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cDNA Cloning of a Novel *CYP*3A from Rat Brain[1]

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One full length cDNA clone, designated 3aH15, was isolated from a control male rat brain cDNA library. 3aH15 encoded a protein composed of 503 amino acid residues. The deduced amino acid sequence of 3aH15 was 92% identical to *Cyp*3a-13 and had a 68.4% to 76.5% homology with the other reported *CYP*3A sequences. Clone 3aH15 was thus named *CYP*3A9. No significant induction of the *CYP*3A9 expression in rat brain by dexamethasone was observed by Northern blot analysis. *CYP*3A9 cDNA was expressed in *E. coli* and the expressed P450 3A9 is active in the demethylation of erythromycin as well as benzphetamine.

The Cytochrome P450 superfamily consists of heme-containing proteins which are found at high levels in the liver. P450s belonging to the *CYP*3A subfamily have been shown to be involved in the metabolism of a wide variety of important steroid hormones, drugs, and carcinogens (1–7). So far, four cDNAs of the *CYP*3A subfamily, *CYP*3A1, *CYP*3A2, *CYP*3A18, a cDNA named cDEX; and a *CYP*3A1 allelic variant have been identified in rat liver (8–12). The genomic clone of *CYP*3A2 was also characterized (13). These sequence data indicate the existence of multiple *CYP*3A genes in the rat.

Since many neuroactive drugs are substrates of the P450 monooxygenase system, we are interested in P450-mediated metabolism in brain, the target organ of these drugs. Several lines of evidence, including P450 isoform identification studies using PCR techniques (14–16), cDNA cloning (17–19), and activity studies using brain microsomal systems (20–22), illustrate the presence and function of multiple forms of cytochrome P450 in brain. Recently, in our laboratory, a new cDNA clone belonging to the *CYP*2D subfamily and three new forms of *CYP*4F cDNA (18, 19) were isolated from a rat brain cDNA library, illustrating the brain specific P450 monooxygenase system.

In 1987, Sugita *et al.* (24) reported that partially purified rat brain P450 was active in estradiol hydroxylation. This report of estradiol hydroxylation suggests the existence of functionally active P450 3A isozymes in rat brain. P450 3A has been shown to be one of the major enzymes catalyzing the major pathway of imipramine metabolism, N-demethylation in human liver (7). Recently, Sequeria and Strobel demonstrated that brain microsomes were capable of metabolizing imipramine to both hydroxylated and N-demethylated products and N-demethylation of imipramine could be greatly inhibited by ketoconazole, a selective inhibitor of P450 3A (21, 22, 25). The evidence provided by these studies suggests that the existence and function of the P450 3A subfamily in the metabolism of the tricyclic antidepressants in the brain. Therefore, we screened a cDNA library of rat brain using a PCR-generated cDNA fragment of *CYP*3A2 as a probe, resulting in the isolation of a novel *CYP*3A clone whose deduced amino acid sequence shows only 68.4% to 72.6% homology with all the other reported rat *CYP*3A sequences. Furthermore we have expressed in *E. coli* the *CYP*3A9[2] protein which shows catalytic activity in the demethylation of erythromycin as well as benzphetamine.

[1] The nucleotide sequence in this paper has been submitted to GenBank under Accession No. U46118.

[2] The deduced amino acid sequence of 3aH15 was identical to *CYP*3A9 except one amino acid difference at position 457 (Val→Phe in *CYP*3A9). The *CYP*3A9 sequence was submitted to the P450 nomenclature committee (23) as a personal communication by Dr. Patrick Nef, but not otherwise reported.

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MATERIALS AND METHODS

Materials. [α^{32}P]dCTP (3000 Ci/mmol) was purchased from ICN (Irvine, CA); pure nitrocellulose membranes and Nytran filters, from Schleicher and Schuell (Keene, NH); Dexamethasone 21-phosphate, from Sigma (St. Louis, MO). All the restriction enzymes were purchased from Stratagene or Promega (Madison, WI).

Treatment of animals and RNA isolation. Male Sprague-Dawley rats (Harlan Labs, Houston, TX), weighing 200 to 225 g were used. Animals received daily intraperitoneal injections of 200 mg/kg body weight dexamethasone 21-phosphate in saline for three days and were sacrificed by decapitation after an overnight fast. Brains and livers were removed and immediately frozen in liquid nitrogen. Total RNA and poly(A)$^+$ RNA was isolated.

Preparation of probes. RT-PCR was performed with total RNA isolated from untreated male liver using the primers and methods described previously (15, 16). The PCR product was subcloned into the pCR II vector (Invitrogen) and sequenced. The plasmid DNA was digested with *Eco*RI and the 592 bp *Eco*RI fragment of *CYP*3A2 cDNA (nucleotides 177 to 768) was used as a probe for the first round of screening. The second round of screening was carried out using an *Apa*I fragment of clone 3aH2 (1702 bp) as a probe.

cDNA cloning and DNA sequence analysis. The cDNA library from untreated male rat brain was screened by the plaque hybridization method. Deletion clones were prepared and sequenced as described previously (18, 19). All sequencings were performed in both directions.

Northern blot analysis. The poly(A)$^+$ RNA was denatured, electrophoresed and transferred onto a Nytran filter. The filter was hybridized with the ^{32}P labeled *Not*I-*Nco*I fragment (nucleotides 1 to 302) of 3aH15 and washed twice in 0.2 × SSC and 0.1% SDS at 65°C for 30 min.

Expression of CYP*3A9 cDNA in E. coli and reconstitution assays. CYP*3A9 cDNA was expressed in *E. coli* DH5α cells using N-terminal modification and the pCWOri+ expression vector (a generous gift from Dr. M.R. Waterman) (26, 27). The membrane fractions were prepared as described previously (5) and solubilized with Renex-690. Reconstitution assays were performed using 100 pmol P450 in a final volume of 300 μl with 100 mM Tris buffer (pH 8.0), saturating NADPH-P450 reductase and cytochrome b_5, 0.15 mM L-α-phosphatidyl choline dilauroyl, 1 mM NADPH and 0.4 mM erythromycin or 1 mM benzphetamine HCl. The reaction was initiated by the addition of NADPH and incubated at 37°C for 30 min. The colorimetric measurement of HCHO formation was carried out as described previously (28).

RESULTS

First, a partial clone, designated 3aH2, was isolated and completely sequenced after screening approximately 500,000 clones from our control rat brain library using a PCR-generated cDNA fragment of *CYP*3A2 as a probe. In order to get the full length cDNA, an *Apa*I fragment (1702 bp) of 3aH2 was used as a probe for the second round of screening. By screening approximately 1,000,000 clones, 26 positive clones were obtained after tertiary screening. Among these, one full length cDNA clone was found and designated 3aH15. The nucleotide and deduced amino acid sequence of 3aH15 is shown in Figure 1A. Clone 3aH15 contained 2119 nucleotides, including 279 bp 5′-untranslated and 309 bp 3′-untranslated sequences and the poly (A) tail which was composed of 19 adenines. The open reading frame of 3aH15 spanning nucleotides 280 to 1791 encoded a protein composed of 503 amino acid residues. The HR2 region which contains a conserved cysteine residue was also found in the deduced amino acid sequence.

The nucleotide sequence of 3aH15 showed 71.3% homology with *CYP*3A1(8) and cDEX(11), 70.3% homology with *CYP*3A2(9) and *CYP*3A18(10), but 91.6% homology with mouse *Cyp*3a-13(29). Interestingly the high sequence homology between 3aH15 and *Cyp*3a-13 extended to both the 5′ and 3′ untranslated region. The deduced amino acid sequence of 3aH15 showed 92% identity with mouse *Cyp*3a-13. With the exception of *Cyp*3a-13, the amino acid sequence identities between 3aH15 and other reported mammalian *CYP*3A sequences ranged from 68.4% to 76.5% (Table 1). When we reported the 3aH15 sequence to Dr. D.R. Nelson who collects cytochrome P450 sequences for P450 nomenclature, we were informed that a sequence matching our clone (with the exception noted above) had been received as a personal communication from Dr. Patrick Nef in September 1990 and assigned the name *CYP*3A9. However, the sequence of *CYP*3A9 has not been published nor released from any database. Therefore, this is the first publication concerning the cDNA cloning of *CYP*3A9.

Clone 3aH2 was 2198 bp in length including 18 adenines in the poly (A) tail. The sequence between nucleotides 446 and 2180 of 3aH2, which contained a region coding for amino acids 25

A

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AGCAGCCTTATATGTATCAAGATGCATTTTATTAAGTCTTCATGGGTATCTGGGGAAGAATAAATTGTTTGTTTGGCCTGAGCACCAATT   90
AGTGTCCACATCTCTGAGAAAGAACATGGATCTACAACTATCCGTGAAAGCCCAATGCATGGGGGCAACATCAATTAGTACTCGTGCCGA  180
ATTCGGCACGAGGGGAGCTCAGCACGTTCAGCCCTGCAAAGGGCAGTACACAAAATTGAGAGTAAAGCTCGAAGAGAGACTTGTTTAAAG  270
                                                                          ▼
AAAACGGCAATGGATTTGATCCCAAACTTTTCCATGGAAACCTGGCTGCTCCTGGTTATCAGCCTGGTGCTCCTCTACCTATATGGAACT  360
          M  D  L  I  P  N  F  S  M  E  T  W  L  L  L  V  I  S  L  V  L  L  Y  L  Y  G  T   27

CATTCACATGGAATTTTTAAAAAGTTGGGAATTCCTGGGCCCAAACCTTTGCCTTTCTTGGGGACGATTCTTGCTTACAGGAAGGGCTTC  450
H  S  H  G  I  F  K  K  L  G  I  P  G  P  K  P  L  P  F  L  G  T  I  L  A  Y  R  K  G  F   57

TGGGAATTTGACAAATACTGCCATAAAAAATATGGGAAATTATGGGGGTTGTACGATGGTCGACAGCCTGTGCTAGCGATCACGGATCCA  540
W  E  F  D  K  Y  C  H  K  K  Y  G  K  L  W  G  L  Y  D  G  R  Q  P  V  L  A  I  T  D  P   87

GACATAATCAAAACAGTGCTGGTGAAGGAATGTTACTCTACCTTCACAAACCGACGGAACTTTGGTCCAGTGGGTATTTTGAAAAAAGCC  630
D  I  I  K  T  V  L  V  K  E  C  Y  S  T  F  T  N  R  R  N  F  G  P  V  G  I  L  K  K  A  117

ATCTCCATCTCTGAGGATGAAGAATGGAAGAGAATTCGAGCCCTGCTGTCTCCAACCTTCACCAGTGGGAAGCTCAAGGAGATGTTCCCC  720
I  S  I  S  E  D  E  E  W  K  R  I  R  A  L  L  S  P  T  F  T  S  G  K  L  K  E  M  F  P   147

ATCATTAACCAGTATACAGATATGTTGGTGAGAAACATGAGGCAGGGATCGGAGGAAGGCAAGCCCACCAGCATGAAAGACATCTTTGGG  810
I  I  N  Q  Y  T  D  M  L  V  R  N  M  R  Q  G  S  E  E  G  K  P  T  S  M  K  D  I  F  G   177

GCCTACAGCATGGATGTGATCACAGCCACCTCATTTGGAGTGAATGTTGATTCCCTTAACAACCCACAGGACCCTTTTGTGGAAAAAGTC  900
A  Y  S  M  D  V  I  T  A  T  S  F  G  V  N  V  D  S  L  N  N  P  Q  D  P  F  V  E  K  V   207

AAGAAGCTCTTAAAATTTGATATCTTTGATCCATTGTTCCTCTCAGTGACACTTTTTCCATTCCTTACCCCACTATTTGAAGCACTAAAT  990
K  K  L  L  K  F  D  I  F  D  P  L  F  L  S  V  T  L  F  P  F  L  T  P  L  F  E  A  L  N   237

GTCTCCATGTTTCCAAGAGATGTCATTGACTTTTTTAAAACTTCAGTAGAACGAATGAAAGAGAATCGCATGAAAGAGAAAGAAAAGCAA 1080
V  S  M  F  P  R  D  V  I  D  F  F  K  T  S  V  E  R  M  K  E  N  R  M  K  E  K  E  K  Q   267

AGAATGGACTTTCTTCAGCTGATGATAAACTCCCAGAATTCCAAAGTCAAAGACTCTCATAAAGCATTATCCGATGTGGAGATTGTGGCC 1170
R  M  D  F  L  Q  L  M  I  N  S  Q  N  S  K  V  K  D  S  H  K  A  L  S  D  V  E  I  V  A   297

CAGTCAGTTATCTTCATTTTTGCCGGCTATGAGACCACTAGCAGTGCTCTTTCCTTTGTTTTGTATTTGCTGGCCATTCACCCTGATATA 1260
Q  S  V  I  F  I  F  A  G  Y  E  T  T  S  S  A  L  S  F  V  L  Y  L  L  A  I  H  P  D  I   327

CAGAAGAAACTGCAGGATGAAATTGATGCAGCTCTCCCCAATAAGGCACATGCCACCTATGATACCCTGCTACAAATGGAGTATCTAGAT 1350
Q  K  K  L  Q  D  E  I  D  A  A  L  P  N  K  A  H  A  T  Y  D  T  L  L  Q  M  E  Y  L  D   357

ATGGTGGTGAATGAAACCCTCAGATTATATCCAATTGCTGGAAGGCTTGAGAGGGTCTGTAAGACAGATGTTGAAATCAATGGGGTGTTC 1440
M  V  V  N  E  T  L  R  L  Y  P  I  A  G  R  L  E  R  V  C  K  T  D  V  E  I  N  G  V  F   387

ATTCCCAAAGGGACTGTGGTGATGATACCAACCTTTGCTCTTCACAAAGACCCGCATTACTGGCCAGAGCCTGAGGAATTCCGCCCTGAA 1530
I  P  K  G  T  V  V  M  I  P  T  F  A  L  H  K  D  P  H  Y  W  P  E  P  E  E  F  R  P  E   417

AGGTTCAGCAAGAAGAATCAGGATAACATCAATCCTTATATGTACCTGCCCTTTGGGAATGGACCCAGGAACTGTATTGGCATGAGGTTT 1620
R  F  S  K  K  N  Q  D  N  I  N  P  Y  M  Y  L  P  F  G  N  G  P  R  N  C  I  G  M  R  F   447

GCTCTCATGAACATGAAAGTTGCTCTTGTCAGAGTCCTGCAGAACTTCTCCTTCCAACCTTGTAAGGAAACTCAGATCCCTTTAAAATTG 1710
A  L  M  N  M  K  V  A  L  V  R  V  L  Q  N  F  S  F  Q  P  C  K  E  T  Q  I  P  L  K  L   477

AGCAAACAAGGACTTCTTCAACCAGAAAAGCCACTGCTTCTAAAAGTTGTGTCCAGAGATGAGACTGTGAATGGAGCTTGATGTAGAATG 1800
S  K  Q  G  L  L  Q  P  E  K  P  L  L  L  K  V  V  S  R  D  E  T  V  N  G  A  *            503

GCCTATAGTGGTGTTGTATCACATGGACCAGAAACCAGTTCATATTGTGAATGAATCCAGAGATGAAGATTGCAGTAATTTTGTTCACTC 1890
CTGGGGGTTTTGGGTGTTCTTTGAGCTTTCTCAGCCTCTGTGCAAGTTGTTTTCTGTGGAACATAAGGATCTAATGACATAAATCCAAGA 1980
TAAATATATTCAACTCGTGGTTCTTGTGGGATGGCCTATGTTACCTTCATTGTTACCACCAAATACTCTGATTTCTGATGAGAAAATAAA 2070
TAAACATCAATTACTAGTTCTATCACCATTAAAAAAAAAAAAAAAAAAA
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B

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CCTTTTGGCTCCTTTCCTCTGTGGTTGATGCTTCTGCTTGGAGTTGCCAGGATGAAATTGGATATTGGTTCTAGAAGACACATATGGGAT  90
CTGCTGATGGGGGGCGTCACAGTTGCACAAAGGAGGCCCAAATTTCTAGATCTCCTGGAGCTGGAGTTACTTGTCACCTGTGGAGAGTGCT 180
GGAAACTGAATTCTAATTTTCTTCAAGGACAGAAAACATGCCCTGTGTAGTCGCCCATGTCTTTCATCCCAGCACTTGGGAGGCAGAGGA 270
ATACAGGAAAGGATGTGTCAACAATCCTGTGCAGCCATACCTGATGTAGGAGAACAGCAGATAAGACATTTCCAGAGGCGTTAAGATTGA 360
CACATGAAGAAACCAATGATAGAAGGATGGAACCCCCTCATTCTTAGTTGCTGCCCAACTACTGTGAAGAGACACCATGGCCAAG      445
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FIG. 1. (A)Nucleotide and deduced amino acid sequences of 3aH15. The numbers on the right indicate the positions of nucleotides and amino acids. The HR2 region is marked by the underlined amino acid residues. ▼ and ▲ indicate the starting and ending nucleotides of the region which has identical nucleotide sequence to 3aH2. The 15 bp insertion at the 3′ end is double underlined. The *Apa*I site used for making the probe for second round screening is indicated by the dashed line. (B) 445 bp unknown sequence at the 5′ end of 3aH2.

to 503 of *CYP*3A9 and the 294 bp 3′-untranslated sequence, is exactly the same as that in 3aH15 (nucleotides 351 to 2085), but upstream of that region was a 445 bp unknown sequence which showed no significant homology with any of the sequences recorded in GenEMBL. 3aH2 cDNA was not expressible when measured by an *in vitro* translation assay using the TNT3 Coupled Reticulocyte Lysate Systems (Promega) (data not shown). The 5′ unknown sequence of 3aH2 is

TABLE 1
Amino Acid Sequence Identities between *CYP*3A9 and Other Members of *CYP*3A Subfamily

cDNAs	Species	Sequence identities (%)	Reference
*CYP*3A1	rat	72.6	8
*CYP*3A2	rat	72.0	9
*CYP*3A3	human	75.4	30
*CYP*3A4	human	76.5	31, 32
*CYP*3A5	human	75.1	33
*CYP*3A6	rabbit	74.4	34, 35
*CYP*3A7	human	73.0	36
*CYP*3A8	monkey	75.3	37
*CYP*3A10	hamster	68.6	38
*Cyp*3a-11	mouse	73.8	39
*CYP*3A12	dog	76.1	40
*Cyp*3a-13	mouse	92.0	29
*Cyp*3a-16	mouse	69.2	41
*CYP*A18	rat	68.4	10
cDEX	rat	72.6	11

shown in Figure 1B. Another interesting difference between 3aH2 and 3aH15 is that 3aH15 contains a 15 bp insertion at the 3′ end.

The results of Northern blot analysis using a 302 bp *Not*I-*Nco*I fragment of 3aH15 as a probe, which is specific to mature *CYP*3A9 mRNA, is shown in Figure 2A. Two bands were detected in both control and dexamethasone treated rat brains. The lower band which was also detected in dexamethasone treated rat liver (data not shown) corresponds in size to the mRNA of *CYP*3A9. The upper band might be caused by cross hybridization of the probe with another mRNA related to this novel P450. No significant change was observed in the expression level of *CYP*3A9 in the brain after dexamethasone treatment.

*CYP*3A9 was expressed in *E. coli* DH5α cells. The expressed P450 3A9 protein was detected in the *E. coli* membrane fractions by a protein immunoblot using anti-rat *CYP*3A2 serum (Daiichi Pure Chemical Co., LTD.) (data not shown) and able to catalyze the demethylation of erythromycin as well as benzphetamine. The catalytic activities of expressed P450 3A9 towards the demethylation of erythromycin and benzphetamine were 84.7 and 57.2 pmole/min/nmole P450, respectively.

DISCUSSION

The full length *CYP*3A9 cDNA clone was isolated from a control rat brain cDNA library. The deduced amino acid sequence of *CYP*3A9 showed 68.4% to 72.6% homology with all the reported

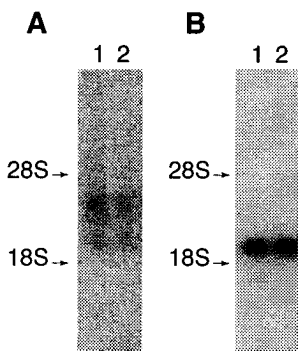

FIG. 2. Northern blot analysis of the poly $(A)^+$ RNA (0.5 μg) from rat brain. (A) *Not*I–*Nco*I fragment of 3aH15 cDNA as a probe (7 days exposure). (B) 2.0 kb human β-actin cDNA as a probe (12 hours exposure). Lane 1: mRNA from control male rat brains; Lane 2: mRNA from dexamethasone treated male rat brain.

rat *CYP*3A sequences. Interestingly a 92% amino acid sequence identity was observed between *CYP*3A9 and *Cyp*3a-13 which was cloned from mouse liver by Yanagimoto *et al.* (29). The nucleotide sequence of *CYP*3A9 also showed high homology (91.6%) with *Cyp*3a-13 and the homology extended to both the 5′- and 3′-untranslated regions. The strong similarity between *CYP*3A9 and *Cyp*3a-13 may suggest that *CYP*3A9 comes from a counterpart gene of mouse *Cyp*3a-13.

With the exception of a 455 bp unknown sequence at the 5′ end and the poly (A) tail, the rest of the 1735 bp nucleotide sequence of 3aH2 is 100% identical to nucleotides 351 to 2085 of *CYP*3A9. This suggests that 3aH2 and *CYP*3A9 come from the same gene. The 15 bp insertion contained in *CYP*3A9 at the 3′ end may also suggest that possibly two *CYP*3A9 mRNA species are expressed in rat brain. P450 mRNA species with different lengths of 3′ untranslated regions have been reported in rabbit liver by Dalet *et al.* (34) and in rat brain by Kawashima *et al.* (18). One possible explanation for the 445 bp unknown sequence at the 5′ end of 3aH2 may be that alternative splicing or unfinished mRNA processing produced an immature mRNA of *CYP*3A9 which contained some intron sequence in the middle of the mRNA sequence and the reverse transcription reaction did not reach the 5′ end when making the cDNA library. Consistent with this explanation was the fact that the 445 bp sequence ended with AG. This follows the GT/AG rule at splice junctions. Whether mRNA splicing or processing is involved in the regulation of *CYP*3A9 expression in brain will require further studies.

The inducibility of *CYP*3A mRNA in the liver by dexamethasone is a well established phenomenon (9–11, 29, 39). However, no significant induction of *CYP*3A9 mRNA by dexamethasone was observed in rat brain by Northern blot analysis. This was consistent with the report by Jayyosi *et al.* (20) which showed that the metabolism of testosterone by rat brain microsomes was not inducible by the known *CYP*3A inducer, pregnenolone-16α-carbonitrile.

Using preliminary catalytic studies of the solubilized *E. coli* membrane fraction, we can show that the expressed P450 3A9 is able to catalyze the demethylation of erythromycin as well as benzphetamine, which are reported as the catalytic activities of *CYP*3A (3, 42). *Cyp*3a-13 was reported to be involved in the activation of aflatoxin B_1 in *in vitro* studies (29), but the substrate specificity and the functions of *Cyp*3a-13 are still not clear. According to the results of *in vitro* studies of imipramine metabolism by brain microsomes in this laboratory (21, 22), it's quite possible that CYP3A9 may be involved in the *in situ* metabolism of imipramine in the brain. More careful catalytic studies, especially of the metabolism of imipramine, are planned using the purified P450 3A9 protein expressed in *E. coli* to characterize the P450 3A9 protein function further.

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