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A Frameshift Mutation and Alternate Splicing in Human Brain Generate a Functional Form of the Pseudogene Cytochrome P4502D7 That Demethylates Codeine to Morphine*

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A frameshift mutation 138delT generates an open reading frame in the pseudogene, cytochrome P4502D7 (*CYP2D7*), and an alternate spliced functional transcript of *CYP2D7* containing partial inclusion of intron 6 was identified in human brain but not in liver or kidney from the same individual. mRNA and protein of the brain variant *CYP2D7* were detected in 6 of 12 human autopsy brains. Genotyping revealed the presence of the frameshift mutation 138delT only in those human subjects who expressed the brain variant *CYP2D7*. Genomic DNA analysis in normal volunteers revealed the presence of functional *CYP2D7* in 4 of 8 individuals. In liver, the major organ involved in drug metabolism, a minor metabolic pathway mediated by CYP2D6 metabolizes codeine (pro-drug) to morphine (active drug), whereas norcodeine is the major metabolite. In contrast, when expressed in Neuro2a cells, brain variant CYP2D7 metabolized codeine to morphine with greater efficiency compared with the corresponding activity in cells expressing CYP2D6. Morphine binds to μ-opioid receptors in certain regions of the central nervous system, such as periaqueductal gray, and produces pain relief. The brain variant CYP2D7 and μ-opioid receptor colocalize in neurons of the periaqueductal gray area in human brain, indicating that metabolism of codeine to morphine could occur at the site of opioid action. Histiospecific isoforms of P450 generated by alternate splicing, which mediate selective metabolism of pro-drugs within tissues, particularly the brain, to generate active drugs may play an important role in drug action and provide newer insights into the genetics of metabolism.

Cytochrome P450 (EC 1.14.14.1; P450)[1] and associated monooxygenases, a family of heme proteins, are the principal class of drug-metabolizing enzymes. A supergene family encodes them, and the member proteins exist as multiple forms having distinct yet overlapping substrate specificities. Multiple forms of P450, which are selectively induced or inhibited by a variety of drugs, are known to exist in liver, the major organ involved in P450-mediated metabolism (1). However, the potential to generate active metabolite(s) at the site of action has generated interest in extrahepatic P450. This has prompted extensive investigations into the xenobiotic metabolizing capability of extrahepatic organs, such as lung, kidney, skin, and nasal epithelium, and the far reaching consequences of such metabolism, *in situ*, within specific cells in target organs have been recognized in laboratory animals (2) and humans (3). The preferential localization of drug-metabolizing enzymes within specific cell types in these organs renders such cells significantly capable of metabolizing drugs (4). Thus, even minor metabolic pathways of xenobiotic metabolism can produce major effects if they take place at the site of action. These observations have prompted investigation into P450-associated monooxygenases in brain with an effort to determine the capability of the brain to metabolize psychoactive drugs (5, 6). P450-mediated metabolism of psychoactive drugs directly in the brain can lead to local pharmacological modulation at the site of action and result in variable drug response.

The interindividual variability in hepatic metabolism of drugs caused by genetic polymorphism exhibited by some forms of P450 such as P4502D6 are reflected in the plasma levels of administered drugs. But plasma drug levels often show poor correlation with therapeutic effect (7), suggesting that metabolism within the brain could influence the therapeutic outcome regardless of hepatic clearance and plasma drug levels. A moderate difference in the pharmacokinetics of psychoactive drugs often leads to dramatic pharmacodynamic effects, again suggesting that metabolism *in situ* within the brain could play a significant role (8).

Over the past decade studies from our laboratory and others have demonstrated the presence of a competent microsomal P450 system in the rodent (2, 9, 10) and human (3, 11) brain and its ability to metabolize a variety of xenobiotics. The appearance of multiple forms of P450 in brain and their selective inducibility by a variety of drugs and xenobiotics have also been identified (9, 12–14). *CYP2D* is one of the major forms of P450 present in both rat (15) and human brain (16). Significant differences are seen in the regulation and function of the isoforms of brain P450 compared with the hepatic forms (17–19). For example, drugs such as alprazolam are metabolized differently in liver and brain wherein a relatively larger amount of the active metabolite is generated in the brain compared with liver (19). These observations have indicated the possible existence of unique P450 isoforms in brain that are different from

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY220845.

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[1] The individual human cytochrome P450 gene is represented as *CYP* followed by an Arabic number denoting family and an alphabetical letter designating subfamily (*CYP2D*). An Arabic number is used to represent genes within a subfamily (*e.g. CYP2D6*). Cytochrome P450 protein is represented with a prefix P450 (P4502D6) in a similar manner.

the well characterized hepatic P450s. We report here the presence of a unique form of *CYP2D7* that is generated by alternate splicing in human brain but not in liver or kidney.

EXPERIMENTAL PROCEDURES

Human brain was obtained from Human Brain Tissue Repository, NIMHANS, India. The tissue was collected at autopsy from male and female traffic accident victims with no known neurological or psychiatric disorders. The average age of the individuals was 35.4 years (3–70 years), and postmortem delay between death and autopsy was 7.3 ± 3.7 h. A region representing exon 6–9 of *CYP2D6* (333 bp) was amplified by RT[2]-PCR with forward and reverse primers 5′-TGATGAGAACCTGCGCATAG-3′ and 3′-ACCGATGACAGGTTGGTGAT-5′, respectively, using human cortex mRNA and used for screening a human cortex cDNA library constructed in our laboratory using Lambda ZAPII library construction kit (Stratagene, La Jolla). Three positive clones of ~1.2, 1.5, and 1.8 kb were isolated and sequenced. The 1.8-kb clone referred to as brain variant *CYP2D7* (GenBank accession number AY220845) containing an open reading frame was used for further investigations.

Expression of Brain Variant CYP2D7—In vitro translation was carried out using a TNT T3-coupled reticulocyte system (Promega, Madison, WI) and brain variant *CYP2D7* cDNA. The translated products were examined by immunoblot using antibody to CYP2D6 (Gentest, San Jose, CA) (19). Luciferase cDNA provided by the manufacturer was used as a positive control. The cDNA of brain variant *CYP2D7* was subcloned into pcDNA3.1 (+/−), sequence verified, and transfected into Neuro2a cells using FuGENE 6 (Roche Applied Science). Positive clones were selected using neomycin. Cells were harvested, and a membrane preparation containing both mitochondria and microsomes was prepared as described earlier (16). Neuro2a cells were also grown in chamber slides and processed for immunohistochemistry using antibody to CYP2D6. Immunostaining was visualized by incubation with biotinylated secondary antibody followed by streptavidin-fluorescein. cDNA to brain variant *CYP2D7* ligated in the reverse orientation was used as a control in transfection experiments.

RT-PCR and Genomic DNA Amplification—RT-PCR was carried out using cDNA synthesized from total RNA of 12 autopsy human brain samples. PCR was performed using the forward and reverse primers, 5′-GGCCAAGGGGAACCCTGAGA-3′ and 5′-GGTCATACCCAGGGGGACGA-3′, respectively. Two PCR products of 340 bp (809–1148 bp of brain variant *CYP2D7*) and 282 bp (843–1125 bp of *CYP2D6*, GenBank accession number M20403) were amplified.

Genomic DNA was extracted from human brain cortex or blood and PCR amplified using forward and reverse primers, 5′-CTTCCTGCTCCTGGTGGA-3′ and 5′-CACCCCCTTCATCCTCGA-3′, respectively, spanning 506 bp of the partial sequences of exon 1 and intron 1 of the genomic sequence of *CYP2D7* (45–551 bp relative to the ATG start codon of the genomic sequence of *CYP2D7*; GenBank accession number NG_002362). PCR products were examined by electrophoresis and sequenced.

Localization of Brain Variant CYP2D7 by in Situ Hybridization—The 340-bp RT-PCR product was ligated into pCRII vector, and digoxigenin-labeled sense and antisense riboprobes were generated. *In situ* hybridization was performed using paraffin-embedded sections of brain, liver, and kidney from the same individual. Digoxigenin-labeled riboprobes were also generated from the cDNA to *CYP2D6* (GenBank accession number M20403). Human brain cortex, liver, and kidney obtained at autopsy from the same individual were fixed in buffered paraformaldehyde (4%, w/v) prior to paraffin embedding. Serial sections (8–10 μm thick) were cut under RNase-free conditions. Sections were dewaxed, hydrated in graded ethanol, acetylated, and treated with proteinase K. The sections were then rinsed in phosphate-buffered saline and dehydrated using graded ethanol. Sections were hybridized overnight with the digoxigenin-labeled riboprobes. After hybridization sections were washed, incubated with blocking reagent (0.5%, w/v, PerkinElmer Life Sciences) followed by antibody to digoxigenin conjugated to horseradish peroxidase. After washing, sections were incubated with biotinylated tyramine followed by streptavidin-fluorescein. Finally the sections were washed, dried, and mounted prior to examination under fluorescence microscope.

Preparation of Antiserum to Brain Variant CYP2D7—An antigen derived from the 19-amino acid peptide (GRRVSPGCSPIVGTHVCPV) representing 57 bp of intron 6 of brain variant *CYP2D7* was conjugated to bovine serum albumin at the N-terminal end and used to generate antiserum to brain variant CYP2D7 in rabbits. The antiserum was verified for lack of cross-reactivity with P4502D6 and used for immunoblotting.

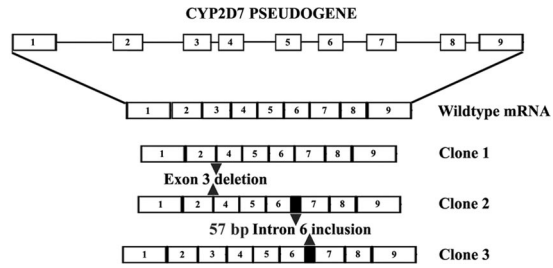

FIG. 1. **Structure of splice variants of *CYP2D7* gene identified in human brain.** A schematic representation of the normal spliced *CYP2D7* and the alternatively spliced forms of *CYP2D7* isolated from human brain is shown. Clone 1 contains an exon 3 deletion, clone 2 has an exon 3 deletion and 57-bp intron 6 inclusion, and clone 3 (brain variant *CYP2D7*) contains an inclusion of 57 bp of intron 6.

Immunoblotting with Membrane Preparations from Human Brain—Brain membrane preparations containing microsomal and mitochondrial protein (200 μg each) isolated from the cortex obtained at autopsy from the 12 human subjects were analyzed by SDS-PAGE (19). The blots were incubated sequentially with antiserum to brain variant CYP2D7 (1:200 dilution) overnight at 4 °C and anti-rabbit IgG conjugated to alkaline phosphatase (1:1,000 dilution) for 2 h at room temperature. Color was developed using chromogenic substrates for the alkaline phosphatase.

Assay of P450 and Metabolism of Codeine—Brain variant *CYP2D7* was expressed in Neuro2a cells as described above, and the membrane preparation containing both mitochondria and microsomes was isolated. Total cytochrome P450 content in membrane preparation was measured from a carbon monoxide reduced minus oxidized difference spectrum (9). Membrane preparation (150 μg of protein) was incubated at 37 °C in Tris-HCl (50 mM, pH 7.4) containing 20 mM $MgCl_2$, 1 unit of purified liver NADPH-cytochrome P450 reductase (20), and 0.01–1 mM codeine. The reaction was initiated by the addition of 1 mM NADPH and stopped after 30 min by addition of acetonitrile (10% of total volume). The samples were centrifuged, filtered, and used for HPLC analysis. Blank reactions did not contain NADPH. HPLC analysis was performed using mobile phase consisting of 40 mM potassium phosphate (pH 7.4), acetonitrile, and tetrahydrofuran in the ratio of 89:10:1 (v/v). Formation of metabolites was detected using a fluorescence detector set at 286 and 360 nm for excitation and emission wavelengths, respectively. Standard morphine, codeine, and norcodeine obtained from the National Institute of Drug Abuse was used for quantitation of metabolites. Brain variant CYP2D7 ligated in the reverse orientation was also transfected in Neuro2a cells and used as control.

Localization of Brain Variant P4502D7 and μ-Opiate Receptor in Human Brain—Serial transverse sections (10 μm thick) were cut from a paraffin-embedded periaqueductal gray region of a human brain and processed for immunohistochemistry as described earlier (16). Sections were incubated with antisera to brain variant CYP2D7, μ-opioid receptor (Sigma), or nonimmune serum and immunostaining was detected using an ABC Elite Kit (Vector Laboratories).

RESULTS

Identification of Splice Variants of CYP2D7 from a Human Brain cDNA Library—We investigated the presence of brain-specific P450 by screening a human brain cortex cDNA library using a 333-bp amplicon generated by RT-PCR of human brain mRNA representing exons 6–9 of *CYP2D6*. Three positive clones were selected, and DNA sequencing identified them as splice variants of *CYP2D7* having exon 3 deletion, partial inclusion of intron 6, or both (Fig. 1). Exon 3 deletion results in a premature stop codon (TGA) at 606 bp relative to the ATG start codon, and therefore the clones having exon 3 deletion were not investigated further. The third clone, 1776 bp long (GenBank accession number AY220845) with an open reading frame of 1551 bp, had an additional 57 bp from intron 6 and an intact exon 3 of the *CYP2D7* genomic sequence (GenBank accession NG_002362). A frameshift mutation 138delT (relative to ATG)

[2] The abbreviations used are: RT, reverse transcription; HPLC, high performance liquid chromatography.

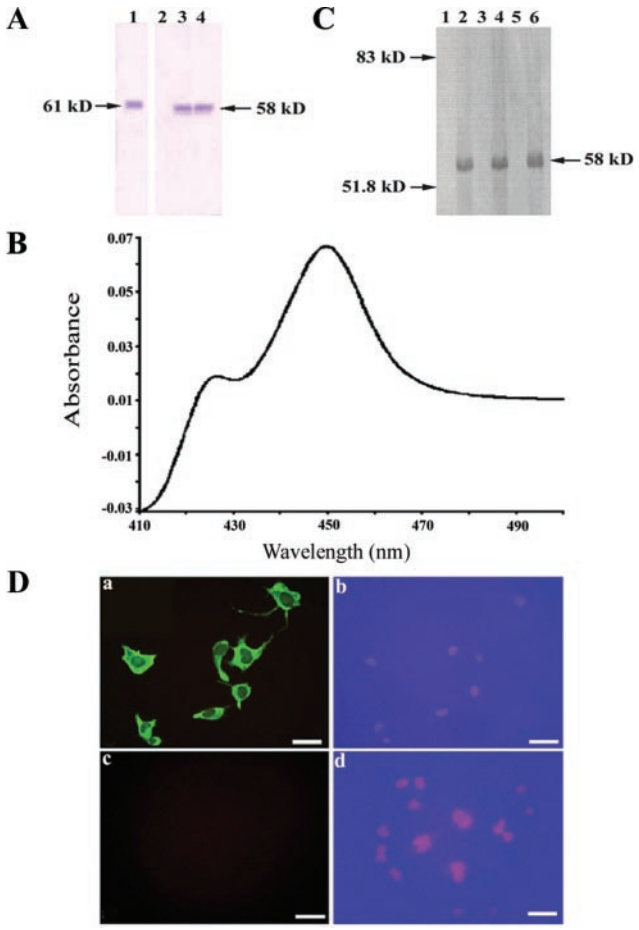

FIG. 2. **Expression of brain variant CYP2D7 and assay of P450 level.** *A*, brain variant *CYP2D7* cDNA was expressed in a rabbit reticulocyte system. *Lane 1* contained a positive luciferase control stained with anti-luciferase antibody. *Lane 2* was loaded with an expression system containing the vector alone, and *lanes 3* and *4* contained an expression system containing cDNA to brain variant *CYP2D7*. The blot was immunostained with antibody to CYP2D6. *Arrows* indicate the molecular mass of the immunostained protein. *B*, dithionite-reduced carbon monoxide binding spectrum of membrane preparations from Neuro2a cells expressing brain variant CYP2D7 protein. An absorption maximum at 450 nm is characteristic of cytochrome P450 proteins. The specific content of cytochrome P450 was 1.03 nmol of P450/mg of protein. *C*, expression of brain variant CYP2D7 protein in Neuro2a cells. *Lanes 1*, *3*, and *5*, which contain mitochondria and microsomes from cells transfected with vector containing the cDNA to brain variant *CYP2D7* in reverse orientation, do not show any immunostaining. Immunostained bands were seen in *lanes 2*, *4*, and *6* containing mitochondria and microsomes from different batches of Neuro2a cells expressing the brain variant CYP2D7 protein. Blots were immunostained with antibody to CYP2D6. All lanes contained 50 μg of protein. *D*, immunohistochemical localization of CYP2D7 in Neuro2a cells expressing brain variant CYP2D7. Cells expressing brain variant CYP2D7 showed intense cytosolic staining (*a*). Cells transfected with expression vector containing cDNA to brain variant *CYP2D7* in the reverse direction showed no staining (*c*). Corresponding nuclear staining with DAPI is depicted in the *right panel* (*b* and *d*), respectively. *Bars* = 50 μm.

modifies the stop codon TGA to GAG enabling the complete translation of the pseudogene *CYP2D7*.

Expression and Functional Activity of Brain Variant CYP2D7—The clone, which we named brain variant *CYP2D7*, translated *in vitro* in a rabbit reticulocyte system into a 58-kDa protein, which was immunochemically similar to hepatic P4502D6 (Fig. 2*A*). The brain variant *CYP2D7* was transfected in Neuro2a cells, and the expressed protein was assessed for P450 activity. The reduced carbon monoxide difference spectrum of the expressed protein had an absorption maximum at 450 nm characteristic of P450 (Fig. 2*B*; specific content = 1.03 nmol of P450/mg of protein). The expressed protein could also be detected by immunoblotting (Fig. 2*C*) and localized by immunohistochemistry using antibody to P4502D6 (Fig. 2*D*). Neuro2a cells, *per se*, and those transfected with the cDNA in the reverse orientation did not have any detectable P450 as observed by immunohistochemistry (Fig. 2*D*) or by the reduced carbon monoxide difference spectrum (data not shown).

RT-PCR and Immunoblotting Show the Presence of Brain Variant CYP2D7 in a Subset of Individuals—A 340-bp fragment representing a partial sequence of exons 6–7 including the additional sequence of 57 bp of intron 6 representing 843–1182 bp of brain variant *CYP2D7* was amplified using RT-PCR in 12 samples of human brain cortex obtained at autopsy. The generation of the 340-bp amplicon indicated the expression of the brain variant *CYP2D7*. Expression of *CYP2D6* represented by the 282-bp amplicon was seen in all 12 human brain samples examined (see Fig. 3*A*). The primers used for RT-PCR amplified both *CYP2D6* and *CYP2D7* because of the considerable similarity (98%) between *CYP2D6* and *CYP2D7* in the exon 6 region. However, the 57-bp sequence of intron 6 present in brain variant *CYP2D7* shares only 77% similarity with the corresponding region in *CYP2D6*. Thus, the 340-bp RT-PCR product represents the brain variant *CYP2D7* exclusively and was seen in 6 of the 12 samples examined.

We generated an antiserum to the 19-amino acid peptide representing 57 bp of intron 6 present in the brain variant *CYP2D7*. The peptide used to generate the antiserum did not share homology with any known P450 enzyme, and the antiserum did not cross-react with P4502D6. Expression of brain variant CYP2D7 protein as assessed by immunoblotting was seen in 6 of the 12 brain samples (see Fig. 3*B*) that were positive for the presence of brain variant *CYP2D7* mRNA by RT-PCR (Fig. 3*A*), indicating the concordance between the RT-PCR and immunoblot results.

Localization of Brain Variant CYP2D7 by in Situ Hybridization—The 340-bp RT-PCR amplicon generated as described above was ligated into the pCRII vector for riboprobe synthesis to localize the brain variant *CYP2D7* in human tissues using *in situ* hybridization. Brain variant *CYP2D7* mRNA was detected in cortical neurons in human brain but not in the liver or kidney of the same individual (Fig. 4, *A*, *B*, and *C*, respectively). However, *CYP2D6* mRNA was detected in brain, liver, and kidney when *in situ* hybridization was performed using the cDNA to *CYP2D6* (Fig. 4, *D*, *E*, and *F*, respectively).

T-Deletion in the Pseudogene CYP2D7 Generates a Functional Transcript—Because the brain variant *CYP2D7* is present only in about 50% of the human brains examined, we developed a genotyping assay to detect the 138delT relative to ATG. The genomic DNA isolated from the 12 human brain samples used for RT-PCR and immunoblot experiments was used as the template. The region spanning 45–551 bp (506 bp) relative to the ATG start codon of the genomic sequence of *CYP2D7* was amplified using PCR. The 138delT (CC**T**GC) was found only in those samples in which the mRNA and protein of the brain variant *CYP2D7* were detected (Fig. 3*C*); others had complete sequence similarity with the pseudogene *CYP2D7* (CC**TT**GC).

Brain Variant CYP2D7 Metabolizes Codeine Predominantly to Morphine—Monooxygenase activity of brain variant CYP2D7 was ascertained by examining the metabolism of codeine to norcodeine and morphine. Membrane preparations containing both mitochondria and microsomes were prepared from Neuro2a cells transfected with the cDNA of brain variant *CYP2D7* because P450-mediated xenobiotic metabolism takes place in both microsomes and mitochondria in rat and human brain (13, 21). The expressed brain variant CYP2D7 metabo-

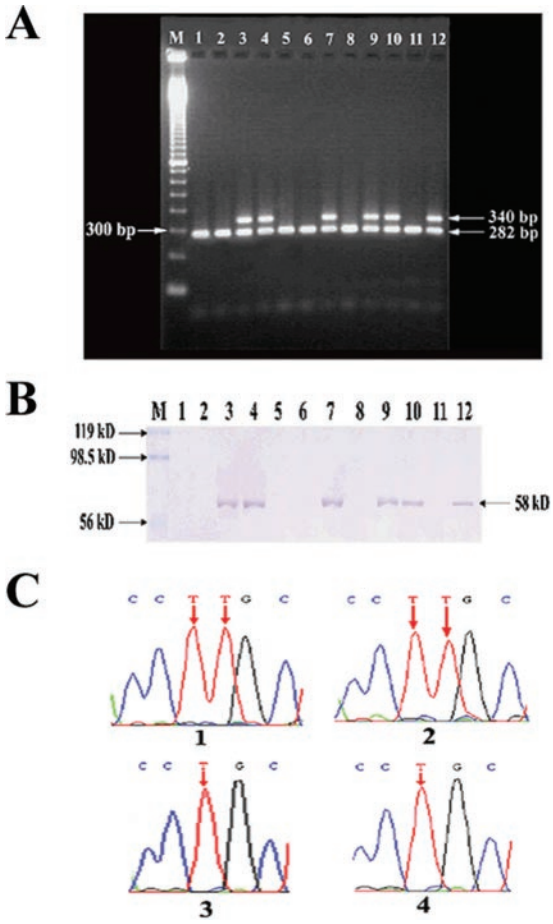

FIG. 3. **Expression of brain variant *CYP2D7* and *CYP2D6* in human brain.** *A*, RT-PCR analysis of brain variant *CYP2D7* in 12 human brain autopsy samples (*lanes 1–12*). Brain variant *CYP2D7* could be detected in 6 samples wherein a band of 340 bp was detected. The PCR-amplified product of 280 bp representing *CYP2D6* was detected in all the 12 samples. *M* represents the DNA molecular mass markers. *B*, membrane preparations from the 12 human autopsy brain samples (*lanes 1–12*) were immunoblotted and stained with antiserum to brain variant CYP2D7. Brain variant CYP2D7 protein could be detected only in 6 brain samples, which also showed the expression of the transcript by RT-PCR. *M* represents protein molecular mass markers. *C*, electropherogram of PCR-amplified genomic DNA from 4 brain samples (represented in *lanes 1–4* in *A* and *B*). In brain variants 1 and 2 *CYP2D7* is not expressed, and the genomic sequence revealed the presence of the pseudogene *CYP2D7*, as represented by the sequence CC**TT**GC. In brain variants 3 and 4 *CYP2D7* is expressed, and the genomic sequence CC**T**GC was seen.

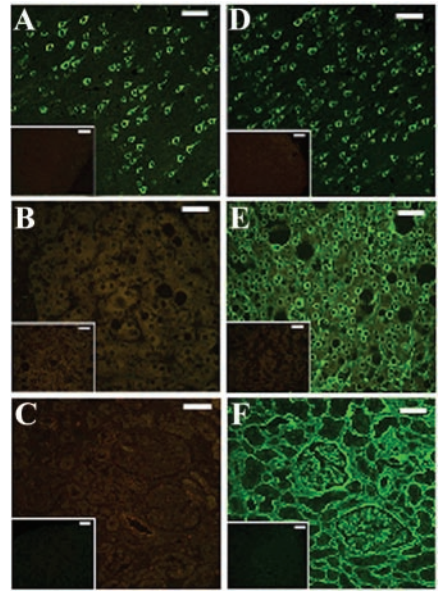

FIG. 4. **Localization of brain variant *CYP2D7* and *CYP2D6* in human tissue.** The brain variant *CYP2D7* transcript was localized in cortical neurons in human brain, but it was absent in liver and kidney sections from the same individual (*A*, *B*, and *C*, respectively). The transcript representing the full-length *CYP2D6* gene was detectable in brain, liver, and kidney (*D*, *E*, and *F*, respectively). *Insets* depict the corresponding control sections hybridized with the sense riboprobes. *Bar* = 50 μm except in the *inset*, where it = 100 μm.

lized 10 μM codeine to morphine alone, and norcodeine could not be detected (Fig. 5). In membranes from cells transfected with cDNA of liver *CYP2D6*, norcodeine was the major metabolite (62%) and morphine, the minor metabolite (38%; Fig. 5*B*). No metabolite of codeine was detected in cells transfected with vector alone or vector containing the cDNA in reverse orientation (data not shown). Typically, codeine is administered at doses ranging from 60 to 120 mg/day. The plasma concentration of codeine in humans after a single dose of 120 mg is about 474 μg/liter (1.1 μM); we therefore used 10 μM codeine as substrate concentration (22). When the codeine concentration was increased to 200 μM, a small amount of norcodeine (11% of total metabolite) was formed. The V_{max}/K_m values for the metabolism of codeine to morphine and norcodeine by brain variant CYP2D7 were 13.5 and 0.6 nmol of product formed/min/nmol P450/mM substrate concentration, respectively. The corresponding values with CYP2D6 were 0.8 and 1.5 nmol of product formed/min/nmol of P450/mM substrate concentration, respectively. Metabolism of codeine to morphine could be inhibited by antiserum to P4502D6 and quinidine, a selective inhibitor of CYP2D (Fig. 5*C*), indicating that this biotransformation was indeed mediated by a P4502D enzyme.

Brain Variant CYP2D7 and μ-Opioid Receptor Colocalize in Neurons—To determine whether the metabolism of codeine to morphine takes place at the site of action of the morphine, namely the neurons in periaqueductal gray that contain μ-opiate receptors, we performed immunohistochemistry using antisera to brain variant CYP2D7 and μ-opiate receptor on serial sections of periaqueductal gray from human brain. Brain variant CYP2D7 and μ-opiate receptor colocalized in the neurons of periaqueductal gray (Fig. 6, *A–D*).

Incidence of CYP2D7 Genetic Polymorphism—DNA isolated from blood samples of eight volunteers was used as a template, and the region spanning 45–551 bp (506 bp) relative to the ATG start codon of the genomic sequence of *CYP2D7* was amplified using PCR. The 138delT (CC**T**GC) was found only in 4 of 8 samples analyzed. The others had complete sequence similarity with the pseudogene *CYP2D7* (CC**TT**GC). The representative electropherogram of 6 samples is depicted in Fig. 7. Thus, the functional *CYP2D7* is presumably present in 50% of the samples analyzed.

DISCUSSION

Several P450 enzymes, such as CYP2D (16), CYP3A (19), and CYP2B (11), are present in human brain and localize predominantly in neurons, the site of action of most drugs (19). However, drug metabolism in human brain is poorly understood, and human brain-specific P450 enzymes remain to be identified. The presence of unique, tissue-specific P450 enzymes generated through alternate splicing provides a mechanism by which active metabolites can be potentially formed at the site of action of drugs within the target organ, such as the brain.

P4502D6 is an important human P450 enzyme that metabolizes a number of substrates (23). It shows a high degree of interindividual variability, which is primarily caused by the extensive genetic polymorphism that influences its expression and function. P4502D6 is a constitutive form of hepatic P450,

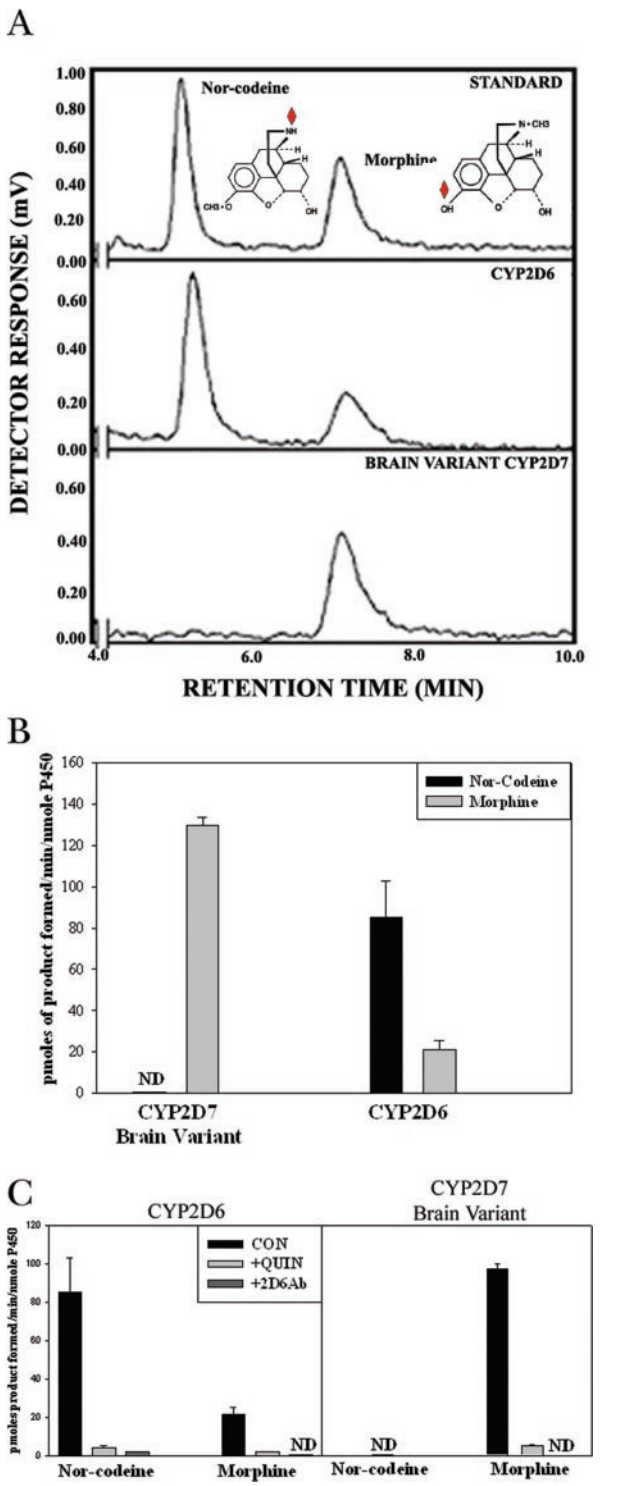

FIG. 5. **Metabolism of codeine by heterologously expressed brain variant CYP2D7 and CYP2D6.** *A*, chromatogram depicting retention times of standard norcodeine and morphine (*top panel*). CYP2D6 metabolizes codeine to norcodeine and morphine (*middle panel*), whereas brain variant CYP2D7 metabolized codeine only to morphine (*bottom panel*). The codeine concentration was 10 μM. *B*, the rate of formation of morphine and norcodeine from codeine (10 μM) by mitochondria and microsomes from cells expressing CYP2D7 brain variant and CYP2D6. *C*, effect of 50 μM quinidine (*QUIN*, an inhibitor of P4502D) and antiserum to P4502D6 on metabolism of codeine by Neuro2a cells transfected with CYP2D6 and brain variant CYP2D7. The K_m and V_{max} for the formation of norcodeine and morphine by CYP2D6 were 1.1 and 0.5 mM and and 1.6 and 0.4 nmol of product formed/min/nmol of P450, respectively. The K_m and V_{max} for the formation of norcodeine and morphine by brain variant CYP2D7 were 1.2 and 0.1 mM and and 0.7 and 1.4 nmol of product formed/min/nmol of P450, respectively. The S.E. was less than 5%.

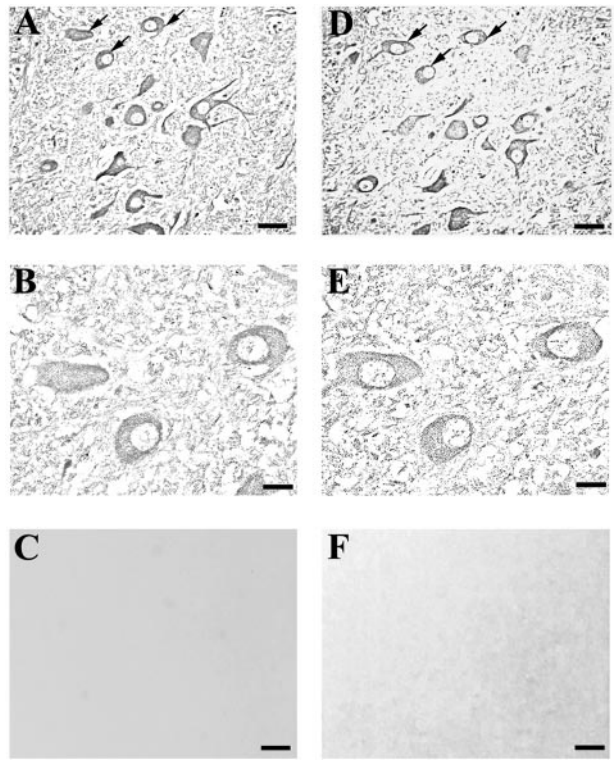

FIG. 6. **Localization of brain variant CYP2D7 and μ-opioid receptors in periaqueductal gray of human brain.** *A* and *B*, antiserum to brain variant CYP2D7 labels neurons in periaqueductal gray of human brain. *Bar* = 25 and 10 μm, respectively. *C*, control section treated with nonimmune rabbit serum. *Bar* = 10 μm. *D* and *E*, serial section shows staining in the same neurons as in *A* and *B* when treated with antiserum to μ-opioid receptor. *Bar* = 25 and 10 μm, respectively. *F*, control sections immunostained with normal rabbit serum. *Bar* = 10 μm.

where it mediates metabolism of several commonly used psychoactive drugs, such as imipramine, amitriptyline, chlorimipramine (24), and haloperidol (25). The human *CYP2D* locus on chromosome 22 consists of the three homologous genes, *CYP2D8*, *CYP2D7*, and *CYP2D6*, which are located within a contiguous region of about 45 kb (26). *CYP2D8* and *CYP2D7* are pseudogenes. *CYP2D8* has multiple deletions and insertions and no open reading frame, whereas the *CYP2D7* gene has a single inactivating mutation, an insertion (T138) in the first exon causing a frameshift resulting in premature termination of translation of *CYP2D7* (27). It has been speculated that CYP2D7 may be expressed as a functional protein as result of repair; however, no evidence has been presented so far (27). Here we present evidence for the 138delT mutation that converts the *CYP2D7* pseudogene to a functional gene in about half the samples analyzed in the present study. The mutation 138delT was seen in DNA isolated from brain tissue as well as blood (Fig. 7), indicating that the presence of functional *CYP2D7* in these individuals. It is to be determined whether the functional *CYP2D7* is indeed present in the liver of individuals with the 138delT mutation of the *CYP2D7* gene.

In individuals having the 138delT mutation, brain-specific splicing led to the formation of a functional *CYP2D7* brain variant enzyme. Thus, in 6 of the 12 brains examined from the Indian population genotyping revealed the presence of the 138delT mutation, and RT-PCR and immunoblot experiments showed the presence of the brain variant *CYP2D7* mRNA and protein in these samples (Fig. 3). It is to be seen whether this mutation exists in other population leading to the presence of functional CYP2D7 and if it does, it is to be determined whether the autopsy brain samples from these population ex-

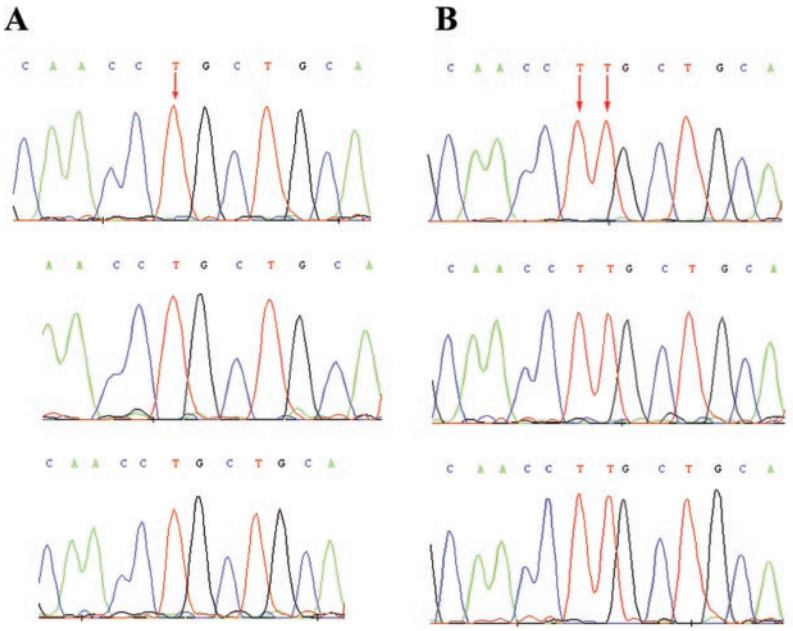

FIG. 7. **Electropherogram of PCR-amplified genomic DNA from blood samples of six individuals.** Electropherogram of PCR-amplified genomic DNA from six blood samples is represented in *A* and *B*. *A*, the genomic sequence CAACC**T**GCTGCA was seen, indicating the presence of functional *CYP2D7*. *B*, the genomic sequence CAACC**TT**GCTGCA indicates the presence of the pseudogene *CYP2D7*.

press the brain variant *CYP2D7* that is generated by brain-specific alternate splicing.

The brain variant *CYP2D7* has a partial inclusion of intron 6 (57 bp) in the transcribed mRNA sequence. This was observed only in human brain and not in liver or kidney of the same individual, indicating that generation of alternate spliced form is a brain-specific event. Earlier studies have shown the presence of an alternate spliced form of flavin-containing monooxygenase (FMO4) with an exon 4 deletion in rat brain but not in other tissues (28). The nervous system has a propensity for generating alternate spliced forms, and splicing defects observed in individuals are not related to differences in the genomic sequence but may be regulated by mechanisms involving spliceosomal complex and RNA-binding proteins, which are poorly understood (29).

The tendency for human brain to generate alternate spliced genes is seen in the present study wherein three alternate spliced variants were identified in the human brain, namely, exon 3 deletion, partial inclusion of intron 6, and a third having both the deletion and inclusion. The alternate spliced variants containing exon 3 deletion have a premature stop codon, which prevents their translation into functional gene products. Thus, estimation of P450 isoforms by examining gene expression using Northern blotting, RT-PCR, and *in situ* hybridization (16, 30, 31) would represent contributions from functional and nonfunctional genes and would potentially overestimate the expression of a particular isoform. The well characterized *CYP2D6* exhibits genetic polymorphism, and the gene is absent in the "poor metabolizer (PM)" phenotype (32). None of the individuals whose brain samples were studied lacked the *CYP2D6* gene as discerned by the presence of the 282-bp amplicon, whereas the brain variant *CYP2D7* form was expressed in 6 but not in others.

Plasma levels of morphine show poor correlation with pain relief provided by codeine (33) because the amount of morphine formed in liver through P4502D6 metabolism of codeine is insufficient to account for the analgesic effect of codeine (34). It has been speculated that human brain-specific metabolic pathways, which can metabolize codeine to morphine, exist which are yet to be identified (35, 36). Thus, even if a very small amount of codeine is metabolized to morphine by brain P450 at the site of action, it could mediate pain relief (37). The results presented herein provide evidence for the formation of morphine from codeine by brain-specific alternate spliced gene product leading to a metabolite(s) profile that is different from liver.

In cells transfected with *CYP2D6*, norcodeine was the major metabolite and morphine, the minor metabolite. Earlier studies had indicated that the metabolism of codeine to norcodeine is mediated by P4503A4 (38); however, in the present study it is seen that P4502D6 also metabolizes codeine principally to norcodeine. However, brain variant CYP2D7 metabolizes codeine largely to morphine, and norcodeine is formed in lesser amounts and only at higher substrate concentration. Morphine is exclusively formed at a lower concentration of codeine (10 μM) which is closer the physiological concentration achieved after administration of a pharmacological dose of codeine (22).

For effective pain relief it would be ideal if morphine were formed in the brain where it can directly bind to μ-opioid receptors. Opiates mediate their central analgesic effects by acting on neurons within brain regions such as the midbrain periaqueductal gray (39). We localized brain variant P4502D7 and μ-opiate receptor by immunohistochemistry in the periaqueductal gray region of a human brain obtained at autopsy. The brain variant CYP2D7 and μ-opiate receptor are colocalized in the neurons of periaqueductal gray, indicating that morphine could potentially be formed from codeine in pain centers of the central nervous system. Biochemical estimation of enzyme activity represents the average in a tissue containing several distinct cell types. In heterogeneous tissues, such as the brain, low levels of enzyme activity may be observed. However, because enzymes such as P450 are localized in selective cell population (Figs. 2*D* and 6), which are the site of action of drugs, it is not the total specific content of the enzyme that would determine the outcome, but the site specificity and the amount of enzyme present in individual cell types. The total P450 levels in human brain are typically only 4–10% of the corresponding levels in the liver (40); however, the presence of brain-specific isoforms of P450 in specific cell population indicates that they may play an important role in metabolism of drugs, *in situ*, at the site of action. Although genetic polymorphism of *CYP2D6* is one of the important determinants of interindividual variation in drug response, functional polymorphism of *CYP2D6* does not always correlate with therapeutic efficacy (8). We now present evidence for the existence of a pathway that can potentially mediate pharmacological action

of codeine at the site of action by mechanisms that are dissimilar from known pathways in liver. Identification and characterization of novel histio-specific isoforms of P450 generated by alternate splicing of known genes or as yet unidentified genes may help predict the pharmacological outcome of drugs that act at sites remote from liver.

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A Frameshift Mutation and Alternate Splicing in Human Brain Generate a Functional Form of the Pseudogene Cytochrome P4502D7 That Demethylates Codeine to Morphine

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