of the alternatively spliced CYP2A7 in human liver and a skin fibroblast cell line

To investigate the extent of alternative splicing of CYP2A7, five human liver RNA samples and total RNA from cultured human skin fibroblast cells were assayed by RT-PCR. The expected wild-type PCR product is a fragment of 750 bp, and the product from the alternatively spliced mRNA is 600 bp (Figure 4a). An ethidium bromide-stained band of 750 bp appeared in four out of five liver RNA samples, whereas a weak band of 750 bp and a much stronger 600 bp band existed in the PCR product of the skin-fibroblast-cell RNA (Figure 4b). After transfer to a Hybond-N nylon membrane and hybridizing with a 0.78 kb probe (spanning exons 1-5 of CYP2A7), four of five samples, including sample L15 (after longer exposure), contained alternatively spliced CYP2A7 mRNA. Variability in the relative level of this mRNA as compared with normal CYP2A7 mRNA was found between samples (Figure 4c). Indeed, in RNA sample L15 the level of the alternatively spliced mRNA was 3–4-fold higher than the correctly spliced transcript. However, in the other samples the normal transcript was the predominant mRNA species. Interestingly, the major CYP2A7 mRNA species in the skin fibroblast cell line appeared to be the alternatively spliced mRNA, with only a very low amount of the normal transcript (Figures 4b and 4c, track F).

Functional analysis of CYP2A transcripts

To investigate whether these mRNAs could be translated, the CYP2A6, CYP2A7 and alternatively spliced CYP2A7 cDNAs were subcloned into the vector pCMV, and transfected into COS cells. Western-blot analysis of the membrane fractions showed that all three cDNAs gave protein products (Figure 5, tracks 2–4). The molecular masses of CYP2A6 and CYP2A7 proteins were identical (49 kDa), and they showed a mobility identical with that of the major immunostained band identified in human liver microsomes with anti-(rat CYP2A1) antibody. The alternatively spliced CYP2A7 gave two protein bands (molecular masses 44 kDa and 42 kDa; Figure 5, track 4). Since the abnormal mRNA often leads to an unstable protein product, the 42 kDa protein could be a degradation product. A protein band which co-migrated with 44 kDa protein was also found in the crude membrane fraction of the human skin fibroblast cells, but not in the human liver microsomal sample. No detectable 49 kDa protein was observed in the fibroblast cell line (Figure 5a, track 5).

In order to determine whether the expressed proteins are catalytically active, coumarin hydroxylase activity in the transfected cells was measured by h.p.l.c. (Figure 5b). Relative to controls, cells expressing CYP2A6 had considerable activity. However, no activity could be measured in cells transfected with the different forms of CYP2A7.

Figure 2  (a) Structures of the normal and alternatively spliced CYP2A7 mRNAs, and (b) deduced amino acid sequences of CYP2A7 and CYP2A7AS

(a) Exon 1A contains exon 1 plus the first 10 bp of intron 1. (b) In CYP2A7AS the 54 amino acids of exon 2 are replaced by the three amino acid residues Val-Ser-Gin.

Figure 3  Sequence covering the alternatively spliced region of CYP2A7

The alternative splice site is indicated by a vertical broken line. The exon sequences are designated by upper-case letters; intron sequences are indicated by lower-case letters.
and in one case CYP2A7AS was the more abundant of these two mRNA species. In the human fibroblast cell line, CYP2A transcripts were detected, but the major product was CYP2A7AS. The basis for the variability in alternative splicing of CYP2A7 mRNA is not known and could be determined by either genetic and/or environmental factors.

It is intriguing that aberrantly spliced mRNAs have also been reported for several other genes in the CYP2 family. For example, two mRNAs are derived from the rat CYP2C6 gene by alternative splicing in exon 8. This, like many other examples, leads to a disruption of the open reading frame. Although the aberrantly spliced mRNA can be translated into a truncated protein, its haem-binding capacity is lost and therefore the protein cannot function as a P-450 mono-oxygenase [26]. The transcript of human CYP2B6, whose expression may be co-regulated with CYP2A [12], is also alternatively spliced, and at least four mRNA species are derived from this gene [25]. The relative levels of different mRNAs are subject to considerable individual variability. Similar to the findings here, a variant of CYP2B6 is generated using a cryptic ‘non-conforming’ 5′-splice site, G/gcaag. Alternative splicing has also been described for human CYP2D genes [4,27]. Taken together, these results suggest that alternative splicing is an important determinant in the expression of many P-450 genes, and this effect will contribute to the inter-individual variation in the enzyme levels. In addition, alternative splicing using cryptic non-GT-conforming 5′-splice site is considered as a rate-limiting regulation for some genes [24].

CYP2A7AS produces a truncated protein of molecular mass 44 kDa. This protein still contains the conserved P-450 haem-binding region and could conceivably still function as a mono-oxygenase enzyme. However, CYP2A7AS does not contain exon 2, which might form a potential transmembrane domain [28] and contains specific amino acids responsible for substrate recognition [29]. The results showed that the CYP2A7AS protein was associated with the crude membrane fraction (Figure 3a). No detectable CYP2A7AS protein was found in the crude supernatant fraction (results not shown). These results agree with
recent membrane-topology models of P-450, suggesting that only exon 1 of the P-450s codes for the membrane anchor [30]. Since the expresional level of CYP2A7AS in COS-7 cells was too low to establish whether the truncated protein still binds haem or not, studies to establish whether this is the case using other expression systems are in progress.

CYP2A6 is reported to be the major enzyme catalysing coumarin 7-hydroxylation in human liver [7,8]. This conclusion was based on the observed correlation between the level of CYP2A6 protein and the enzyme activity in human livers. Our finding that CYP2A7 had exactly the same mobility as CYP2A6 on SDS/PAGE indicated that CYP2A7 may also contribute to this activity. However, cDNA-directed expression of CYP2A6 and CYP2A7 in COS-7 cells indicated that only CYP2A6 had coumarin 7-hydroxylation activity, although the cellular expression of this protein was much lower than that of the CYP2A7 protein. The lack of correlation of the level of CYP2A6 protein with coumarin hydroxylase activity in some individual samples could be explained if CYP2A7 were the major protein present.

Enzymes in the CYP2A subfamily play a role in the metabolic activation of promutagens, such as nitrosamines [31,32], benzo[a]pyrene and aflatoxin B1 [9,33–35]. To date no substrates for CYP2A7 have been identified. Analysis of mRNA levels indicates that in certain samples, CYP2A7 is expressed at higher concentration than CYP2A6. It will therefore be important to determine the substrates for this enzyme.

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