Structural Analysis of Cloned cDNAs for Polycyclic Hydrocarbon-Inducible Forms of Rabbit Liver Microsomal Cytochrome P-450

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Two cDNA clones, pHPa1 and pHPa2, encoding polycyclic hydrocarbon-inducible forms of rabbit liver microsomal cytochrome P-450 were isolated and their nucleotide sequences were determined. The inserts of pHPa1 and pHPa2 contained open reading frames specifying the entire primary structures of cytochrome P-450s, consisting of 518 and 516 amino acid residues, respectively. The deduced amino acid sequences for pHPa1 and pHPa2 are 76 and 73% homologous with rat P-450c and P-450d, respectively, and 96% homologous with rabbit P-450 forms 6 and 4, respectively. We conclude that pHPa1 and pHPa2 encode the rabbit counterparts of rat P-450c and P-450d, respectively. A region highly conserved in all species of cytochrome P-450 so far examined, called the HR2 region, can be detected in the pHPa1 and pHPa2 primary structures, but another conserved region, HR1, cannot be observed. Northern hybridization analysis of total RNAs from livers of untreated and drug-treated rabbits demonstrated that the pHPa1 and pHPa2 genes are expressed in untreated animals, induced considerably by administration of 3-methylcholanthrene or β-naphthoflavone, and suppressed by phenobarbital and isosafrole.

Multiple forms of cytochrome P-450 exist in mammalian liver microsomes and play a crucial role in the metabolism of endogenous lipids and a large variety of xenobiotics, and administration of drugs to animals induces the synthesis of a specific form(s) of cytochrome P-450, depending on the drug administered (1). A number of different forms of cytochrome P-450 have so far been purified from liver microsomes of untreated and variously drug-treated animals, and characterized in detail. These studies have led to the conclusion that liver microsomal cytochrome P-450s can be classified into several groups, such as constitutive, phenobarbital (PB)-inducible, and polycyclic hydrocarbon-inducible ones. More recently, cDNAs encoding a number of cytochrome P-450s have been cloned and sequenced. One of the outcomes of these efforts is the finding that two PB-inducible forms of rat liver cytochrome P-450, i.e. P-450b

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Abbreviations: BNF, β-naphthoflavone; bp, base pairs; kb, 1,000 bp; MC, 3-methylcholanthrene; PB, phenobarbital; 1 x SSC, 0.15 M NaCl, 0.015 M sodium citrate, pH 7.5.
and P-450e, are 97% homologous with each other, even though they are encoded by distinct genes (2, 3). Such genetic microheterogeneity has also been reported for PB-inducible forms of rabbit liver cytochrome P-450 (M. Komori, Y. Imai, and R. Sato, submitted). Injection of polycyclic hydrocarbons such as 3-methylcholanthrene (MC) into rats has been shown to lead to co-induction of at least two forms of liver cytochrome P-450, i.e. P-450c and P-450d (4). These two forms are immunologically related, but exhibit different substrate specificities and are encoded by different genes (5). No polycyclic hydrocarbon-inducible forms of cytochrome P-450 other than P-450c and P-450d have been purified from rat liver microsomes, suggesting that the number of genes encoding these forms of cytochrome P-450 is not large. One major form of cytochrome P-450, called P-448!, (6) or P-450LM4 (7), has been purified from liver microsomes of MC- or 3-naphthoflavone (BNF) treated rabbits. This form is similar in spectral properties and substrate specificity to rat P-450d. However, rabbit liver cytochrome P-450 resembling rat P-450c has been purified only in very low yields from liver microsomes of polycyclic hydrocarbon-treated rabbits (8). This paper reports the isolation of two cDNA clones encoding polycyclic hydrocarbon-inducible forms of rabbit liver microsomal cytochrome P-450, and their sequence determinations. It is also reported that these two cDNAs code for the rabbit counterparts of rat P-450c and P-450d. Evidence obtained by Northern hybridization analysis indicates further that the level of mRNA encoding the P-450c-type protein is higher than that of the P-450d-type one in livers of both untreated and polycyclic hydrocarbon-treated rabbits, in contrast to the results of purification studies.

MATERIALS AND METHODS

A cDNA clone encoding rat P-450d, pcP-450mc3, was kindly supplied by Dr. Y. Fujii-Kuriyama of the Cancer Institute, Japanese Foundation for Cancer Research, Tokyo. A homogeneous preparation of rabbit P-448i, a generous gift from Dr. T. Aoyama of this laboratory, was used to raise anti-P-448i antibodies in a guinea pig. The antibodies were purified by chromatography on a protein A-Sepharose 4B column (9). Total RNA was prepared from the liver of an MC-treated rabbit by the guanidine thiocyanate method (10) or from liver microsomes of a BNF-treated rabbit by the phenol-chloroform-isoamyl alcohol method (11). Poly(A)+ RNA was enriched from the total RNA by two cycles of oligo(dT)-cellulose column chromatography and subjected to sucrose density gradient centrifugation (5 to 25% sucrose). Fractions containing P-448, mRNAs were detected by translation in each fraction in a wheat-germ cell-free system (12), followed by immunoprecipitation with anti-P-448, antibodies (13).

A cDNA library (MC-library) was constructed from the enriched mRNA isolated from the liver of an MC-treated rabbit by the method of Okayama and Berg (14) using Escherichia coli strain DH1 as recipient cells. For construction of a second cDNA library (BNF-library), cDNA was synthesized from the mRNA enriched from liver microsomes of a BNF-treated rabbit as described by Gubler and Hoffman (15). The 3'-ends of cDNA were oligo(dC)-tailed and annealed to PstI-cut, oligo(dG)-tailed pBR322. The plasmid cDNA was used to transform E. coli strain HB 101. The cDNA libraries were screened for clones bearing cDNAs encoding polycyclic hydrocarbon-inducible cytochrome P-450s by in situ colony hybridization (16) using as a probe a “HR2”-containing 647-bp PstI/Sau96I fragment excised from pcP-450mc3, a rat P-450d cDNA clone (17). Since the 10 positive clones thus obtained from the MC-library were found to carry only short inserts, the insert of one of the positive clones was used as the secondary probe to screen the same library, resulting in the isolation of pHPah1 and pHPah3. Screening of the BNF-library, on the other hand, yielded pHPah2. Plasmid DNA was isolated for restriction enzyme analysis by the alkaline lysis method (16). Various restriction fragments were subcloned into vectors M13 mp10 and mp11 (or mp18 and mp19) and sequenced by the dideoxy method (18) using [α-35S]dATPαS as a radioactive label, essentially according to the instructions provided by the kit supplier. The optimally matched alignment of a pair of protein sequences was kindly performed by H. Nakajima of Kanazawa University.

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RESULTS AND DISCUSSION

Nucleotide Sequences of cDNA Encoding Polycyclic Hydrocarbon-Inducible Cytochrome P-450s—By using a "HR2"-containing fragment of rat P-450d cDNA as a primary probe, it was possible to isolate three positive clones, termed pHPa1, pHPa2, and pHPa3, from the two cDNA libraries constructed from rabbit liver mRNA. As shown in Fig. 1, the restriction map of the insert of pHPa1 (2.3 kb long) was clearly different from those of pHPa2 and pHPa3 (1.6-1.7 kb long), whereas the maps of the inserts of the latter two clones were apparently identical with each other. The nucleotide sequences of the inserts of these cDNA clones were determined by adopting the strategy shown in Fig. 1. The complete nucleotide sequence of the insert of pHPa1 thus determined is depicted in Fig. 2. This sequence contains an open reading frame (1,554 bp) together with a 96-bp 5'-untranslated region and a 675-bp 3'-noncoding region plus a poly(A) stretch. In the 3'-noncoding region, a poly(A) addition signal, AATAAA, is seen 13 bp upstream from the poly(A) addition site. As shown in Fig. 3, the cDNA insert of pHPa2 consists of a 47-bp 5'-leader sequence, a 1,548-bp open reading frame, and a 11-bp 3'-noncoding sequence. It is certain that this cDNA lacks most of the 3'-trailer sequence including the poly(A) stretch. The insert of pHPa3, on the other hand, contains an incomplete coding sequence (1,230 bp) lacking the region for the NH2-terminal portion of the polypeptide, but does contain a 481-bp 3'-noncoding sequence including the poly(A) sequence. It is to be noted that this noncoding region contains two overlapping poly(A) addition signals, AATAAATAAA, just before the poly(A) addition site. The coding sequence of pHPa3, except for a 38-bp stretch at its 5'-extreme (open box in Fig. 1), is in perfect match with the corresponding region of pHPa2. The 38-bp 5'-terminal sequence shows no homology with any other cDNAs encoding cytochrome P-450s so far sequenced and, therefore, does not seem to code for a portion of the primary structure of a cytochrome P-450. It appears that an artifact was somehow introduced during the cDNA construction. Another possibility is that this cDNA was synthesized with an unspliced mRNA as the template. This latter possibility seems more likely because an AG splicing acceptor site is present at nucleotides 37 and 38 and Okino et al. (19) have reported a similar observation for a cDNA encoding rabbit P-450 form 4. Despite the presence of the 38-bp stretch, it is reasonable to conclude that pHPa2 and pHPa3 were derived from the same mRNA and the 3'-trailer sequence of the pHPa3 insert covers the region missing in pHPa2. In Fig. 3, therefore, the two nucleotide sequences,

![Image of restriction maps and sequencing strategy for cDNAs in pHPa1, pHPa2, and pHPa3. The restriction enzymes used were: P, Pst; E, EcoRI; H, HindIII; S, ScaI; S, Sau3a; Hp, HpaII; N, NarI; B, Ball; BgII; X, XciI; PvuII; and T, TagI. Thick and thin lines denote the coding and noncoding regions, respectively, of the cDNA inserts. The open box in pHPa3 is an unusual sequence (see the legend to Fig. 3). Arrows indicate the direction and extent of sequencing.](http://www.journals.uchicago.edu/journal/jb/article-pdf/101/6/1487/710270/jb101_6_1487.pdf)
Fig. 2. The nucleotide sequence of pHPahl cDNA and the amino acid sequence deduced therefrom. The conserved "HR2" region is underlined.
**STRUCTURAL ANALYSIS OF P-450 cDNAs**

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ATG GCC ATG TCC CCA GCC CTC GCC CTC GTC ATC AGT CTC CTC CTC CTC GCC TTC GGC CTC GTC TGC TGC TTC TGC TGC TTC TGC TGC TTC GTC CTC CTC

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TGC AAC TGC GCC ACT TGC TGC TGC TGC TGC TGC TGC TGC TGC TGC TGC TGC TGC TGC TGC TGC TGC TGC TGC TGC TGC TGC TGC TGC TGC TGC TGC

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CTC GCC CAC CTC CTC TTC GCC TCC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC

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CCA GCC TAC TTC GCC CTC GCC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC

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**Fig. 3.** The composite nucleotide sequence of pHpaH2/ah3 cDNA and the amino acid sequence deduced there-from.

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**Note:** The regions covered by pHpaH2 and pHpaH3 cDNAs are indicated. The nucleotide sequences of these two cDNAs are in perfect match in their overlapping regions, except for the 38-bp 5' -terminal stretch in pHpaH3 cDNA (open box in Fig. 1, tcatgtcctgccctctgtagaagggaggacgctagAGGG...). The "HR2" region is underlined.
except for the 38-bp stretch in pHPah3, are combined and the composite sequence is shown. The total size of pHPah2/ah3 cDNA (2,076 bp) is similar to that of its mRNA estimated by RNA blot hybridization (2.2 kb, see Fig. 4), suggesting that the cDNA in pHPah2 covers most of the 5'-noncoding sequence of the mRNA.

**Deduced Amino Acid Sequences**—The primary structures deduced from the nucleotide sequences of the pHPah1 and pHPah2 (pHPah3) are shown in Figs. 2 and 3, respectively. The pHPah1 polypeptide is composed of 518 amino acid residues (molecular weight, 58,200), whereas the pHPah2 protein consists of 516 residues (molecular weight, 58,300). These two proteins are 73% homologous with each other. As shown in Table I, among the rat cytochrome P-450s examined, P-450c (20) and P-450d (17), both polycyclic hydrocarbon-inducible, exhibit the highest homology with the pHPah1 (76%) and pHPah2 (74%) proteins, respectively, whereas two PB-inducible forms, P-450b (2) and P-450e (3), and a pregnenolone 16-carbonitrile-inducible form, P-450PCN (21), are only 26–29% homologous. It may be concluded that the inducible form, P-450PCN are only 26–29% identical with each other. As shown in Table I, among the rat cytochrome P-450s examined, P-450c (20, 27) and P-450d (17, 28), there is little possibility that the two proteins are the products of different genes, as in the case of rat P-450b and P-450e (2, 3). It is, therefore, highly likely that these two sets of proteins are actually identical with each other and the apparent minor differences were caused by technical errors during sequencing. However, the possibility cannot be ruled out that the differences are due to allelic polymorphism among rabbit strains.

A form of cytochrome P-450, called P-448, purified by us from liver microsomes of MC-treated rabbits is closely similar in molecular, spectral, and catalytic properties to P-450 form 4 (6). Although the primary structure of P-448 is not yet known, it is highly likely that it is identical.

**TABLE I. Homology comparison of the deduced primary structures of the pHPah1 and pHPah2 protein with those of several mammalian cytochrome P-450s.**

<table>
<thead>
<tr>
<th>Source</th>
<th>Form</th>
<th>pHPah1 (%)</th>
<th>pHPah2 (%)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>pHPah1</td>
<td>72.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pHPah2</td>
<td>72.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P-450 form 1</td>
<td>31.2</td>
<td>29.3</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>P-450 form 2</td>
<td>29.4</td>
<td>26.9</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>P-450 form 3b</td>
<td>30.3</td>
<td>26.8</td>
<td>24</td>
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<tr>
<td></td>
<td>P-450 form 4</td>
<td>71.5</td>
<td>96.3</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>P-450 form 6</td>
<td>95.9</td>
<td>66.5</td>
<td>19</td>
</tr>
<tr>
<td>Rat</td>
<td>P-450b</td>
<td>27.5</td>
<td>27.1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>P-450c</td>
<td>76.3</td>
<td>66.3</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>P-450d</td>
<td>63.9</td>
<td>73.8</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>P-450e</td>
<td>27.3</td>
<td>26.8</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>P-450PCN</td>
<td>28.5</td>
<td>21.9</td>
<td>21</td>
</tr>
<tr>
<td>Bovine</td>
<td>P-450c21</td>
<td>26.3</td>
<td>25.4</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>P-450c21</td>
<td>27.4</td>
<td>23.5</td>
<td>32</td>
</tr>
</tbody>
</table>

* The available portion of this primary structure was compared.
with the pHPaH2 protein. As mentioned above, the pHPaH1 protein can be regarded as the rabbit counterpart of rat P-450c. Three closely similar forms of cytochrome P-450, i.e. P-448 (8), P-450LM6 (7), and P-450 form 6 (26), have been purified from liver microsomes of untreated, isosafrole-treated, and 2,3,7,8-tetrachlorodibenzo-p-dioxin-treated rabbits, respectively, and shown to resemble rat P-450c in many respects. Since the deduced primary structure of P-450 form 6 is practically identical with that of the pHPaH1 protein, it is likely that P-448 and P-450LM6 are also identical with the pHPaH1 protein.

Gotoh et al. (29) have reported that all species of cytochrome P-450 possess at least two highly conserved amino acid sequences, called “HR1” and “HR2,” which are located in the NH2-terminal half and near the COOH-terminus, respectively. A region assignable as “HR2” is actually detectable in both the pHPaH1 (residues 454-473) and pHPaH2 (residues 451-470) sequences. These regions contain the conserved cysteinyl residue (residue 461 and 458 in pHPaH1 and pHPaH2, respectively) that is thought to act as the fifth ligand to the heme iron (29). We could not, however, pinpoint any region qualified as “HR1” in the two sequences deduced in this study. Although the implication of the absence of the “HR1” region remains to be explored, it may be said that this region is not required for the functions of all microsomal cytochrome P-450s.

Expression of pHPaH1 and pHPaH2 Genes in Rabbit Liver—To study the drug inducibility of the expression of the pHPaH1 and pHPaH2 genes, total RNA was prepared from livers of untreated and variously drug-treated rabbits and examined by Northern blot analysis. A fragment of pHPaH1 cDNA covering most of the 3’-noncoding region and a pHPaH3 fragment corresponding to about 40% of the coding region and part of the 3’-non-coding sequence were used as probes. When total RNA from MC-treated rabbit liver was examined, the pHPaH1 probe hybridized with mRNA of about 2.4 kb (Fig. 4, lane a), whereas the pHPaH3 probe detected mRNA of about 2.2 kb (Fig. 4, lane c), indicating that the mRNAs transcribed from the two genes could be detected separately under the stringent conditions employed and that each of the two probes hybridized only with mRNA having a single size. As shown in Fig. 5A, under the same stringent conditions the pHPaH1 probe hybridized most strongly with the total RNA from the MC-treated rabbit, followed by that from the BNF-treated animal. The RNA from the untreated animal also gave a fairly strong signal, but the signals were very weak when the RNAs from PB- and isosafrole-treated rabbits were examined. It was, therefore, concluded that the pHPaH1 gene is expressed in the liver of untreated rabbits.

Fig. 4. Northern blot analysis of total RNA from MC-induced rabbit liver. Total RNA (20 $\mu$g) was subjected to electrophoresis on a 1.0% agarose gel containing 1.1 M formaldehyde and transferred to a nitrocellulose filter as described (16). Hybridization was performed overnight at 42°C in the presence of 45% formamide. The filters were washed twice with 0.2 x SSC containing 0.5% sodium dodecyl sulfate at 68°C for 60 min. The probes used were a 630-bp Xcy/PvuII fragment of pHPaH1 cDNA (nucleotides 1664-2293) and a 1,105-bp NarI/BglII fragment of pHPaH3 cDNA (nucleotides 537-1641). The probes were 32P-labeled by nick translation. Since the hybridization signal obtained with the pHPaH1 probe was much stronger than that obtained with the pHPaH3 probe, the radioactivity of the pHPaH1 probe (2 x 107 cpm) was reduced to one-tenth that of the pHPaH3 probe (2 x 108 cpm). The sizes of mRNAs were estimated by comparing their mobilities with those of HindIII fragments of phage DNA. Lane a, hybridization with the pHPaH1 probe; lane b, hybridization with a mixture of the two probes; lane c, hybridization with the pHPaH3 probe.
A salient finding was that, in all five types of liver examined, the hybridization signals observed with the pHPlh3 probe were considerably weaker than those obtained with the pHPlh1 probe, indicating that the levels of mRNA coding for the pHPlh2 protein were notably lower than those of mRNA for the pHPlh1 protein in all cases. These results are apparently inconsistent with the experience obtained in protein purification studies. As reported by Imai et al. (6), and Haugen and Coon (7), a form of cytochrome P-450, called P-448, or P-450 form 4, can be purified in the largest yield from liver microsomes of MC- or BNF-treated rabbits and, therefore, is thought to be the major polycyclic hydrocarbon-inducible form. As discussed above, P-448, or P-450 form 4 is thought to be identical with the pHPlh2 protein. On the other hand, P-448, which is apparently identical with the pHPlh1 protein, has not yet been purified from the same source, though it has been isolated from liver microsomes of untreated rabbits in an extremely low yield (8). Nevertheless, the RNA blot analysis indicates that in the polycyclic hydrocarbon-treated livers the pHPlh1 mRNA is present at considerably higher concentrations than the pHPlh2 mRNA. Although no satisfactory explanation is available for this discrepancy, it is conceivable that the translation efficiency in vivo of the pHPlh2 mRNA is much higher than that of the pHPlh1 mRNA for unknown reasons. It is also likely that P-448 (pHPlh1 protein) is degraded in vivo at a much higher rate than P-448 (pHPlh2 protein). Another apparent contradiction is that no measurable amount of the pHPlh2 mRNA was detectable in the liver of PB-treated rabbits, whereas P-448, or P-450 form 4 has been purified from liver microsomes of rabbits treated with the same drug in substantial yields (6, 7). This discrepancy may also be explained by the above possibilities. In any case, much further work is required to resolve these apparent inconsistencies.

The authors wish to thank Dr. Y. Fujii-Kuriyama for providing a P-450d cDNA clone, pcP-450mc3. We also thank Dr. T. Aoyama, for a generous gift of purified P-448, Dr. Y. Imai and Dr. M. Komori for useful discussions, and Mr. H. Nakajima for performing the homology search.

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