Volume 17 Number 20 1989 Nucleic Acids Research

Alternative splicing in the human cytochrome P450IIB6 gene generates a high level of aberrant messages

J.S.Miles, A.W.McLaren and C.R.Wolf*

Imperial Cancer Research Fund, Laboratory of Molecular Pharmacology and Drug Metabolism, University Department of Biochemistry, Hugh Robson Building, George Square, Edinburgh EH8 9XD, UK

Received August 18, 1989; Accepted September 15, 1989 EMBL accession no. X13494

ABSTRACT

Polymorphisms within the human cytochrome P450 system can have severe clinical consequences and have been associated with adverse drug side effects and susceptibility to environmentally linked diseases such as cancer. Aberrant splicing of cytochrome P450 mRNA has been proposed as a potential mechanism for these polymorphisms. We have isolated aberrantly, as well as normally, spliced mRNAs (cDNAs) from the human P450IIB6 gene which either contain part of intron 5 and lack exon 8 or which contain a 58-bp fragment (exon 8A) instead of exon 8. Sequence analysis of the P450IIB6 gene demonstrates the presence of cryptic splice sites in intron 8 which will account for the generation of exon 8A. The mRNAs were therefore generated by alternative splicing. These data gain significance as the mRNAs will not encode a functional P450 enzyme and appear to represent a high proportion of the P450IIB6 mRNA population. Analysis of mRNA from fifteen individual human livers and cDNA libraries constructed from a variety of human tissues using the polymerase chain reaction shows that the aberrant splicing occurs in all cells and all individuals tested. This suggests a high level of infidelity in the processing of P450IIB6 mRNAs and demonstrates that the presence of abnormal transcripts does not imply the presence of a functionally inactive gene.

INTRODUCTION

Cytochrome P450 enzymes play a major role in the metabolism of foreign compounds (1) as well as in the synthesis and modification of endogenous compounds such as steroid hormones (2). Several multigene families encode these proteins (3,4) and the multiplicity within this enzyme system may have arisen because of the selective advantage gained by the increased capacity to metabolize potentially harmful chemicals. Genetic differences in the P450-mediated metabolism of a wide variety of drugs has been clearly demonstrated (5), and by inference these may serve as markers for altered susceptibility to environmental toxins and carcinogens (3,6).

While expression of certain P450 genes appears to be constitutive and is for example regulated by hormones (7), the expression of other P450 genes can be dramatically affected by exogenous compounds including barbiturates such as

©IRL Press

phenobarbital (8). The major phenobarbital-inducible forms of P450 (P450IIB) have been intensively studied in the rat where at least six genes have been identified (9). Recently we have cloned cDNAs (encoding P450IIB6) from a single liver library representing the human P450IIB gene subfamily and have shown that it contains two or three members located on chromosome 19q12-->13.2 (10). One of these cDNA clones was derived from an apparently normally-spliced mRNA. The other lacked a 142-bp segment, which we proposed corresponded to exon 8, and retained all or part of a putative intron 5. This message would not be capable of encoding an active P450 enzyme. Several other groups have isolated P450 cDNAs which could not encode a functional P450. These observations have been directly linked to genetic polymorphisms in the human P450 system (11), however the origin of many of these cDNAs has not been established. In this report we have demonstrated that in the case of P450IIB6, mRNAs incapable of coding for a cytochrome P450 are derived by alternative splicing and not as a consequence of genetic polymorphism. The relative rate of generation of normal versus abnormal transcript may be a factor in the regulation of these highly inducible proteins in man.

{We have used the P450 nomenclature system proposed by Nebert *et al*. (12,13) throughout}.

MATERIALS AND METHODS

Preparation of radioactive probes

Restriction fragments for use as probes were isolated from low gelling temperature agarose or made using the polymerase chain reaction (PCR; 14) and radioactively labelled with [α-^{32}P]dCTP (3000 Ci mmol^{-1}) by random primer extension (15). Oligonucleotides were labelled with [γ-^{32}P]ATP (3000 Ci mmol^{-1}) and T4 polynucleotide kinase.

Isolation of human P450IIB6 genomic and cDNA clones

A 338-bp *Eco*RI fragment from pMP10 containing a putative intron 5-containing sequence of a human P450IIB cDNA (10) was used to screen plaques from a human leucocyte DNA library cloned in EMBL3 (obtained from Clontech, Palo Alto, CA) by the method of Benton and Davies (16). An exon 2-containing *Eco*RI-*Sal*I fragment of the genomic clone (pMP58; Figure 1), and a cDNA fragment encoding exons 6,7 and 8 of a closely-related gene (pMP17, 96% identical to P450IIB6, unpublished observations) were used to screen a human liver λgt11 cDNA library (17). Positive clones were purified, the phage DNA isolated and the *Sal*I (EMBL3) and *Eco*RI (λgt11) fragments subcloned into pUC19.

Restriction mapping and Southern blotting

A restriction map of the genomic clone (λMP11) was derived using digests with a combination of the enzymes *Sal*I, *Eco*RI and *Xba*I. Digested DNA was transferred from gels to Hybond-N nylon membranes (18) using the manufacturer's recommended conditions (Amersham International). The membranes were probed (and reprobed after stripping of radioactivity) with oligonucleotides (used previously in sequencing some human P450IIB6 cDNAs, 10), pMP10 and with a 550-bp *Bam*HI-*Hin*dIII cDNA fragment containing exons 1,2 and 3 from the rat P450IIB1 gene (a gift of Dr M. Adesnik). This was sufficient to order the fragments and derive the map shown in Figure 1.

DNA sequence analysis

The dideoxy chain termination method was used with [α-^{35}S]thio-dATP (400 Ci $mmol^{-1}$) to sequence DNA cloned in M13 (19,20). For the cDNA clones overlapping sequences were derived using a series of synthetic oligonucleotides and the subcloned *Eco*RI fragments. For the genomic clone all of the *Eco*RI and *Eco*RI-*Sal*I fragments were cloned into both M13mp18 and M13mp19. Oligonucleotides, whose positions were assumed from the organization of the rat P450IIB1 gene, were used to prime sequencing reactions within each exon (except for exons 2 and 6 which contain *Sal*I and *Eco*RI sites, respectively). Sequence data across each exon-intron junction were obtained, complementary oligonucleotides were synthesised and used to sequence the second strand from each intron back to the adjacent exon. Sequences were compiled and analysed using Staden Plus software implemented on a DCS286 computer (21).

RNA isolation and Northern blot analysis

Total RNA was isolated from human livers obtained from kidney transplant donors by the guanidine HCl method (22), 15μg samples were run on denaturing agarose gels and then transferred to Hybond-N (Amersham International; 23). Hybridization conditions were the same as those described by Meehan *et al*. (24) and the final wash was in 2xSSC at 65°C for 30min (1XSSC is 0.15M NaCl, 0.015M Na_3 citrate).

cDNA synthesis and polymerase chain reaction (PCR)

Copy DNA was made selectively using the oligonucleotide primers PCR1 (CCGCTCGGGCGATGCCTTCACC) and PCR3 (CTAAGGAGAAGGGGATAAAAGC, Figure 2). Total RNA (10μg) and PCR primer (3μg) were hybridized at 37°C for 30min in reverse transcriptase buffer (50mM Tris-HCl pH8; 6mM $MgCl_2$; 10mM dithiothreitol; 100mM NaCl). All four deoxynucleotides were added to a final concentration of 200μM and 18U of AMV reverse transcriptase used to initiate the reaction. Primer

extension was for 60min at 37°C. RNA was removed by RNaseA treatment (1µg/ul) followed by phenol and $CHCl_3$ extraction and cDNA precipitation was with ethanol. The DNA pellet was dissolved in PCR buffer recommended by the enzyme supplier, 3µg each of the PCR primer pairs PCR1 + PCR2 (GATTCAGAGATTTTCCGACCTT) or PCR3 + PCR2 (Figure 2) and 2U *Taq* DNA polymerase were added. The chain reaction was initiated by denaturing DNA at 95°C for 1min, annealing by cooling to 55°C for 2min and polymerizing at 70°C for 1.5min (14). Twenty cycles were performed, the product DNA ethanol precipitated, and analysed by electrophoresis on 6% polyacrylamide followed by ethidium bromide staining. DNA size markers were either a *Hae*III digest of ΦX174 replicative form, or PCR-generated fragments derived from 0.5ng of cloned target cDNA following the above protocol.

PCR was also carried out on a variety of human tissue cDNA libraries cloned in λgt11 (from Clontech) using the procedure described by Friedman *et al*. (25), and the oligonucleotide pairs PCR1 + PCR2, PCR3 + PCR2 and PCR5 (CCAAGGTCCTGCTTGCCAAGAT; Figure 3) + PCR2.

PCR-generated DNA was transferred from polyacrylamide gels to Hybond-N. Hybridization with ^{32}P-labelled oligonucleotide S33 (Figure 2) was in 6xSSC, 10x Denhardt's solution and 0.2% sodium dodecyl sulfate and the final wash was in 6xSSC for 10 min at 51°C, 3°C below the estimated T_m (26). Denhardt's solution is 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone and 0.02% Ficoll.

Some of the PCR-generated fragments were blunt-end ligated into the *Sma*I site of M13mp13 and sequenced with universal primer.

Reagents

Restriction enzymes, T4 DNA ligase, DNA polymerase I (Klenow fragment) and AMV reverse transcriptase were from Boehringer Ltd; [α-^{32}P]dCTP, [γ-^{32}P]ATP and [α-^{35}S]thio-dATP were from Amersham International and *Taq* DNA polymerase was from Stratagene (Stratagene, La Jolla, CA) or Anglian Biotechnology (Colchester, U.K.). Other reagents were from the usual suppliers. Oligonucleotides were made on an Applied Biosystems 380A DNA synthesizer.

RESULTS

Isolation and characterization of P450IIB6 genomic and cDNA clones

In order to identify the origin of the abnormal P450IIB6 mRNA (cDNA) containing part of intron 5 and lacking exon 8 (λMP1; 10) a genomic P450IIB6

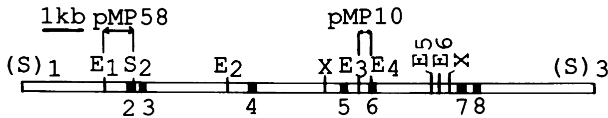

Fig.1. Restriction map of the DNA insert of λMP11 containing exons 2-8 of the P450IIB6 gene. Exons are marked by black boxes and intervening sequences by open lines. Restriction sites used for mapping and subcloning into pUC and M13 are E, EcoRI; S, SalI and X, XbaI. (S) indicates SalI sites within the cloning site of EMBL3. The position of the intron 5-containing cDNA fragment (pMP10) used to clone λMP11 is shown, as is the exon2-containing fragment (pMP58) used to clone λMP12.

clone (λMP11; Figure 1) was isolated. The derived sequence confirmed the identity of the putative intron 5 and exon 8 sequences (Figure 2). The genomic clone contained most of the P450IIB6 gene but exons 1 and 9 were not present in λMP11 (Figure 2). (Sequence data reported in this paper has been deposited in the EMBL Sequence Databank under the accession number X13494.)

In order to isolate further cDNA clones the 0.7-kb EcoRI-SalI fragment from pMP58 (Fig. 1) containing exon 2 (based on homology with rat P450IIB1), and also a fragment containing exons 6,7 and 8 from a closely-related gene (pMP17, unpublished observations) were used to screen an individual human liver cDNA library cloned into λgt11 (17). Two clones were isolated: λMP8 and λMP12 (Figures 2 and 3). λMP12 contained 0.8-kb and 0.4-kb EcoRI fragments which hybridized to both probes pMP58 and pMP17, respectively and contains sequence from exon 1-8 (Figure 2). Comparison with the rat P450IIB1 gene shows that it lacks the first 69 nucleotides of coding region. λMP8 only hybridized to pMP17 and contains sequences corresponding to exons 6, 7 and 9, but instead of exon 8 it contains a 58-bp sequence which was found to be derived from part of intron 8 (Figures 2 and 3). The sequence of λMP8 extends 246bp beyond the end of the normal coding region within exon 9 in the previously published sequence of λMP1 and λMP2 (10).

Taken together, these data demonstrate that human liver P450IIB6 mRNA can be alternatively spliced (Figure 3) to give (A) an apparently normal message as exemplified by λMP2 and λMP12 or (B) a message in which intron 5 remains and exon 8 is spliced out (λMP1) or (C) a message in which exon 8 is replaced by a cryptic exon (exon 8A) derived from intron 8 (λMP8).

Recently a full-length normally-spliced P450IIB6 mRNA (cDNA) has been cloned and expressed to give an active P450 protein product (F. J. Gonzalez, personal communication).

EXON 1

EXON 2

EXON 3

EXON 4

EXON 5

EXON 6

INTRON 6

Alu

PCR2

EXON 7

S33

PCR4

PCR3

EXON 8

PCR5

EXON 8A

PCR1

EXON 9

Organization of the human P450IIB6 gene

The positions of the exon-intron junctions in the human P450IIB6 gene are in the same relative place as in other published P450II gene family sequences, namely rat IIB1 and IIB2 (27), rabbit IIC2 (28), human IID1 (11) and rat IIE1 (29). These observations are consistent with the proposed evolutionary relationship within the P450II gene family. The size of the introns of P450IIB6 (1,>2.5kb; 2,132bp; 3,~2.5kb; 4,~2.1kb; 5,~550bp; 6,~2.0kb; 7,188bp; 8,~2.8kb) are similar to those of the rat P450IIB1 and P450IIB2, but are markedly different to those of the rat P450IIE1 gene; the significance of this observation is unclear.

All of the splice sites contain the highly conserved 5' GT and 3' AG sequence motifs, except for the 5' splice site flanking exon 8A which has a GC (Table I). It is worthy of note that all other "non-conforming" 5' splice sites (i.e. those not possessing a 5' GT) have the same G/gcaag sequence as seen in exon 8A (30). The 3' splice site serving exon 8A agrees well with the consensus (30).

Cytochrome P450IIB6 sequence

An almost full length nucleotide sequence can be compiled from the cDNA clones λMP12 and λMP2 (Figure 2), which exhibits 78.5% identity with the rat P450IIB1 cDNA sequence over the coding regions. Translation of the coding region (Figure 2) and comparison with various other members of the P450IIB subfamily (not shown) indicates that the sequence does not encode the first twenty three amino acids. The human P450IIB6 protein shares the highest homology with rabbit P450IIB4 (76% identity) compared to 75% with rat P450IIB1, 71% with mouse P450IIB9 and 68% with rat P450IIB3 (not shown). The alternatively-spliced product of the P450IIB6 gene containing intron 5 (λMP1) does not contain an open reading frame and therefore could not encode a cytochrome P450. Similarly a termination codon occurs very soon within exon

Fig.2. Nucleotide sequence of the human P450IIB6 gene. The sequences were compiled from λMP11 (exons 2-8 and flanking introns), λMP12 (exon 1) and λMP1 (exon 9). Exon sequences are shown in upper case and their translation is shown above; intron sequences are shown in lower case. The sequence of the cDNA clones λMP1 and λMP2 were published previously (10). The regions covered by λMP1, λMP2, λMP8 and λMP12 are marked as dashes below the corresponding exons; a single base change in exon 9 of λMP8 is shown. The cryptic exon (exon 8A) in λMP8 is located within intron 8. An Alu sequence in intron 6 is marked, and oligonucleotides used in the polymerase chain reaction (PCR1-5) are shown by arrows underlying the sequence, as is S33 which was used as a hybridization probe.

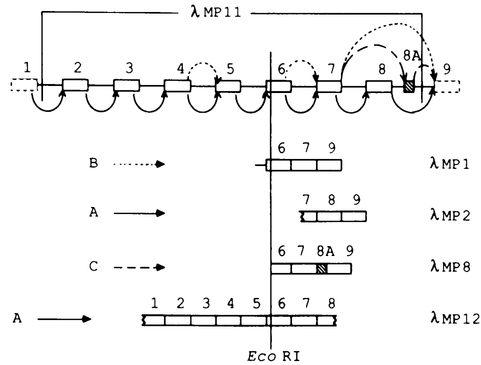

Fig.3. Diagrammatic representation of the P450IIB6 gene and its alternative splicing pathways. Open boxes represent the exons and single lines the introns (not to scale). The cDNA clone λMP2 and λMP12 are derived from normally-spliced mRNAs using pathway A; λMP1 is derived from a mRNA containing part of intron 5 but lacking exon 8 (pathway B) and λMP8 is derived from an mRNA in which exon 8 is replaced by a cryptic exon 8A found within intron 8 (pathway C). A common EcoRI site within exon 6 is shown for reference. λMP1 and λMP2 are from one individual liver cDNA library, and λMP8 and λMP12 are from a second.

8A (Figure 2) and again a cytochrome P450 could not be encoded by the mRNA corresponding to λMP8.

RNA analysis

The isolation of these alternatively (or aberrantly) spliced mRNA (cDNA) species raises the question of at what frequency do they arise, and do they arise by a tissue-specific mechanism? Northern blot analysis of 4 human liver RNA samples identified two mRNA species of approximately 1.65kb and 3.0kb which are probably the transcripts of two or more P450IIB genes (Figure 4a). The 3.0-kb transcript is likely to correspond to the P450IIB6 gene based on the size of overlapping cDNA clones (λMP2 and λMP12; Figure 2, 10). When the same RNA samples are probed with a DNA fragment containing only exon 8 of the P450IIB6 gene (made using PCR with oligonucleotides PCR3 and PCR4; Figure 2) the 3.0-kb transcript hybridizes strongly (Figure 4b) suggesting that a normal P450IIB6 mRNA containing exon 8 is present. This transcript was observed in a further 11 individuals however a large inter-individual variation in level was observed (Figure 4 and not shown).

In order to confirm the presence of a normal P450IIB6 transcript and to determine the frequency of the alternatively spliced mRNAs we decided to undertake an analysis of the RNA from 15 individual human livers. Because of

Table I

3' Splice site	Exon	5' Splice site
	1	AGG
GGATGTGATTGGC**AG**T	2	ATG**GT**GAGA
TCCCCTGCACCCC**AG**G	3	AGG**GT**GAGT
GCTGCTTCTTCCT**AG**G	4	CAG**GT**CAGG
CCTTCTTTCTTGC**AG**C	5	AAA**GT**GGGG
CTACTGTGGACGC**AG**G	6	CAG**GT**GGGC
TTCTTTTCTGTAC**AG**A	7	AAG**GT**AAGA
GTGATCCTCCCTC**AG**G	8	TAG**GT**AAGC
CTACAACCCTATA**AG**G	8A	TGG**GC**AAGT
G	9	
YYYYYYYYYYYNC**AG**G	Consensus	NAG**GT**RAGT

Splice sites flanking each exon of the P450IIB6 gene compared to the consensus sequence. Conserved **AG** and **GT** motifs are highlighted. The 5' splice site flanking exon 8A contains a **GC** rather than a **GT**.

potential allelic variation and the presence of other closely related P450IIB genes which would interfere with nuclease protection experiments, we chose to use the polymerase chain reaction (PCR). Copy DNA was made selectively from total RNA using reverse transcriptase and the oligonucleotide primer PCR1 which hybridizes within exon 9 (Figure 3). DNA spanning the region of interest was amplified using PCR and primers PCR1 and PCR2. The products were analysed by polyacrylamide electrophoresis. The sizes of the DNA which hybridizes within exon 9 (Figure 3). DNA spanning the region of interest was amplified using PCR and primers PCR1 and PCR2. The products were analysed by polyacrylamide electrophoresis. The sizes of the DNA fragments generated from the variant mRNAs can be predicted from the cDNA sequence (Figure 2). Variant A (λMP2 and λMP12; Figure 3), containing

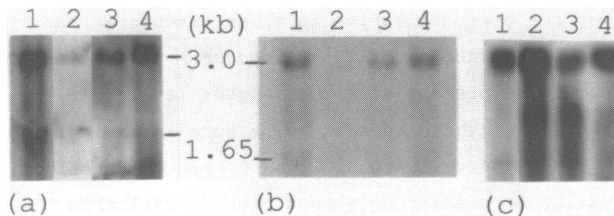

Fig. 4. Northern blot analysis of RNA from four individual human livers. 15μg of total RNA (tracks 1-4) was used in each case and probed with (a) pMP17, containing exons 6,7 and 8 of a cDNA clone 96% identical to P450IIB6; (b) exon 8 of P450IIB6; and (c) a mouse actin cDNA probe as a control for equal loading. Sizes are in kb. Based on its size and stronger hybridization to exon 8, the 3.0-kb transcript is believed to be P450IIB6.

correctly spliced exon 8, would generate a 269-bp fragment; variant B (λMP1), missing exon 8, 127bp; and variant C (λMP8), containing exon 8A instead of exon 8, 185bp. First strand cDNA synthesis was allowed to proceed for 60 min then amplified using primers PCR1 and PCR2 and separated on a 6% polyacrylamide gel (Figure 5a). The gel was blotted and probed with a P450IIB6-specific oligonucleotide which hybridizes within exon 7, common to all the expected variant cDNAs (S33; Figure 2). Three P450IIB6 DNA fragments corresponding to variants A, B and C were identified (Figure 5b). The 127-bp fragment was subcloned into M13 and its sequence was identical to that predicted for variant B (i.e. the same as in λMP1). An oligonucleotide primer (PCR3) which hybridizes to part of exon 8, was then used in conjunction with PCR2 to synthesize cDNA. The expected size of DNA fragment (230bp), corresponding to a cDNA containing exon 8 (variant A), was produced (Figure 5c), which hybridizes to oligonucleotide probe S33 (Figure 5d). An additional fragment of approximately 115bp was also present which does not hybridize to S33. The 230-bp fragment was subcloned into M13 and gave the sequence expected for variant A, whereas the 115-bp fragment had the sequence of the PCR primers at either end, but the intervening sequence was not related to the P450IIB6 gene (data not shown). Thus the 115-bp fragment and those other fragments not hybridizing to S33 have arisen by the fortuitous hybridization of the PCR primers to unrelated RNA species. The fifteen human liver RNA samples examined all contained variants A, B, and C.

All three variants A, B and C were found, by PCR analysis, in the two human liver cDNA libraries used to isolate the four λ clones. Similarly all three species were detected in cDNA libraries constructed from mRNA from human breast, foetal liver, lung, placenta and testes using PCR1 and PCR2 (not shown) indicating the expression of P450IIB6 in these tissues. Although the PCR technique may not be quantitative in these circumstances, it is interesting to note that the normally-spliced mRNA (A) and the variant lacking exon 8 (B) appear to be present at a much higher level than the exon 8A-containing variant (C). There appears to be some inter-individual variation in the relative ratios of variants A, B and C, probably reflecting individual exon 8 (B) appear to be present at a much higher level than the exon 8A-containing variant (C). There appears to be some inter-individual variation in the relative ratios of variants A, B and C, probably reflecting individual differences in the degree of alternative splicing (Figures 5a and 5b).

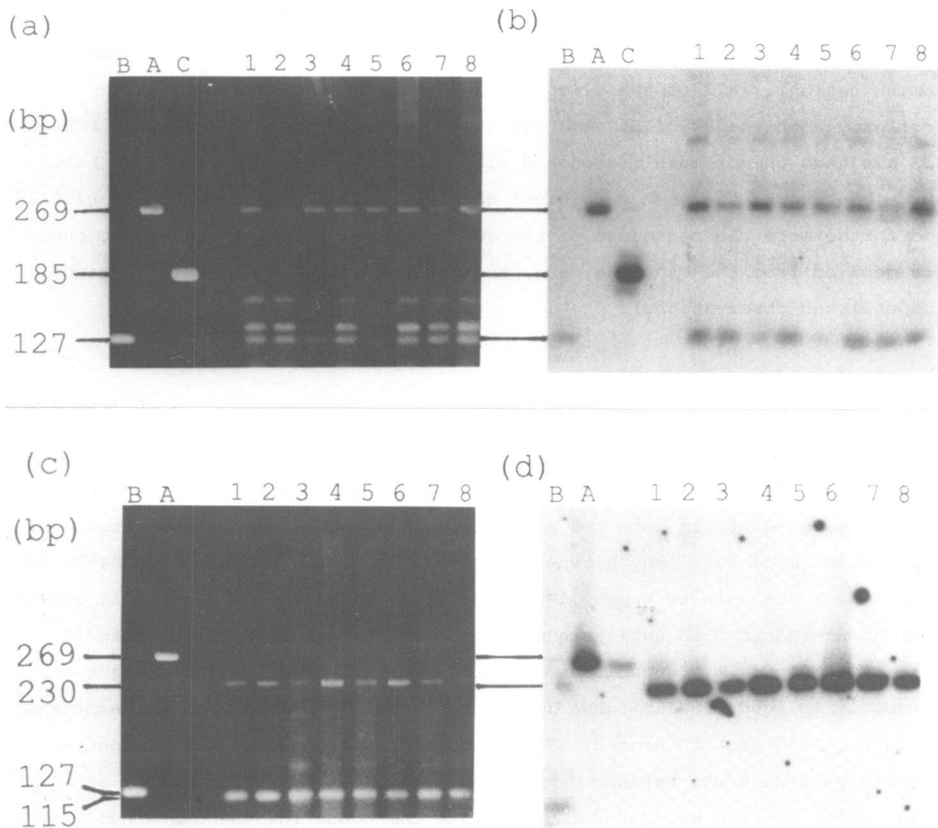

Fig. 5. PCR analysis of P450IIB6 transcripts. (a) cDNA was synthesized for 60min using PCR1 and then amplified using PCR1 and PCR2. Tracks 1-8 show the products from eight individual human liver RNA samples resolved on a 6% polyacrylamide gel. Tracks A, B and C are the amplification products generated by PCR1 and PCR2 with λMP1, λMP2 and λMP8 as controls (127, 269 and 185bp, respectively). (b) Autoradiograph of the same samples as in (a) blotted onto a nylon membrane, probed with the ^{32}P-labeled exon 7-specific oligonucleotide (S33) and then washed at 3°C below its T_m of 54°C. The products corresponding to variants A and B can be seen clearly; the products corresponding to variant C can be seen more clearly on the original autoradiographs. The nature of the larger hybridizing DNA fragment is not known. (c) The PCR products derived from human liver RNAs 1-8 when cDNA synthesis is with PCR3 (hybridizing within exon 8) and amplification with PCR3 and PCR2. The sizes of the two major products are shown in bp. (d) Autoradiograph of the same gel as in (c) blotted onto a nylon membrane, probed with S33 and washed as before. Note that only the 230-bp fragment hybridizes to S33. In controls where either RNA or reverse transcriptase or oligonucleotides were omitted no DNA was generated.

A second human P450IIB gene exists that has ca. 96% sequence identity over exons 7,8 and 9 (unpublished observations; F. J. Gonzalez, personal communication). All of the oligonucleotides used in this study match perfectly with the P450IIB6 gene and the oligonucleotides PCR1, PCR2, PCR4 and S33 all have one or more mismatches with the second P450IIB gene. We have shown that under the wash conditions employed, S33 hybridizes only to P450IIB6 and furthermore the sequences of the PCR-generated fragments show that they are derived from the P450IIB6 gene, and not the second human P450IIB gene (unpublished observations).

DISCUSSION

Evidence is presented that the variant human P450IIB6 mRNA species have arisen by the differential or alternative splicing of the P450IIB6 gene. The structure of the relevant portion of the gene, and identification of the cryptic exon 8A within intron 8 strongly supports this conclusion (Figure 3). Translation of a full length type A mRNA would produce a cytochrome P450, and immuno-blot analysis of human liver microsomes shows that a protein is present that cross-reacts with anti-sera made against the rat P450IIB1 protein (L. Forrester and C.R.W., unpublished observations). Indeed, a full-length correctly spliced P450IIB6 mRNA (cDNA) has recently been cloned and expresses an intact P450 protein product (F. J. Gonzalez, personal communication). Neither variant B nor variant C mRNA would encode a cytochrome P450, as translation of each would terminate before the codon for the cysteinyl ligand required for haem binding which is found at the beginning of exon 9. The possibility that these variants encode proteins with other functions however can not be excluded.

Variants A, B and C were present in all fifteen human livers tested and so the aberrant splicing appears to occur in all individuals and in all tissues where the gene is expressed. The aberrant transcripts appear to occur concomitantly with the generation of a functional mRNA and the sequence identity between the different transcripts shows that they are derived from the same gene. The presence of normal and abnormal RNAs in all of the fifteen samples tested also implies that the transcripts are not the result of a
the same gene. The presence of normal and abnormal RNAs in all of the fifteen samples tested also implies that the transcripts are not the result of a
genetic polymorphism in the P450IIB6 gene. We therefore propose that alternative splicing of the P450IIB6 gene represents a stochastic process in which a proportion of messages are spliced following the different pathways.

Alternative or differential splicing has been suggested to occur in several other cytochrome P450 genes and has been proposed as the basis of genetic polymorphism in the expression of cytochrome P450 in man (11). However, these aberrant mRNAs could arise by similar mechanisms to those described here and therefore may not represent a genetic polymorphism. The mechanism of mRNA processing of the human P450IIB6 gene could explain many of the examples of unusual P450 mRNAs described in the literature. For example, in the rat P450IIC6 gene (31) a 142-bp DNA fragment, corresponding to exon 8, is replaced by a 159-bp fragment which disrupts the open reading frame. Indeed Kimura et al. (32) have recently shown that the 159-bp fragment is derived from intron 8 by a process of alternative splicing of cryptic splice sites. This is similar to the case of P450IIB6 variant C in which exon 8 is replaced by the 58-bp exon 8A. Deletion of a 142-bp DNA fragment thought to correspond to exon 6 in mouse P450IID8 has also been reported (33) and an alternative splicing process which joins exon 5 to exon 7 could account for this observation. Again this parallels the case for P450IIB6 variant B where exon 7 is spliced to exon 9, thus deleting exon 8. Retention of intron 5 or intron 6 has been shown to occur in mRNAs from the human P450IID1 gene (11), similar to P450IIB6 variant B which retains intron 5 (at least in the cDNA clone).

It is interesting to note that most of the proposed unusual splicing products are generated from genes within the P450II family in which the gross exon-intron organization is highly conserved. Also, all of these examples are from mRNAs expressed in the liver, which might suggest a lower fidelity of splicing in this tissue or a tissue-specific splicing effects. However, the finding of all three variant cDNAs in λgt11 libraries made from mRNA derived from a variety of different tissues indicates that this is not the case.

Cytochrome P450 gene expression is subject to large individual variation because many of the genes are regulated at a transcriptional level by a host of environmental factors. This is particularly the case for the rat P450IIB subfamily where 25- to 100-fold increases in mRNA levels are observed following exposure to certain inducing agents (34). The large inter-individual variation in the levels of P450IIB6 mRNA suggests that environmental factors may also play a role in man. It is of interest that in all individuals there appears to be a high level of alternative or aberrant splicing of P450IIB6 mRNA leading to inactive products. Also there appears to be inter-individual differences in the ratios of the three transcripts (Figure 5). These may be important factors in determining the expression of the

P450IIB6 gene and such a mode of regulation could have significant pharmacological and toxicologcal consequences.

ACKNOWLEDGMENTS

We would like to thank Miss A. Seabourne and Mr A. Sinclair for help with isolating and restriction mapping the genomic clone; Dr S. Woo for a human liver cDNA library; Drs U. Meyer and D. Back for some of the human liver samples and Mr I. Goldsmith for synthesizing oligonucleotides.

*To whom correspondence should be addressed

REFERENCES

1. Wislocki, P.G., Miwa, G.T. and Lu, A.Y.H. (1980) In "Enzymatic Basis of Detoxification" (Jakoby, W.G., ed.) Vol. 1, pp. 135-182.
2. Hamberg, M., Samuelsson, B., Bjorkhem, I. and Danielsson, H. (1974) In "Molecular Mechanisms of Oxygen Activation" (Hayaishi, O., ed.) pp. 29-85, Academic Press, New York.
3. Wolf, C.R. (1986) Trends in Genetics 8, 209-214.
4. Nebert, D.W. and Gonzalez, F.J. (1987) Annu. Rev. Biochem. 56, 945-993.
5. Meyer, U.A., Gut, J., Kronbach, T., Skoda, C., Meier, U.T., Catin, T. and Dayer, P. (1986) Xenobiotica 16, 449-464.
6. Ayesh, R., Idle, J.R., Ritchie, J.C., Crothers, M.J. and Metzel, M.R. (1985) Nature 311, 169-170.
7. Skett, P. (1987) Progress in Drug Metabolism 10, 85-140.
8. Conney, A.J. (1967) Pharmacol. Rev. 19, 317-366.
9. Atchison, M. and Adesnik, M. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 2300-2304.
10. Miles, J.S., Spurr, N.K., Gough, A.C., Jowett, T., McLaren, A.W., Brook, J.D. and Wolf, C.R. (1988) Nucl. Acids Res. 16, 5783-5795.
11. Gonzalez, F.J., Skoda, R.C., Kimura, S., Umeno, M., Zanger, U.M., Nebert, D.W., Gelboin, H.V., Hardwick, J.P. and Meyer, U.A. (1988) Nature 331, 442-446.
12. Nebert, D.W., Adesnik, M., Coon, M.J., Estabrook, R.W., Gonzalez, F.J., Guengerich, F.P., Gunsalus, I.C., Johnson, E.F., Kemper, B., Levin, W., Phillips, I.R., Sato, R. and Waterman, M.R. (1987) DNA 6, 1-12.
13. Nebert, D.W., Nelson, D.R., Adesnik, M., Coon, M.J., Estabrook, R.W., Gonzalez, F.J., Guengerich, F.P., Gunsalus, I.C., Johnson, E.F., Kemper, B., Levin, W., Phillips, I.R., Sato, R. and Waterman, M.R. (1989) DNA 8, 1-14.
14. Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1988) Science 239, 487-491.
15. Feinberg, D.P. and Vogelstein, B. (1983) Anal. Biochem. 136, 6-13.
16. Benton, W.D. and Davies, R.W. (1977) Science 196, 180-182.
17. Kwok, S.C.M., Ledley, F.D., DiLella, A.G., Robson, K.J.H. and Woo, S.L.C. (1985) Biochemistry 24, 556-561.
18. Southern, E.M. (1975) J. Mol. Biol. 98, 503-518.
19. Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.J.H. and Roe, B.A. (1980) J. Mol. Biol. 143, 161-178.
20. Biggin, M.D., Gibson, T.J. and Hong, G.F. (1983) Proc, Natl. Acad. Sci. U.S.A. 80, 3963-3965.
21. Staden, R. (1986) Nucl. Acids Res. 14, 217-231.
22. Cox, R.A. (1968) Meth. Enzymol. 12B, 120-129.
23. Thomas, P.S. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 5201-5205.

24. Meehan, R.R., Barlow, D.P., Hill, R.E., Hogan, B.L.M. and Hastie, N.D. (1984) EMBO J. 3, 1881-1885.
25. Friedman, K.D., Rosen, N.L., Newman, P.J. and Montgomery, R.R. (1988) Nucl. Acids Res. 16, 8718.
26. Wallace, R.B., Johnson, M.J., Hirose, T., Miyake, T., Kawashima, E.H. and Itakura, K. (1981) Nucl. Acids. Res. 9, 879-894.
27. Suwa, Y., Mizukami, Y., Sogawa, K. and Fujii-Kuriyama, Y. (1985) J. Biol. Chem. 260, 7980-7984.
28. Govind, S., Bell, P.A. and Kemper, B. (1986) DNA 5, 371-382.
29. Umeno, M., Song, B.J., Kozak, C., Gelboin, H.V. and Gonzalez, F.J. (1988) J. Biol. Chem. 263, 4956-4962.
30. Shapiro, M.B. and Senapathy, P. (1987) Nucl. Acids Res. 15, 7155-7174.
31. Kimura, H., Yoshioka, H., Sogawa, K., Sakai, Y. and Fujii-Kuriyama, Y. (1988) J. Biol. Chem. 263, 701-707.
32. Kimura, H., Sogawa, K., Sakai, Y. and Fujii-Kuriyama, Y. (1989) J. Biol. Chem. 264, 2338-2342.
33. Wong, G., Kawajiri, K. and Negishi, M. (1987) Biochemistry 26, 8683-8690.
34. Hardwick, J.P., Gonzalez, F.J. and Kasper, C.B (1983) J.Biol. Chem. 258, 8081-8085.