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TISSUE-SPECIFIC EXPRESSION AND METHYLATION OF THE HUMAN *CYP2E1* GENE

FLORENCE BOTTO,* ERIC SEREE, SAID EL KHYARI, GEORGES DE SOUSA, ANNICK MASSACRIER,† MICHEL PLACIDI, PIERRE CAU,† WILLIAM PELLET,‡ ROGER RAHMANI and YVES BARRA

INSERM U278, 27 Boulevard Jean Moulin 13385 Marseille Cedex 05; †Laboratoire de Biologie Cellulaire EA DRED 868, 27 Boulevard Jean Moulin 13385 Marseille Cedex 05; and ‡Hôpital Sainte-Marguerite, 270 Bd Sainte-Marguerite 13009 Marseille, France

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Abstract—The level and number of CYP2E1 gene transcripts were investigated by northern blot analysis in various human adult tissues including liver, lung, placenta, skin and neurinoma. Three transcripts of 1.8, 2.6 and 4 Kb were expressed in a tissue-specific manner. The origin of the various transcripts was studied and showed that both 4 and 2.6 Kb mRNAs contained sequences from the 3′ non-translated region of the gene and that the 4 Kb also contained region localized in the 5′ non-translated region. Furthermore, it clearly appeared that a catalytically active CYP2E1 enzyme (as proved by NDMA demethylase activity) was only detected in tissues expressing the 1.8 Kb. The human CYP2E1 was also identified through immunohistochemical techniques. Finally, we observed a relation between the hypomethylation of the human *CYP2E1* gene and the hypoexpression of the corresponding protein.

Key words: gene expression; DNA methylation; cytochrome P450; CYP2E1; *N*-nitrosodimethylamine metabolism, immunochemical evidence

Cytochrome P450s belong to a multigene family of enzymes [1]. These proteins are mostly expressed in the liver and to a lesser extent in numerous extrahepatic tissues including lung [2], small intestine [3], lymphocytes [4], bone marrow [5], kidney [6] and nasal mucosa [6, 7]. Some cytochrome P450s are constitutively expressed in untreated animals and some can be induced by treatment with specific compounds [8]. Cytochrome P450s are involved in the metabolism of exogenous compounds (such as drugs, carcinogens and pesticides) as well as endogenous substances (such as fatty acids, prostaglandins, steroids and ketone bodies produced during fasting). They can either convert xenobiotics to inert polar metabolites or bioactivate them to reactive species. Some cytochrome P450s are modified by physiopathological conditions such as fasting, diabetes, age, sex, stress or by exposure to various chemical compounds (environmental pollutents, drugs etc.).

One of the most widely studied cytochrome P450s is the ethanol-inducible CYP2E1. The regulation of expression of this cytochrome is one of the most complex. Indeed, it involves regulation: (1) at the level of transcription at birth (with an associated demethylation [9, 10]) (2) by mRNA stabilization in the diabetic state and by fasting; (3) by increased translation of existing mRNA; and (4) by inhibition of protein degradation [11]. CYP2E1 is crucial since it is implicated in the biotransformation of chemicals which have toxic effects in humans (styrene, vinyl chloride [12, 13]) and in the bioactivation of precarcinogens (benzene, *N*-nitrosodimethylamine etc. [14]). These reactions are generally dependent on the CYP2E1 protein level, which has been found to be elevated in: (1) people suffering from alcoholism [15]; (2) patients undergoing therapy with isoniazid [16]; (3) diabetic and especially those who do not respond to insulin [4].

The aim of the present study was to analyse the variations in expression of the *CYP2E1* gene (mRNA and protein) in various human adult tissues using enzymatic assay (NDMA§ demethylation), immunohistochemical experiments, northern hybridization and to explain them at least in part (characterization of the different transcripts and gene methylation).

MATERIALS AND METHODS

Reagents. [α-^{32}P]dATP and [γ-^{32}P]ATP were obtained from Amersham (Amersham, U.K.). NDMA was purchased from Sigma (Saint Quentin Fallavier, France). Antibodies conjugated to alkaline phosphatase or horse radish peroxidase were from Biosys (Compiegne, France). The rabbit anti-rat CYP2E1 IgG was kindly provided by F.J. Gonzalez [17]. Restriction enzymes Hpa II, Msp I, Hind III and BamH I were obtained from Boehringer (Mannheim, F.R.G.). Moviol was from Calbiochem (Frankfurt, F.R.G.). All other reagents were of the highest quality available.

Human samples. A variety of human adult tissues were studied including: liver, placenta, skin, lung and neurinoma. The livers were obtained under strict ethical conditions from organ donors. The placenta were from full term pregnancies after

* Corresponding author.

§ Abbreviations: IgG, immunoglobulin; NDMA, *N*-nitrosodimethylamine; NGS, normal goat serum; PCR, polymerase chain reaction; SSC, standard saline citrate

Table 1. CYP2E1 oligonucleotides used in this study

CYP2E1 probe	Length (bp)	Localization	Ref.	Sequence (5′–3′)
B	40	11,503–11,542	34	Complementary to an intronic segment in the 3′ flanking region localized after the AATAAA site: CAA GAT CAT GCC ACT GCA CTC CAT CCT GGT CAA CAA GAG C
C	20	−819–−800	34	Identical to an intronic segment localized in the 5′ flanking region: TAT TGT GCG CCG GGA TCA AC
D	20	11,335–11,354	34	Identical to an intronic segment in the 3′ flanking region localized before the AATAAA site: CTG ATT CCT TTC TTT GCA TA
E	20	−819–−800	34	Complementary to an intronic sequence localized in the 5′ flanking region: GTT GAT CCC GGC GCA CAA TA
F	40	77–116	34	Complementary to a region in the first exon: TGC ACC TGC CTC CAC ATG GAC ACC AGC AGG AGG AAG GCC G
G	20	4801–4820	34	Complementary to a region in the fourth exon: AGG ATG TCG GCT ATG ACG TT
H	20	5101–5120	34	Complementary to a region in the fourth intron: CAC ATC CTG ACG TTA GGA AA
I	20	11,944–11,963	34	Complementary to an intronic segment in the 3′ flanking region localized after the AATAAA site: ACT CCC TTT CGT ATA TAC AT
J	20	828–847	10	Complementary to regions localized in exons 5 and 6: AAT GGA GAA GGA AAA GCA CA
K	17	971–987	10	Complementary to regions localized in exons 6 and 7: GAG CTT CTC TTC GAT CT

normal deliveries. The mothers were healthy, drug-free and non smokers. Placenta were washed with ice-cold sterile physiological solution to eliminate blood. Left acoustic neurinoma and lung biopsies were obtained from surgical resection of tumours. Skin was obtained from women after breast plastic surgery. All tissues were kept frozen within half an hour after removal and stored at −80° until use.

Cloning of the human P450A cDNA probe. Cytochrome P450A cDNA was isolated from a human adult liver cDNA library [18], cloned into the Pst I site of the pBR 322. Screening was done with the R17 (*CYP2B2*) [19] probe under non-stringent conditions. A clone (P450A) was selected according to its highest hybridization with this probe and after sequencing (following the method of Sanger *et al.* [20]) appeared to be part of the *CYP2E1* cDNA (position 749 to 1623 [10]).

Preparation of radioactive probes. ^{32}P-labelled single-strand cDNA probes, GAPDH (glyceraldehyde-3-phosphate deshydrogenase) [21] and probe A, were prepared by incorporation of [α-^{32}P]-dATP by primer extension [22] using the Klenow polymerase. The oligonucleotides used for hybridization analysis are shown in Table 1. Oligonucleotides (6 pmol) were 5′ labelled with γ-^{32}P by incubating with T4 polynucleotide kinase [22].

RNA extraction, electrophoresis and blot analysis. Total cellular RNA was isolated from various human tissues following the procedure described by Sambrook *et al.* [22]. Total RNA (10 μg) was fractionated on a 12.3 M formaldehyde–1.4% agarose gel, transferred to nitrocellulose membrane (0.2 μm, Schleicher and Schuell) as described previously [23] and baked at 80° for 2 hr. Total RNA extracted from normal human liver 3 was run as control in each analysis. The blots were prehybridized 2 hr at 68° in 6 × SSC (20 × SSC is: 3 M NaCl, 0.3 M citric acid; pH = 7.0), 10% dextran sulphate, 5 × Denhardt's (100 × Denhardt's solution is: 2% polyvinyl pyrrolidone, 2% Ficoll, 2% BSA in 3 × SSC), and hybridized at 45° in the same solution containing 10^6 cpm/mL of the labelled oligonucleotide probes. The filters were also prehybridized in 5 × SSC, 10% dextran sulphate, 5 × Denhardt's, 50% freshly deionized formamide and hybridized at 45° in the same solution containing 10^5 cpm/mL of the labelled A or GAPDH probes. The filters were then washed three times at 45° in either 6 × SSC or 2 × SSC. The blots were exposed at −80° with an intensifying screen. The autoradiogram intensities of the 1.8 Kb mRNA band were estimated by scan densitometry and normalized to GAPDH; this was noted REL for Relative Expression Level:REL = Densitometric value of 1.8 Kb CYP2E1 transcript/Densitometric value of GAPDH transcript.

DNA extraction, electrophoresis and blot analysis. Genomic DNA was extracted from human tissues as previously described [22]. Ten micrograms of DNA were digested with mentioned restriction enzymes, fractionated on a 1% agarose gel, transferred onto hybond-N filters according to the procedure of Southern [24] and baked for 2 hr at 80°. The blots were prehybridized 2 hr at 68° in 3 × SSC, 5 × Denhardt's, 0.1% SDS and hybridized in the same solution containing 10^5 cpm/mL of the

labelled A probe. The filters were then washed three times at 68° in 3 × SSC, 0.1% SDS. The blots were exposed at −80° with an intensifying screen.

PCR analysis. One microgram of total RNA was reverse-transcribed in 30 μL of 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 5 mM $MgCl_2$ containing 200 μM each of four desoxyribonucleoside triphosphates, 25 pmol of the downstream primer and 5 U of Moloney murine leukaemia virus reverse transcriptase (Boehringer) at 42° for 30 min. The cDNA product was amplified in 90 μL of the above buffer containing 200 μM each of four desoxyribonucleoside triphosphates, 25 pmol of the primers and 2.5 U of the Taq DNA polymerase (Boehringer). PCR (30 cycles) was carried out as follows: 1 min for denaturation at 94°, 2 min for annealing at 51°, and 2 min for extension at 72°. After amplification, 10 μL of the PCR reaction product was electrophoresed on a 1% agarose gel. The gel was then stained with ethidium bromide.

Preparation and analysis of human tissue microsomes. Human adult tissue microsomes were prepared by a differential centrifugation technique [25], after the tissues had been homogenized as described previously [3], and stored at −80° until use. Protein concentration was estimated as described by Bradford [26]. For nitrosodimethylamine demethylation estimation, the incubation mixture contained 100 mM KH_2PO_4 (pH = 7.4), 40 mM NDMA and 1 mg of microsomal protein. The reaction was started by the addition of NADPH (40 mM final concentration). After a 30 min incubation at 37°, the reaction was terminated by addition of trichloroacetic acid (12.5% final concentration). NDMA demethylase activity was estimated by determination of formaldehyde formation according to Nash [27]. It should be noted that NDMA is a very dangerous compound.

Immunohistochemistry. Tissue sections of 10 μm thickness were cut on a cryostat (Microm) at −20° and stored at −80° before use. After rehydration in PBS, the sections were treated with PBS containing 10% NGS for 10 min and incubated overnight at 4° with rabbit anti-rat CYP2E1 IgG (final concentration 20 μg/mL) or rabbit non immune IgG (final concentration 20 μg/mL) in PBS containing 10% NGS. Sections were then washed with PBS containing 1% NGS and incubated for 1 hr at room temperature with anti-rabbit IgG conjugated to alkaline phosphatase. Subsequently sections were washed with PBS before staining, using Naphtol and Fast red diluted in 100 mM Tris pH 7.4 containing 1 mM $MgCl_2$ and 25 mg/mL levamisole. The slides were then rinsed with PBS.

RESULTS AND DISCUSSION

Tissue-specific expression of the CYP2E1 *gene*

The level and number of the CYP2E1 mRNAs were examined in hepatic and extrahepatic human adult tissues—including skin, placenta, lung and neurinoma. CYP2E1 is implicated in the bioactivation of pre-carcinogens [12–14] and low molecular weight compounds. It is well known that the liver is the

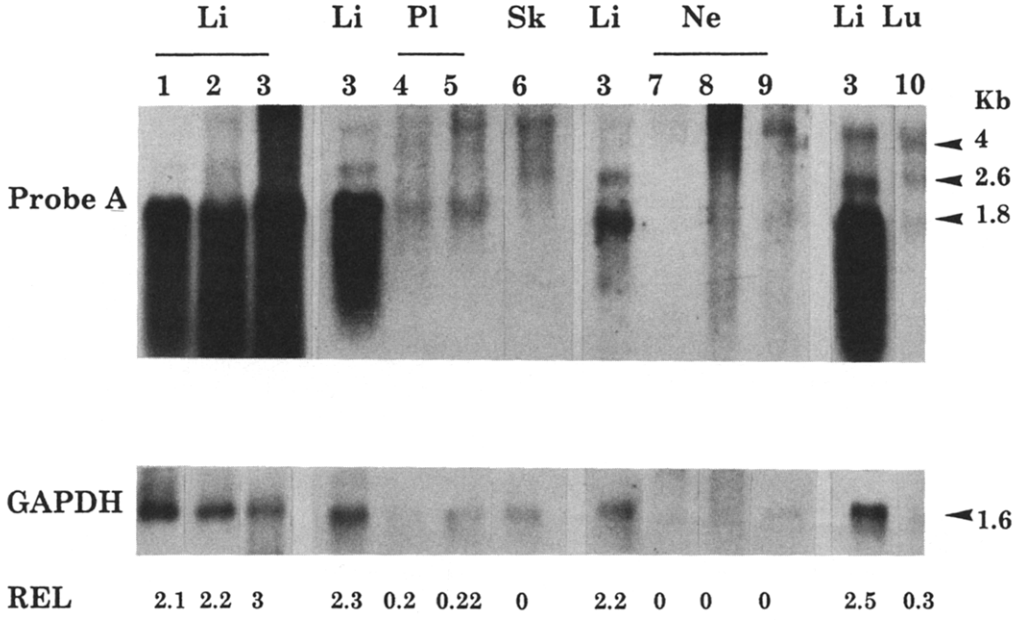


Fig. 1. Tissue-specific expression of the human *CYP2E1* gene. Ten micrograms of total RNAs prepared from human liver (Li), placenta (Pl), skin (Sk), neurinoma (Ne) and lung (Lu) were subjected to northern blot analysis using probe A and reprobed with glyceraldehyde-3-phosphate deshydrogenase (GAPDH), after the previous probe had been stripped from the filter by briefly boiling in water. The intensities of the 1.8 Kb mRNA in the autoradiograms were estimated by densitometry and normalized to GAPDH. This was noted REL for Relative Expression Level. Molecular sizes were derived from ethidium bromide-stained marker RNA (Bethesda Research Laboratories).

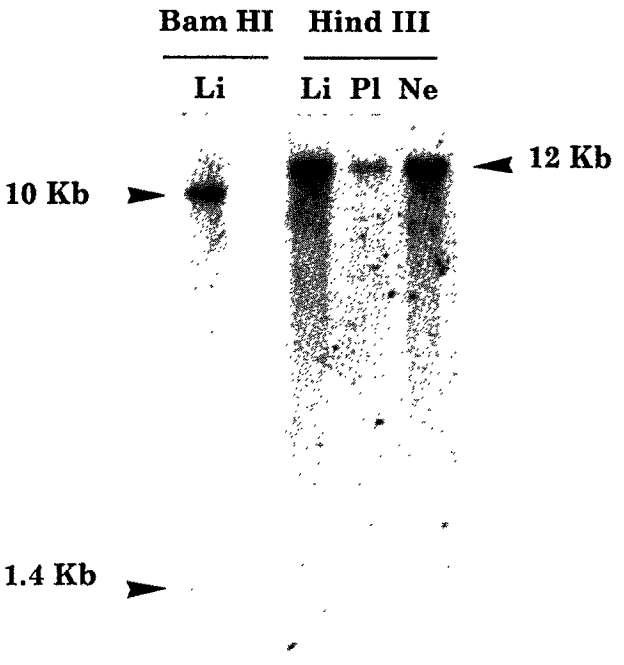


Fig. 2. Southern analysis of human genomic DNA. Ten micrograms of human liver (Li), placenta (Pl) and neurinoma (Ne) genomic DNA were digested with Hind III (A) and 10 μg of human liver (Li) were digested by BamH I (B). The digested DNA were subjected to Southern blot analysis, as described in Materials and Methods, using probe A.

organ with the highest CYP2E1 activity but other tissues may express this isoenzyme, including lung [2], duodenum, jejunum and ileum [3]. We chose to study the lung, the placenta and the skin because they represent a line of defense against toxic compounds and any variation of CYP2E1 level in these tissues could have severe consequences for the adult and/or the foetus. Moreover, the presence of cytochrome P450 in the human nervous system is of interest to neurobiologists as well as to toxicologists and to our knowledge no data concerning CYP2E1 are available. For ethical reasons we only had access to neurinoma.

The results of the northern blot analysis (shown in Fig. 1) enable us to make three observations: (i) when the same tissue from various individuals was considered (liver, placenta and neurinoma), the pattern of expression of the *CYP2E1* gene was similar. (ii) Three transcripts of 1.8, 2.6 and 4 Kb were observed in liver; their coexistence in human hepatic tissues had previously been detected by Miles *et al.* [28], Wrighton *et al.* [15] and Jones *et al.* [9]. In the rat liver, Song *et al.* [10] obtained a single band corresponding to the 1.8 Kb mRNA, indicating the presence of this CYP2E1 orthologue in the rat and humans. In the rabbit, Khani *et al.* [29] observed the presence of two transcripts of 1.9 and 1.6 Kb corresponding to two genes. In human hepatic tissues, the 1.8 Kb mRNA corresponds well with the size expected from the cDNA clone [10], however, the origin of the 4 and 2.6 Kb transcripts and their relationship to the 1.8 Kb mRNA are as yet unclear. (iii) The highest steady state level of 1.8 Kb was found in the liver (REL approx. 2.4) compared to all other extrahepatic tissues (REL around 0.2, 0, 0 and 0.3 for placenta, skin, neurinoma and lung, respectively). In the liver, the 1.8 Kb transcript was overexpressed compared to the 2.6 and 4 Kb mRNAs. In the lung, the three transcripts were equally detected. The two transcripts of 4 and 1.8 Kb were expressed with the same intensity in the placenta. In the skin a low expression of the 4 and 2.6 Kb was observed, the 1.8 Kb mRNA being undetected even after lengthy exposure time (data not shown). Only the high molecular weight mRNA was detected in the neurinoma. The above results showed a tissue specific expression of the *CYP2E1* gene in the human tissues.

Significance of the elongated transcripts

The three transcripts observed (Fig. 1) may arise as a result of transcription either from a single gene or from separate but closely related genes. Using probe A, we detected either a single band (12 Kb) or two bands (10.5 and 1.4 Kb) when genomic DNA was digested with Hind III and BamH I, respectively (Fig. 2). The size of these bands was compatible with the finding of Song *et al.* [10]. These observations are consistent with the existence of only one *CYP2E* gene in the human genome. We focused our attention

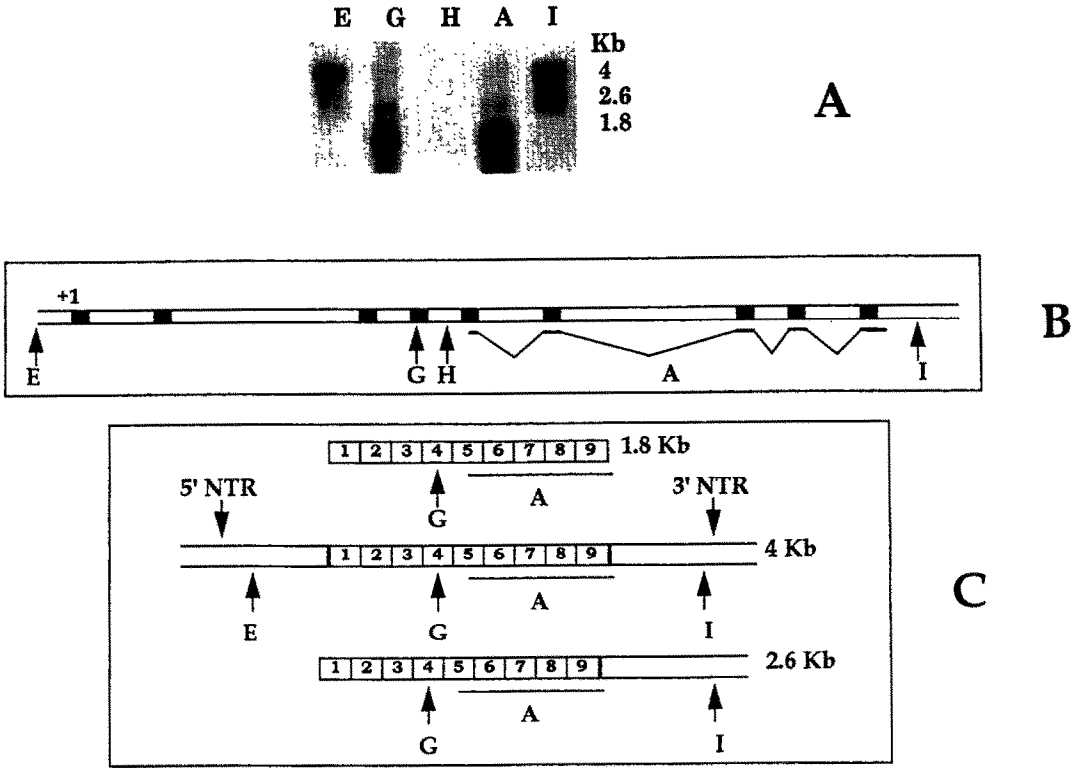


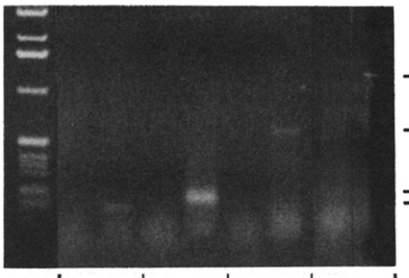
Fig. 3. Significance of the various transcripts. Total human liver RNAs (10 μg per lane) were subjected to northern analysis (A) using various probes localized along the length of the genome (B). The hypothesis of the origin of the various transcripts is represented in panel C.

on the 2.6 and 4 Kb transcripts because of their prevalence relative to the 1.8 Kb mRNA in certain extrahepatic tissues such as lung, skin and neurinoma. In order to clarify the nature of the elongated transcripts, we undertook northern blot analysis using probe A and oligonucleotides (E, G, H, I) hybridizing with various sequences localized throughout the *CYP2E1* gene (Fig. 3B and Table 1). The results of the hybridization are shown in Fig. 3A. As expected, the exonic probes A and G allowed detection of the three transcripts. When using probe E, localized in the 5′ non-coding region, we only detected the 4 Kb mRNA, the 1.8 Kb not being observed. This suggests that the 4 Kb transcript may arise from the use of an upstream putative site of initiation of transcription. With probe I, only the 4 and 2.6 Kb mRNAs were detected even after lengthy exposure time (data not shown), suggesting that these transcripts were CYP2E1 mRNAs extending beyond the normal cleavage point. With probe H, localized in intron 4, the various transcripts were not observed, but no conclusion could be drawn concerning the implication of other intronic sequences in the extended transcripts. All these observations lead us to suggest that both 4 and 2.6 Kb mRNAs contain sequences from the 3′ non-translated region of the gene and that the 4 Kb also contains region localized in the 5′ non-translated region (Fig. 3C).

In order to confirm the presence of these non-coding sequences in CYP2E1 mRNAs, PCR was undertaken (Fig. 4) with human liver 3 RNA and pairs of primers (described in Table 1) extending either the 5′ (primers C-F) or the 3′ (primers D-B and D-I) non translated regions of that gene. With primers J-K used as a positive control, a 163 bp amplified fragment was observed (Fig. 4 lane 2). These primers were chosen to span an intron and therefore did not amplify genomic DNA, which may be present as a contaminant in RNA preparations. Using primers C-F we obtained a band with exactly the expected size (932 bp) (Fig. 4 lane 8) suggesting the presence of fragments localized in the 5′ non coding region in CYP2E1 transcripts. The primers D-B and D-I amplified fragments of 210 bp and 630 bp respectively (Fig. 4 lanes 4 and 6), implying that region localized after the polyadenylation site of *CYP2E1* may be part of the elongated transcripts of that gene. Negative controls in which RNA were not reverse transcribed (Fig. 4 lanes 1, 3, 5 and 7) were used with each pair of primers.

CYP2E1 enzyme activity and immunohistochemical analysis

The demethylation of NDMA has been shown to be a good metabolic marker for the presence of CYP2E1 [14, 30, 31]. As shown in Table 2 hepatic microsomes were approx. three to four times more



Primers	J-K		D-B		D-I		C-I	
Lane	1	2	3	4	5	6	7	8
RTase	-	+	-	+	-	+	-	+

Fig. 4. PCR of total RNA from human liver 3. Total RNA were either reverse transcribed (lanes 2, 4, 6 and 8) or not (lanes 1, 3, 5 and 7). The product obtained was amplified with primers J, K (lanes 1, 2) D–B (lanes 3, 4), D–I (lanes 5, 6) and C–F (lanes 7, 8), respectively.

Table 2. Cytochrome CYP2E1 enzyme activity in microsomes isolated from various human adult tissues

Parameter	Liver			Placenta		Lung	Skin	Neurinoma
Sample number	1	2	3	4	5	10	6	Pool
HCHO formation (nmol/min/mg)	0.53 ± 0.05	0.53 ± 0.03	0.59 ± 0.05	0.14 ± 0.03	0.12 ± 0.02	0.20 ± 0.03	ND	ND

CYP2E1 catalytic activity was expressed as nmol of formaldehyde/min/mg of protein. Assays were done on three different preparations of microsomes. Values are expressed as means ± SD. ND = non detected values.

active in oxidizing NDMA than microsomes from human lung and placenta, respectively. The results of NDMA metabolism in our human liver samples agreed with earlier values [15]. Skin and neurinoma showed no detectable NDMA demethylase activity. It should be noted that NDMA activity was present only in tissues where the 1.8 Kb mRNA was detected. A similar variation between the level of CYP2E1 mRNA and protein may be the consequence of transcriptional regulation of the human *CYP2E1* gene. A similar mechanism of regulation of the *CYP2E1* gene had previously been observed in cultured rabbit hepatocytes treated with acetone. Indeed, Kraner *et al.* [32] demonstrated increased rates of *de novo* CYP2E1 protein synthesis as well as enhanced levels of CYP2E1 mRNA. To investigate whether enhanced NDMA activity was accompanied by increases in CYP2E1 protein, immunohistochemical detection was performed in liver, lung, and neurinoma samples with an antibody raised against the rat orthologue CYP2E1. Light microscopic examination of the human tissue sections is shown in Fig. 5. A strong specific immunoreactivity (as checked by comparing serial sections incubated with anti-CYP2E1 antibody and non-immune IgG) was present in liver (Fig. 5E). In the lung (Fig. 5C), the staining was essentially localized in the alveolar macrophage cytoplasm; a slight labelling was also observed in endothelial cells as well as in some epithelial cells delineating the alveoli. No immunoreactivity was detected in the neurinoma (Fig. 5A) section. As expected, no specific staining was observed with the non-immune IgG (Fig. 5B, D, F). The above results reveal that the difference in NDMA demethylase activity was accompanied by changes in CYP2E1 protein concentration, as visualized by the immunohistochemical analysis.

Methylation status of the CYP2E1 *gene*

To assess the role of DNA cytosine methylation in the expression of the *CYP2E1* gene, we performed Southern blot analysis of genomic DNA from liver, lung and placenta, using the same probe as in Fig. 1. The two isoschizomers Hpa II and Msp I,

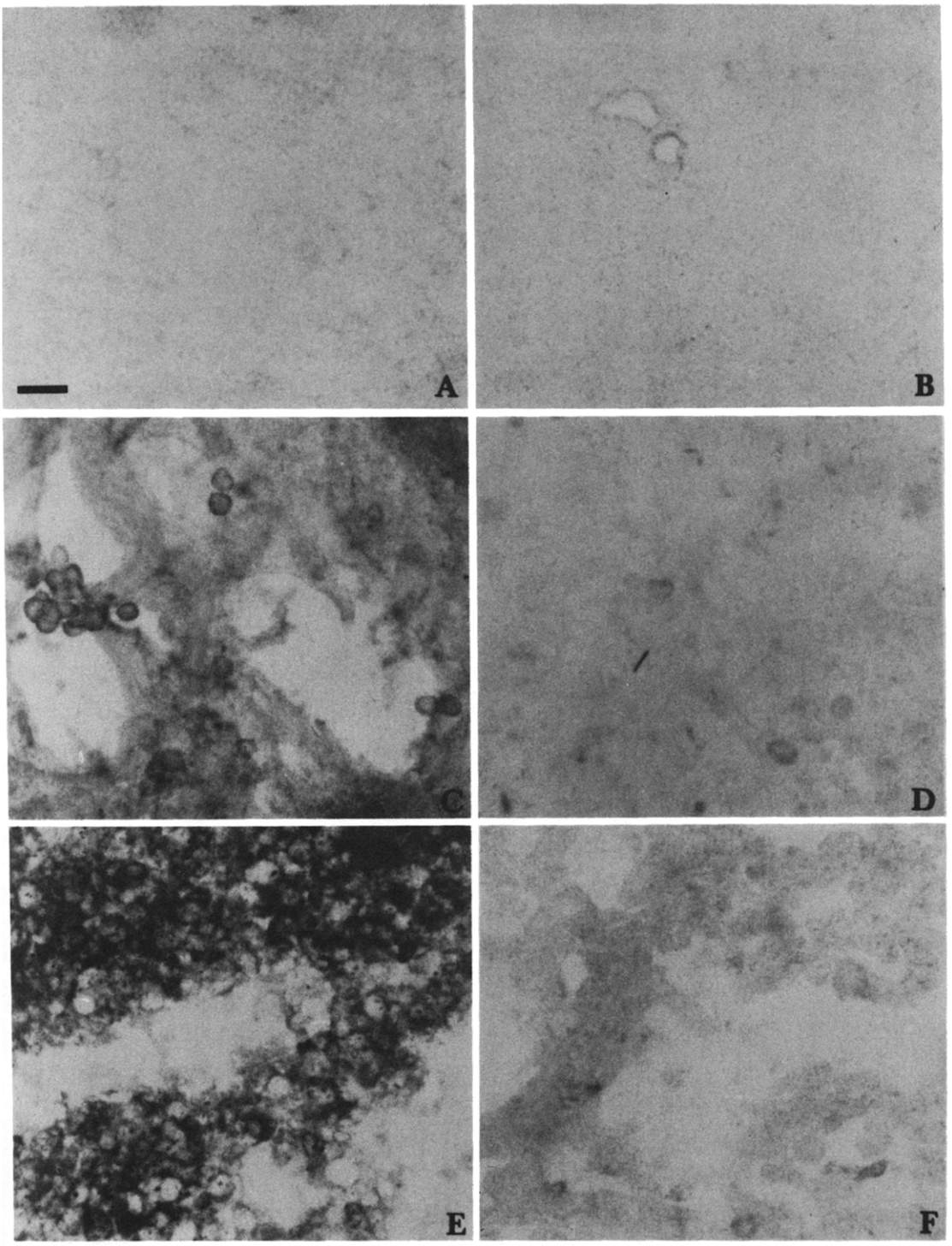


Fig. 5. Immunolocalization of CYP2E1 in various human tissues. Human neurinoma (A, B), lung (C, D) and liver (E, F) sections were incubated either with antibodies raised against the rat orthologous CYP2E1 (A, C, E) or with rabbit non-immune IgG (B, D, F), then reacted with anti-rabbit antibodies conjugated with alkaline phosphatase. Bar = 50 μm.

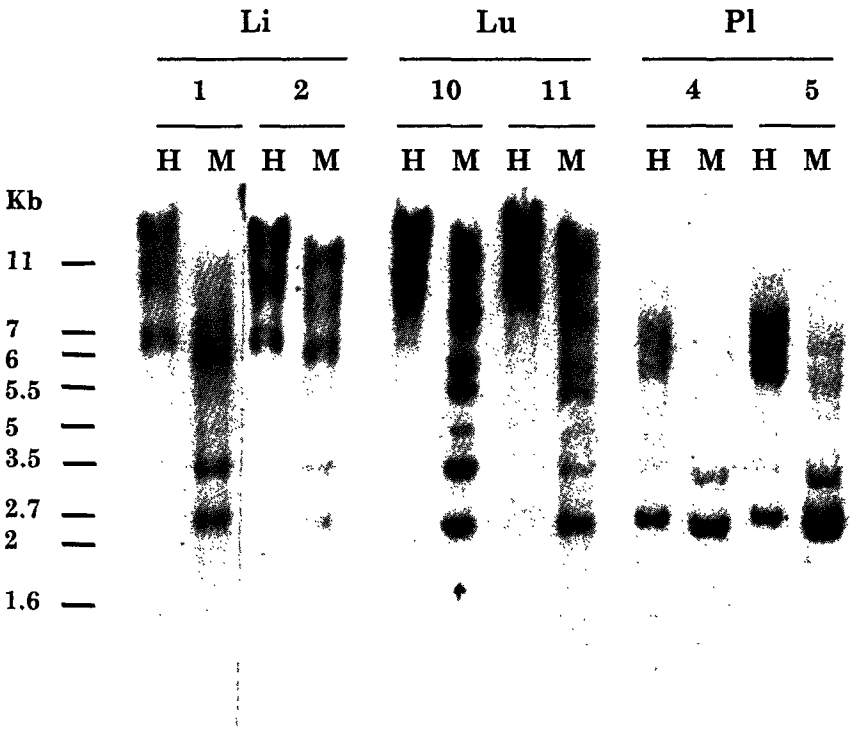


Fig. 6. Methylation status of the 3′ end of the human *CYP2E1* gene. Ten micrograms of genomic DNA, prepared from human liver (Li), placenta (Pl) and lung (Lu), were digested with Hpa II (H) or Msp I (M). Digested DNA were electrophoresed on a 1% agarose gel, blotted onto Hybond N-filters, hybridized with probe A as described in Materials and Methods and exposed to autoradiographic film. Molecular weights are expressed in Kilobases (Kb).

which recognized the same sites 5′-CCGG-3′ (C, cytosine; G, guanine) were used. It is well known that CG methylation allows digestion by Msp I which cuts the sequence only if the external cytosine residue is unmethylated, regardless of the state of methylation of the internal cytosine. CC methylation allows digestion by Hpa II, which cleaves the sequence only if the internal cytosine is unmethylated, regardless of the state of methylation of the external cytosine [33]. The result shown in Fig. 6 clearly reveals that the pattern of methylation of the *CYP2E1* gene was identical within the same tissue of various humans. A similar result was obtained by Jones *et al.* [9] in liver DNA of human adult and foetuses of different gestational ages. After Msp I digestion, two major components of 2.7 and 3.5 Kb were detected in liver, lung and placenta. These two bands were only detected in placenta DNA digested with Hpa II, suggesting that a CG-type demethylation of the *CYP2E1* gene exists in this tissue. Liver and lung DNA, Msp I digested, give qualitatively the same pattern. However, in liver compared to lung, the 5 and 5.5 Kb bands are weaker with a relative intensification of a 6 Kb band. Furthermore, the same DNA digested by Hpa II showed, in addition to the bands detected in both liver and lung, the presence in liver of an extra signal at 7 Kb. These results suggest that CC and CG-type demethylations of the *CYP2E1* gene are present in lung compared to liver. In conclusion we observed that the *CYP2E1* gene is methylated differently in the various tissues tested—most in liver and least in placenta. Since we found a similar pattern in CYP2E1 mRNA level and enzymatic activity, we suggest that CC and CG methylations are associated, at least in part, with the expression of this gene.

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