

Gene conversion in a cytochrome P-450 gene family

(cytochrome P-450 diversity/evolution/phenobarbital/DNA sequencing)

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ABSTRACT The mRNAs encoding the two major phenobarbital-inducible forms of cytochrome P-450 of rat liver, P-450b and P-450e, are remarkably similar (98% homologous) in nucleotide sequence, but the distribution of differences within them is not random. While the 5' halves (≈ 1 kilobase) appear to be identical, there are 36 divergent residues in the remaining sequences of the two mRNAs, with 14 differences residing in two short highly divergent segments, which in the P-450e gene are located within exon 7. DNA sequence analysis of portions of a number of P-450b/e-related genes provides strong evidence that at least one of the short divergent segments is the result of a recent gene conversion event between an ancestor to the cytochrome P-450e gene and a related donor P-450 gene of unknown function. The sequence data also suggest that extensive gene conversion has occurred within all the members of this gene family in the region including exons 7 and 8 and the intron between them, with a resultant homogenization of those sequences relative to other portions of the genes. Genomic Southern blotting analysis demonstrates that the presence of an apparent "constant" region in the 5' halves of the P-450b and P-450e mRNAs does not reflect a rearrangement in somatic cells of a germ-line DNA configuration. It is therefore proposed that it, too, is a consequence of a very recent gene conversion event between ancestors of the genes encoding both proteins or of an unequal crossing-over between them. On the basis of these and other data we propose that gene conversion represents an important evolutionary mechanism for the generation of related cytochrome P-450 isozymes in which regions of extraordinary sequence similarity and dissimilarity are intermixed. The gene conversion mechanism would account for some of the overlaps in substrate specificities of distantly related P-450s as well as for substantial differences in catalytic properties between closely related members of the same P-450 protein family.

It has long been recognized (1) that members of a multigene family within a single species are generally much more closely related to each other than to equivalent (orthologous) genes in other species. This concerted evolution of genes that have had at least as long a time to diverge from each other as from their orthologous counterparts may result from unequal crossing-over between tandemly linked genes or from either double reciprocal recombination or gene conversion (1). The latter term refers to a nonreciprocal recombination event in which a segment of one gene replaces the corresponding segment of a related gene (2). The occurrence of homogenizing gene conversion events in the human α -globin (3), human fetal γ -globin (4), oxytocin-vasopressin (5), and immunoglobulin (6) gene families has been inferred from a comparison of DNA sequences.[†] It has recently become apparent that gene conversion may also produce short regions of high diversity within closely related members of a multigene family, when the donor gene for the conversion is

a more distantly related family member. In this way gene conversion has served to enhance the diversity of immunoglobulin genes (7–9) and to increase polymorphism within genes encoding the heavy chains of the major histocompatibility antigens (10, 11).

The microsomal mixed-function oxidase system of mammalian liver contains at least a dozen distinct forms or isoenzymes of the terminal oxidase, cytochrome P-450, which exhibit distinct but broad and overlapping substrate specificities (12). In rat liver, two major closely related phenobarbital-inducible cytochrome P-450 isozymes, cytochromes P-450b and P-450e, have been identified (13, 14). Sequence data for cDNA (15, 16) and genomic clones (17, 18) indicate that the mRNAs encoding these proteins are remarkably similar (98% homologous) but that the distribution of homologous and nonhomologous sequences within them is not random. Thus, while the 5' 936 nucleotides of the two mRNAs seem to be identical, there are 36 nucleotide differences in the remaining halves of the mRNAs. Nearly half of these differences are contained within two short regions of relatively high (23–24%) sequence divergence that account for nearly half of the amino acid differences between the two polypeptides and are located within exon 7 of the cytochrome P-450e gene (17, 18). These flank a more highly conserved segment encoding an "analogous tridecapeptide" observed by Ozols *et al.* (19) in two ostensibly unrelated rabbit liver P-450 isozymes, one constitutive (LM-3b) and one highly induced by phenobarbital (LM-2).

Recently, a series of genes distinct from the P-450e and P-450b genes but having sequence homology to cytochromes P-450b and P-450e cDNAs have been cloned and characterized (18, 20). Hybridization of several of these genes to P-450e cDNA probes combined with heteroduplex analysis revealed that the region of high homology to the cytochrome P-450e gene is limited to exons 7 and 8, and possibly the intron between them (18). In this report we present DNA sequence data for portions of four different genes related to those for P-450b and e, which indicate that gene conversion can account for the unusual arrangement of regions of high and low homology within the P-450b/e polypeptides and suggest that this mechanism has played an important role in the evolution of the cytochrome P-450 superfamily of proteins.

MATERIALS AND METHODS

DNA sequencing by the Sanger dideoxynucleotide method, subcloning of DNA fragments in M13 mp8 and mp9 phage vectors, and Southern blotting were performed as described previously (18).

Abbreviations: bp, base pair(s); kb, kilobase(s).

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[†]It should be noted, however, that formally it is impossible to distinguish by DNA sequence comparisons alone between double reciprocal recombination, with a subsequent evolutionary loss of one parental genotype, and a true gene conversion event.

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RESULTS

Evidence for Gene Conversion Events Between Members of the Cytochrome P-450b/e Family. DNA sequences for exons 7 and 8, intron 7, and portions of introns 6 and 8 were determined for all the members of the cytochrome P-450b/e gene family recently cloned in our laboratory (18). The strategy employed for obtaining these sequences is shown in Fig. 1 and the DNA sequences obtained are presented in Fig. 2. In the second figure, two boxes were drawn that enclose the segments of high divergence between cytochrome P-450b and P-450e mRNAs and the corresponding regions of the other four genes. The segment in the cytochrome P-450e gene that encodes the analogous tridecapeptide, located between the two boxes, is overlined; all six genes contain nearly identical sequences in this region, and only a single conservative amino acid change (Asp-350 to Glu in gene 5) would be found in the encoded peptide segment. In the boxed sequences upstream from those encoding the tridecapeptide, where the P-450e and P-450b cDNAs differ in 9 out of 37 residues, genes 2-5 are nearly identical to the P-450b gene in the first (5') five divergent residues, but these genes are much more similar to the P-450e gene in the last four divergent residues. In contrast, within the boxed sequences downstream from the tridecapeptide-encoding region, the P-450e gene and gene 3 are identical, whereas the four other genes show distinct sequences with

extensive divergences. The region of identity between gene 3 and the P-450e gene extends upstream to nucleotide position 137 and downstream past the intron-exon junction into intron 7 to position 374. In total, the region of identity between these two genes encompasses 236 bp, with 115 bp residing within intron 7. This relatively long region of identity strongly suggests that a gene conversion event has occurred between gene 3 and the P-450e gene.

The DNA sequence comparisons also indicate, quite surprisingly, that within all members of this gene family intron 7 is nearly as highly conserved as are the two flanking exons 7 and 8. It is easily seen that this is not a general property of all introns within this multigene family, since in all of the genes the partial sequences shown for introns 6 and 8 are much less homologous to each other and to the cytochrome P-450e gene. It should be noted that, generally, members of multigene families show much higher sequence conservation in exons than in introns. These data, therefore, are consistent with frequent gene conversion events having occurred between the different genes of the cytochrome P-450b/e family involving the 500-bp regions encompassing exons 7 and 8 and the intervening intron, which resulted in an homogenization of the sequences.

A DNA Rearrangement Does Not Account for the Presence of an Apparently Constant 5' Region in the Cytochrome P-450b and P-450e mRNAs. The region of apparent identity within the P-450b and P-450e mRNAs corresponds to the first five exons of the P-450e gene. The first six exons of the cytochrome P-450e gene are contained in liver DNA within a 14-kilobase (kb) *EcoRI* fragment (17, 18) that can be detected by hybridization to a cDNA clone (R17) containing sequences derived from exons 6 through 9 of the cytochrome P-450e gene (16). Had the liver P-450e gene been formed by a somatic recombination event that linked a germ-line "constant" region containing exons 1 to 5 to a P-450e variable region segment containing exons 6 to 9, then germ-line DNA would not show the 14-kb *EcoRI* segment detected in liver DNA. Similarly, in liver DNA, exons 4 through 9 and 8 kb of 3' flanking sequence are contained within a 13-kb *BamHI* fragment that would not be found in germ-line DNA had the liver P-450e gene been generated by somatic rearrangement. Genomic DNA samples from rat sperm and liver were therefore digested with either *EcoRI* or *BamHI*, followed by Southern blotting and hybridization with the R17 cDNA clone. As shown in Fig. 3, there was no detectable difference in the two diagnostic genomic fragments between sperm and liver DNA. In addition, none of the other *EcoRI* or *BamHI* bands, representing cross-hybridizing restriction fragments of other genes within the cytochrome P-450b/e subfamily, showed any evidence of somatic rearrangement. Thus, somatic recombination events linking a single 5' constant region to either of two 3' variable regions could not account for the identity of sequences in the 5' halves of the cytochrome P-450b and P-450e mRNAs.

Conservation of a Cysteine-Containing Peptide in the Cytochrome P-450b/e Family. It has been suggested that cysteine-436 in cytochromes P-450b and P-450e, which lies within a peptide relatively highly conserved in all P-450 isozymes sequenced to date (21), provides the fifth ligand to the heme iron in the enzyme active site (15, 22, 23). Portions of exon 9 containing the region encoding the conserved peptide were sequenced in two of the related genes (genes 2 and 3) and compared to the corresponding sequence in the cytochrome P-450e gene (Fig. 4). These gene segments, which are overlined in the figure, show very high homology (90%) in all three genes. Downstream from this region, however, in the segments encoding the C-terminal portions of the polypeptides, DNA sequence homology is much lower (approximately 70%). It should be noted that the substantial divergence in the 3' coding regions of these genes is in striking

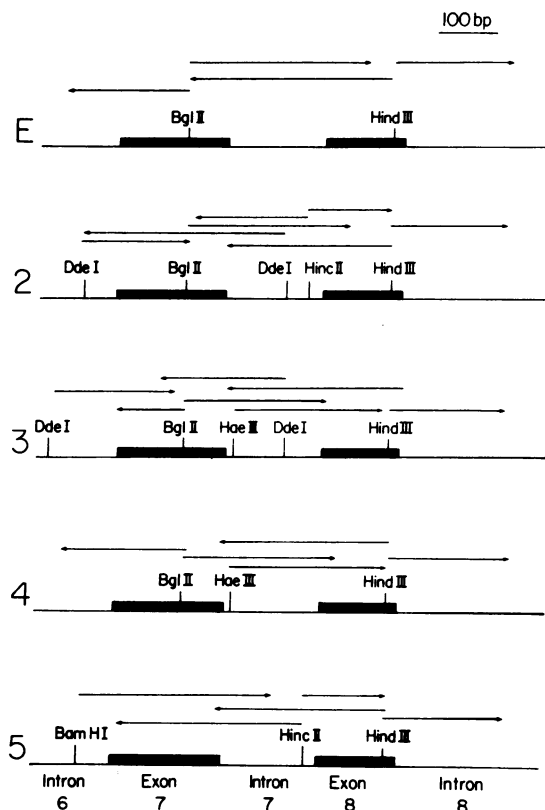


FIG. 1. Strategy for subcloning and sequencing portions of the cytochrome P-450e-related genes. Partial restriction maps for the relevant regions of the P-450e-related genes are shown. Exons 7 and 8 are represented by black boxes, whereas introns 6, 7, and 8 are indicated by lines. The M13 subclones used for sequencing by the Sanger method were obtained by either shotgun cloning or directed cloning of isolated restriction fragments. DNA sequencing was performed in the direction and to the distance indicated by the arrows. Wherever a sequence was read in only one strand, that sequence was determined two to four times. The number to the left of each map represents the gene number according to Atchison and Adesnik (18). bp, Base pairs.

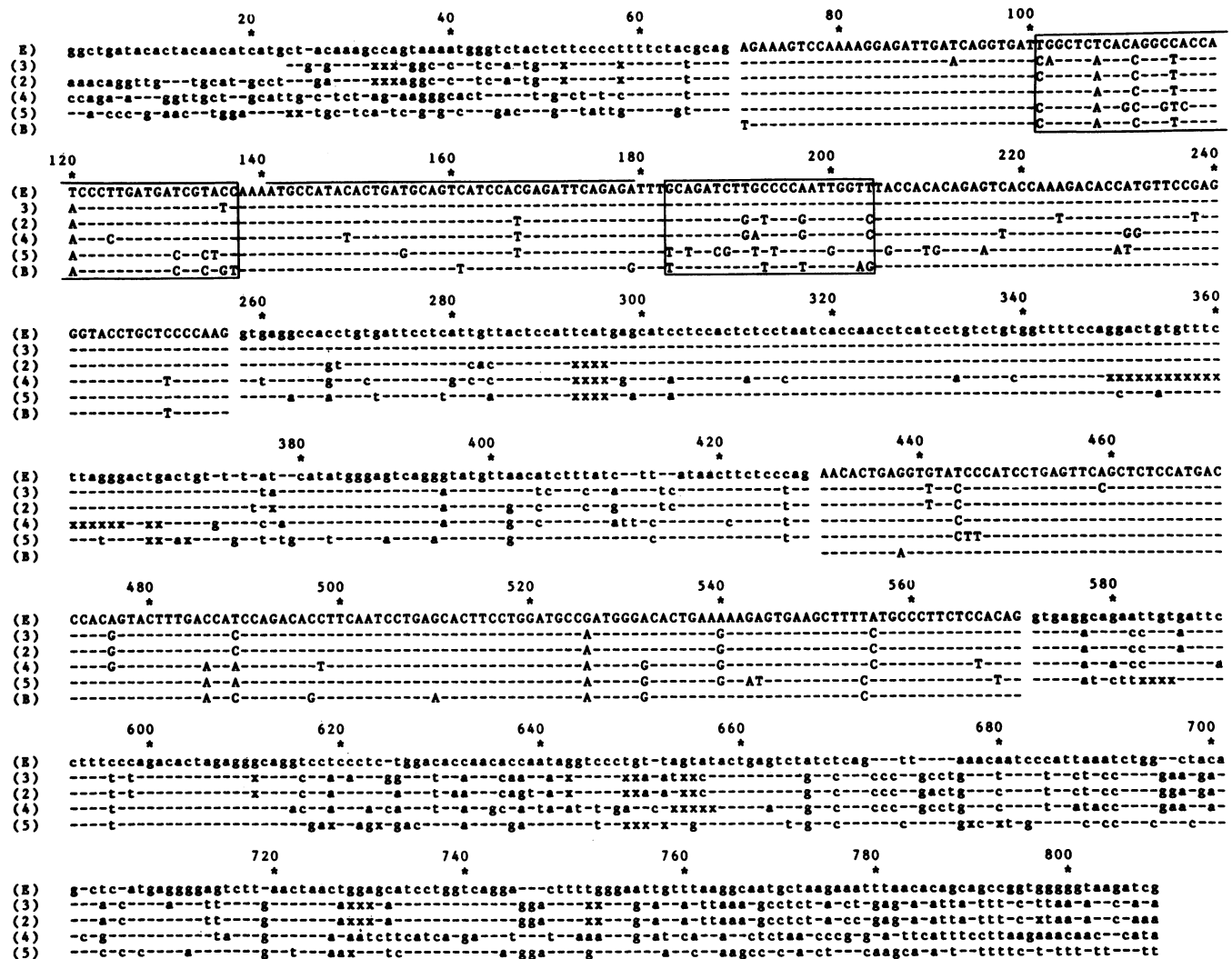


FIG. 2. DNA sequences of highly homologous segments in the regions of exons 7 and 8 of various members of the cytochrome P-450b/e gene family. The DNA sequences of exons 7 and 8, intron 7, and portions of introns 6 and 8 are compared for the cytochrome P-450e gene (E), four P-450b/e-related genes (genes 3, 2, 4, and 5), and the cytochrome P-450b cDNA sequence (B) determined by Fujii-Kuriyama *et al.* (15). The top line corresponds to the P-450e gene sequence. Intronic sequences are indicated by lowercase letters while exonic sequences are denoted by capital letters. The numbering system is arbitrary with position 1 beginning in intron 6, 69 bp 5' to the beginning of exon 7 in the cytochrome P-450e gene. For the other genes, only nucleotides that differ from the corresponding residues in the P-450e gene are shown. Dashes in the P-450e sequence indicate gaps introduced to maximize homology; an x in any sequence indicates a deletion. The analogous tridecapeptide region is overlined in the P-450e gene sequence, and the two highly divergent segments between P-450b and P-450e, as well as the corresponding segments in the other genes, are boxed. The region of identity between the P-450e gene and gene 3 begins at nucleotide position 137 and extends to position 374. A clone (number 29) that appeared to correspond to a sixth related gene was originally isolated with those containing portions of genes 2-5 (18). The DNA sequence of this clone in the region described above was found to be identical to gene 3 over 800 bp of sequence, but the upstream DNA did not contain sequences corresponding to exons 1-5 as assessed by hybridization to mRNA. It, therefore, seems likely that this clone resulted from a cloning artifact involving a deletion in the region upstream of exon 6.

contrast to the high sequence conservation in introns 7 of the same genes.

DISCUSSION

The DNA sequence data in this paper strongly suggest that gene conversion is responsible for the striking sequence divergence observed in a short segment of the two highly homologous cytochrome P-450 isozymes P-450b and P-450e. One gene, among a family of four P-450b/e-related genes, was shown to contain a segment identical to a portion of the P-450e gene that not only includes the highly divergent region but also extends over 236 bp of flanking sequences, 115 of which are within an intron. The P-450b and e genes also contain a second highly divergent segment, located upstream from the region encoding the "analogous tridecapeptide" that characterizes many cytochrome P-450s. Corresponding

segments in the four P-450b/e-related genes resemble in part the P-450b gene and in part the P-450e gene. It is possible that the marked differences between the P-450b and e genes in this region resulted from gene conversion events involving a more distantly related donor gene, perhaps one similar to those described by Leighton *et al.* (25), which would not be expected to hybridize to the P-450e cDNA used to select the genomic clones for genes 2-5.

The DNA sequence data for exons 7 and 8 and the intron between them obtained for all the other members of the cytochrome P-450e gene family that we have cloned (genes 2-5) confirm the results of hybridization experiments (ref. 18 and unpublished observations) and indicate that homology to the P-450e gene in this region is much greater than in other portions of the genes. Indeed, we show that, for at least two genes, intron 7 is much more highly conserved with respect to the P-450e gene than most of the coding region within exon

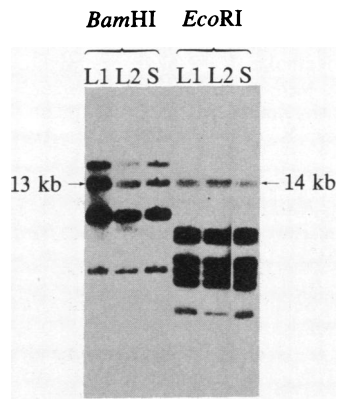


FIG. 3. Similar structure of cytochrome P-450 genes in germ-line and liver DNA. Liver DNAs isolated from two rats (L1 and L2) and DNA from sperm (S) pooled from four rats were digested with either *EcoRI* or *BamHI* restriction endonuclease and electrophoresed on a 0.7% agarose gel. The DNA was then transferred to a nitrocellulose filter and hybridized with the nick-translated R17 cDNA insert, as described (18). A faint band representing an approximately 7-kb fragment present in the *BamHI* digest of L2 DNA but not in the digests of L1 or sperm DNA may be due to polymorphism within this cytochrome P-450 gene family.

9. This extraordinarily high intronic sequence homology may well reflect the occurrence of frequent gene conversion events involving the corresponding regions of the various genes. On the other hand, it is certainly possible that even the conservation of the intronic sequence may be the result of natural selection for sequences that are functionally important. Thus, the intronic sequence could in some way regulate gene expression at the DNA level or play a role in the processing of nuclear precursors of the P-450 mRNAs.

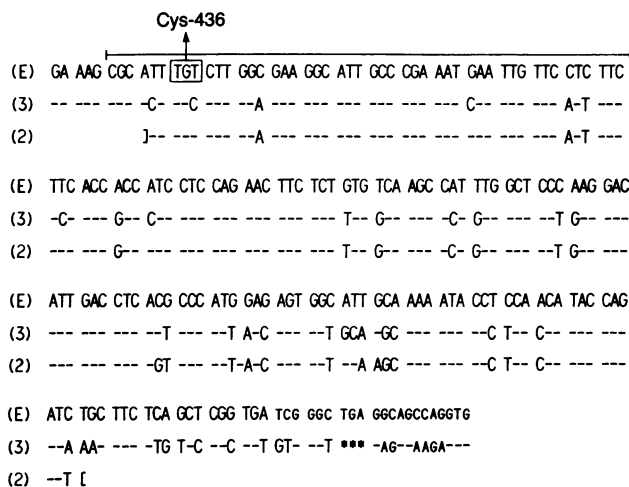


FIG. 4. Conservation of cysteine-containing peptides encoded in exons 9 of the cytochrome P-450b/e-related genes. Genomic bacteriophage clones containing genes 2 or 3 were digested with *HinfI* restriction endonuclease and 500-bp DNA fragments previously detected by hybridization to an exon 9 P-450e gene probe were isolated by polyacrylamide gel electrophoresis. These were then individually subcloned in *Sma* I-cleaved M13 mp8 DNA and the appropriate plaques were identified by plaque hybridization (24). The DNA sequences, obtained from only one strand, were determined two to four times. The top line (E) represents the cytochrome P-450e gene sequence (17, 18), the middle line (3) the gene 3 sequence, and the bottom line (2) the gene 2 sequence. Capital letters represent protein-encoding sequences and small-capital letters represent 3' untranslated sequences. The gene segment encoding the highly conserved cysteine-containing peptide in cytochrome P-450b and P-450e (15) is overlined. The three successive asterisks represent the termination codon in gene 3.

The nearly perfect conservation of the "analogous tridecapeptide" within the proteins encoded by the members of the P-450b/e gene family is particularly striking. This tridecapeptide was recognized (with one amino acid difference) (19) in the ostensibly unrelated rabbit P-450s LM-3b and LM-2 and was subsequently found in rat P-450b and P-450e. It has also recently been observed (with one or two divergent residues) in the protein sequences derived from three distinct rabbit P-450 cDNAs, one of which probably corresponds to LM-3b (25). The sequence of the tridecapeptide is substantially less, albeit significantly, conserved in the major cytochromes P-450 induced by 3-methylcholanthrene, P-450c (26) and P-450d (27) in the rat and P₁-450 (28) and P₃-450 (23, 28) in the mouse. The high conservation within the various P-450 genes of the tridecapeptide sequence strongly supports the notion that this portion of the polypeptide plays an important functional or structural role common to many P-450s.

Several mechanisms can be considered to account for the presence of apparently identical 5' halves within the cytochrome P-450b and P-450e mRNAs. We have shown that neither somatic rearrangement linking one 5' constant region to either of two 3' variable regions (Fig. 3) nor differential splicing of a single transcript containing two variable regions (18) could explain the unusually long stretch of identity. It is attractive to speculate that a very recent unequal crossing-over or gene conversion event is also responsible for this feature of the P-450e and b mRNAs. In this context it is worth noting that gene conversion between the P-450b and e genes has recently been suggested to account for a small number of differences between the sequences of the cloned P-450e gene (18, 20) and various P-450e cDNAs isolated in different laboratories (29).

An examination of the literature suggests that gene conversion may also be responsible for the nonrandom distribution of regions of homology and divergence in other cytochrome P-450 gene subfamilies. One such example is provided by the group of genes corresponding to three rabbit liver cDNAs that encode proteins bearing approximately 50% sequence homology to rabbit P-450 LM2 (25). In the two most related (88% overall homology) cDNA clones there are several regions of extraordinary homology (most notably a segment spanning residues 924-1063 in which there is only one base change) that may be explained as resulting from homogenizing gene conversion events. There is also a short highly divergent segment (14 differences in 35 residues) near the 3' ends (residues 1351-1385) of these two closely related cDNAs. It is reasonable to suggest that this highly divergent region was introduced into one of these genes by conversion from the gene corresponding to the third cDNA clone since the latter shows an overall homology of 72% to the other two cDNAs but in the highly divergent segment differs from one of them in only 3 out of 35 residues. Another example may be provided by the genes for rat liver cytochromes P-450c and P-450d, two immunochemically related isozymes, both markedly induced by 3-methylcholanthrene (30, 31). Although the cDNA coding sequences for these two cytochromes P-450 are 30% divergent, segments 459 bp in length (corresponding to codons 36-188 of P-450c) contain only 5.7% divergent residues (26, 27). Similar, highly homologous segments, with only 2.8% divergence, are present in the mouse P₁-450 (codons 39-191) and P₃-450 cDNAs (23), which correspond to the genes orthologous to the rat P-450c and P-450d genes. We suggest that the presence of these extraordinarily conserved segments reflects the occurrence of a conversion event before the evolutionary divergence of the mouse and rat. The extent of the subsequent divergence of the segments involved in the putative conversion is only slightly less than the overall divergence of the coding sequences of mouse P₁-450 and rat P-450c.

The many examples in which gene conversion can be invoked to explain the existence of regions of high conservation or divergence within related cytochrome P-450s raises the possibility that gene conversion represents a general mechanism that has operated for generating diversity within this superfamily of proteins. Since members of the cytochrome P-450 superfamily of isozymes recognize such a large number of substrates and, in effect, catalyze numerous enzymatic reactions, it would have been advantageous from an evolutionary standpoint for these genes to evolve quickly. Gene conversion may have greatly assisted in this process by allowing genes to procure DNA segments from other distantly related genes and even nonfunctional pseudogenes, thus producing genes encoding proteins with novel functions. It should be noted, however, that genes resulting from initial conversion events can either be fixed in the population or eliminated by natural selection. Indeed, it is very possible that the high frequency with which regions of unusually high sequence conservation or divergence are observed in related cytochrome P-450 genes does not necessarily reflect an unusually high frequency of recombinational conversion events affecting these genes but rather a high probability of their fixation by natural selection (29, 32). On the other hand, the frequency of the recombinational events between P-450 genes may be unusually high, reflecting the presence of regions of homology or other characteristic features of the genes, which promote recombination (4). In this regard, it is possible that the evolutionary pressure to conserve the "analogous tridecapeptide" found in related as well as ostensibly unrelated P-450 isozymes is ultimately responsible for the apparent high frequency of gene conversion in the vicinity of exon 7 of the various P-450b/e gene family members.

Note. A recent report of the structure of the cytochrome P-450b gene (33) shows that its 5' half diverges from that of the P-450e gene, displaying single base changes in exons 1 and 2 and, more strikingly, a 9-kb insertion in intron 2. This directly demonstrates that neither somatic recombination nor unequal crossing-over could account for the apparently identical 5' constant regions in the P-450b and e mRNAs. Furthermore, the absence of even a single base change in the approximately 300 reported residues of intronic DNA flanking exons 2 to 5 supports the hypothesis that the nearly identical 5' halves of the two mRNAs resulted from a conversion event involving a segment extending from a point within intron 2 to a point in intron 5, or perhaps exon 6.

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