Stable Expression of Human Cytochrome P4502E1 in HepG2 Cells: Characterization of Catalytic Activities and Production of Reactive Oxygen Intermediates†

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ABSTRACT: Experiments were carried out to stably and constitutively express the coding sequence of the human cytochrome P4502E1 in HepG2, a human-hepatoma-derived cell line, by recombinant retroviral expression. Southern blot analysis showed a successful integration of a single copy of unaltered viral DNA into the genome of each transduced clone tested. Northern blot analysis showed that the transduced clones produced an RNA species which hybridized to the CYP2E1 cDNA probe. Western blot analysis using anti-human P4502E1 IgG indicated that the transduced clones produced a protein band with molecular weight of 54 000. Microsomes from transduced clones were catalytically active with *p*-nitrophenol, dimethylnitrosamine, aniline, and ethanol as substrates; little or no activity was found with control clones. Oxidation of *p*-nitrophenol was inhibited by anti-human P4502E1 IgG, diethyl dithiocarbamate, 4-methylpyrazole, and ethanol. ESR spectroscopy showed that microsomes from clone MV2E1-9 produced superoxide radical. Rates were an order of magnitude higher than that for control microsomes, most likely reflecting the loose coupling associated with P4502E1. The rate of H_2O_2 production by microsomes from MV2E1-9 was 2-fold greater than that of control clones. The elevated rate of H_2O_2 production in clone MV2E1-9 is about half the rate of superoxide radical production, suggesting that this H_2O_2 is largely derived from superoxide radical dismutation. Microsomal lipid peroxidation was determined using ferric-ATP as the iron catalyst. When the concentration of iron was "high" (0.025 mM), rates of production of thiobarbituric acid reactive components were identical for microsomes from MV2E1-9 and control clones. However, when the concentration of iron was lowered to 0.005 mM, control clones did not display lipid peroxidation, whereas microsomes from MV2E1-9 were reactive. This peroxidation was sensitive to antioxidants such as trolox, propyl gallate, and glutathione but not to catalase or superoxide dismutase. Rates of superoxide and H_2O_2 production and of lipid peroxidation were 7–20-fold higher on a per nanomole of P450 basis with clone MV2E1-9 compared to human liver microsomes, indicating that the human P4502E1 is especially reactive in production of reactive oxygen intermediates and in catalysis of lipid peroxidation.

The cytochromes P450 are a superfamily of hemoproteins that serve as the terminal oxidases in the mixed function oxidase system for metabolizing various endogenous substrates such as steroids and xenobiotics such as drugs, toxins, and carcinogens (Guengerich, 1987). Cytochrome P4502E1 (P4502E1),[1] the ethanol-inducible form, is of interest because of its ability to metabolize and activate many toxicologically important substrates including ethanol, carbon tetrachloride, acetaminophen, and *N*-nitrosodimethylamine (DMN) to more toxic products (Guengerich et al., 1991; Koop, 1992; Yang et al., 1991). The human P4502E1 was isolated by immunoaffinity purification or by chromatography (Wrighton et al., 1986, 1987; Lasker et al., 1987) and shown to have a relative molecular weight of 54 000 and to oxidize typical P4502E1 substrates such as DMN, ethanol, and aniline (Wrighton et al., 1987; Lasker et al., 1987).

P4502E1 from rat and rabbit liver exhibits enhanced NADPH oxidase activity as it appears to be poorly coupled with NADPH–cytochrome P450 reductase (Gorsky et al., 1984; Ekstrom & Ingelman-Sundberg, 1989). P4502E1 was shown to be an effective catalyst for lipid peroxidation, relative to several other forms of P450 (Ekstrom & Ingelman-Sundberg, 1989). Microsomes from ethanol-treated rats, in which P4502E1 is induced, display elevated rates of production of superoxide ($O_2^{\bullet-}$) and H_2O_2 (Ekstrom & Ingelman-Sundberg, 1989; Boveris et al., 1983; Lieber & DeCarli, 1970; Thurman, 1973) and, in the presence of certain iron complexes, enhanced production of hydroxyl radical ($^{\bullet}OH$), increased rates of lipid peroxidation and chemiluminescence, and increased inactivation of metabolic enzymes by oxidative attack (Ekstrom & Ingelman-Sundberg, 1989; Klein et al., 1983; Dicker & Cederbaum, 1987, 1988; Rashba-Step et al., 1993; Ekstrom et al., 1986; Krikun & Cederbaum, 1986; Puntarulo & Cederbaum, 1988a). Elevated NADPH oxidase activity and lipid peroxidation were found to correlate with the content of P4502E1 (Ekstrom & Ingelman-Sundberg, 1989; Castillo et al., 1992). Production of reactive oxygen intermediates by human P4502E1 has not been characterized.

To evaluate the production of reactive oxygen intermediates by human P4502E1 in its microsomal environment, and the consequences of such production, the establishment of a stable

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[1] Abbreviations: PNP, *p*-nitrophenol; DMN, *N*,*N*-dimethylnitrosamine; $O_2^{\bullet-}$, superoxide anion radical; $^{\bullet}OH$, hydroxyl radical; DDC, diethyl dithiocarbamate; 4-MP, 4-methylpyrazole; GSH, glutathione; SOD, superoxide dismutase; TBA, thiobarbituric acid; TCA, trichloroacetic acid; P4502E1, cytochrome P4502E1; CYP2E1, the gene coding for cytochrome P4502E1; LTR, long terminal repeat; ESR, electron spin resonance; NEO, neomycin phosphotransferase gene; DMEM, Dulbecco's modified Eagle's medium; MEM, minimum essential medium; 4-oxo-TEMPO, 4-oxo-2,2,6,6-tetramethylpiperidinyloxy free radical.

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cell line constitutively expressing the enzyme is important. A human liver cell line which does not produce significant amounts of P450 would appear to be desirable for expression of the human P4502E1, free from contributions by other forms of P450. A number of systems have been used to express mammalian cytochrome P450 including bacteria, yeast, insect cells, and mammalian cells mediated by transfection of plasmid DNA or infection of recombinant viruses including *Vaccinia* virus, baculovirus, and retrovirus in either transient or stable fashion (Waterman & Johnson, 1991). The recombinant retrovirus mediated transduction method introduced by Battula seems to be a powerful system to stably express P450 in mammalian cells because of its precise transmission of the recombinant viral DNA sequences into the target cells (Battula, 1989). Using recombinant retroviral infection, Battula described the establishment of a stable NIH3T3 cell line which constitutively expressed mouse CYP1A2 (P_3-450) (Battula, 1989).

The current paper describes the establishment of a cell line which stably and constitutively expresses the coding sequence of the human CYP2E1 into HepG2, a human hepatoma-derived cell line which does not express detectable P4502E1 (Waxman et al., 1991; Patten et al., 1992). Catalytic activity of the expressed enzyme with typical P4502E1 substrates and sensitivity to inhibitors was characterized. The production of reactive oxygen intermediates and iron-dependent lipid peroxidation were measured and results compared to that of human liver microsomes to validate the loose coupling associated with P4502E1.

EXPERIMENTAL METHODS

Materials. Restriction enzymes, DNA ligase, and Klenow enzyme were from New England Biolabs, Inc. Bacto-tryptone, yeast extract, and bacto-agar for bacterial culture were purchased from Difco Laboratories. Media for tissue culture and their supplements including G418 were purchased from Gibco/BRL. Aniline, PNP, fetal calf serum, DDC, and polybrene were from Sigma Chemical Co.; DMN, 4-MP, and 4-oxo-TEMPO were from Aldrich Chemical Co. Ethanol was from Fisher America, Inc., and NADPH and catalase were from Boehringer Mannheim Corp. For the ESR experiments, the phosphate buffer and the water used to prepare solutions were passed through Chelex 100 resin to remove metal impurities.

Cell Lines and Culture Conditions. The NIH3T3-derived amphotropic retrovirus packaging cell line, PA317 (Miller & Buttimore, 1986), was provided by Dr. H. Stuhlmann (Mount Sinai School of Medicine, New York). The HepG2 cell line (Aden et al., 1979) was provided by Dr. G. Acs (Mount Sinai School of Medicine). PA317 and HepG2 were grown in DMEM and MEM, respectively, supplemented with 1% PSN antibiotics mixture (Gibco) and 10% fetal calf serum.

Plasmid and DNA Preparation. Plasmid p91023(B)-IIE1 (Umeno, 1988), containing a full-length human CYP2E1 complementary DNA, was kindly provided by Dr. F. J. Gonzalez (National Cancer Institute, Bethesda, MD). The retrovirus shuttle vector, pMV7 (Kieschmeier et al., 1988), was kindly provided by Dr. R. S. Krauss (Mount Sinai School of Medicine). This Moloney murine leukemia virus based plasmid contains an RNA packaging signal, a modified herpes simplex virus thymidine kinase (TK) promoter driven bacterial NEO gene for selecting stable expression clones, and an *Eco*RI cloning site downstream to the 5′ LTR promoter. All plasmids for transfection were prepared by a standard alkaline lysis method followed by CsCl equilibrium density gradient centrifugation (Sambrook et al., 1989).

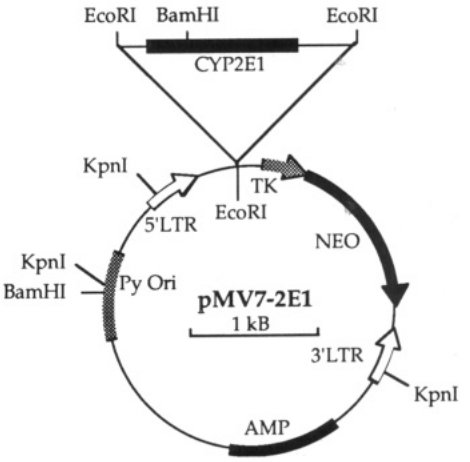

FIGURE 1: Restriction map and construction strategy of plasmid pMV7-2E1. The LTRs of the construct are shown as open arrows. The transcription initiation site and direction of the 5′ LTR (open arrow) and the TK (stippled arrow) promoter are shown. Bacterial NEO gene is shown as a solid arrow. Full-length human CYP2E1 cDNA was inserted into the *Eco*RI site of plasmid, pMV7; solid box represents the coding region.

Transduction of CYP2E1 in HepG2. The full-length human CYP2E1 cDNA insert was released by digesting p91023-(B)-IIE1 with *Eco*RI and was subcloned into the *Eco*RI site in pMV7 (Figure 1). Plasmid pMV7-2E1, the subclone with the cDNA insert in the sense orientation (as assured by restriction mapping), and pMV7 were used to transfect the PA317 packaging cell line to generate stable transfected pools producing MV2E1 virus with the CYP2E1 insert and the control MV virus (lacking the CYP2E1 insert), respectively. Transfection of PA317 was carried out according to the calcium phosphate method (Ausubel et al., 1990) with minor modifications. Briefly, 20 μg of either plasmid DNA was precipitated with 125 mM calcium phosphate in 19 mM HEPES buffer (pH 7.08) and then applied onto about 30% confluent PA317 cells in 90-mm dishes for 5 h. Transfected cells were grown to confluence and then split into selecting DMEM medium which contained 0.5 mg/mL G418. Resistant colonies were formed in about 10 days. Pooled clones were grown to subconfluence before changing to DMEM medium without G418 for 2 days. Virus stock was prepared by harvesting and sterilizing the medium through a 0.45-μm microfilter and was kept at −70 °C.

Virus infection of HepG2 cells was carried out by applying 3 mL of undiluted MV2E1 virus stock or 1:10 diluted MV control virus stock plus 8 mg of polybrene/mL onto 30% confluent HepG2 cells in 90-mm dishes for 3 h. Infected cells were grown to confluence before splitting into selecting MEM medium containing 0.4 mg/mL G418. Resistant colonies were formed in about 2 weeks. Colonies were isolated by ring isolation, grown to large scale, and maintained in MEM medium containing 0.2 mg/mL G418.

Microsomal Preparation. Stable-transduced HepG2 clones were grown in MEM medium containing 0.2 mg/mL G418 in the presence of 2 mM 4-MP until confluence. The 4-MP was added as a ligand to stabilize the expressed P4502E1 protein (Larson et al., 1991a,b). During screening of mixed clones by immunoblots, it was observed that P4502E1 content was higher when 4-MP was present in the medium. Cells were washed once with phosphate-buffered saline (pH 7.4) and harvested by scraping and subsequent sonication for 45 s. Microsomes were prepared by differential centrifugation, resuspended in 0.125 M KCl–0.01 M phosphate (pH 7.4) buffer containing 20% glycerol, and kept at −70 °C.

Blot Analyses. Western blot analysis (Laemmli, 1970; Towbin et al., 1979) was carried out by separating proteins on a 10% running PAGE gel and electrophoretically transferring onto a nitrocellulose membrane. Immunoblots were developed using either anti-rat P4502E1 (Palakodety et al., 1988) or an anti-human P4502E1 polyclonal antibody provided by Dr. J. M. Lasker (Bronx VA Medical Center, New York) and alkaline phosphatase conjugated goat anti-rabbit IgG as the second antibody (Promega). Genomic DNA was extracted from HepG2 clones according to a standard DNA extraction method (Sambrook et al., 1989). Southern blot analysis (Southern, 1975) was carried out by digesting 10 μg of genomic DNA with *Bam*HI or *Kpn*I, electrophoresing on a 0.6% agarose gel, and hybridizing the transferred DNA with the ^{32}P-labeled human CYP2E1 cDNA probe. Poly(A) RNA was isolated using an mRNA purification kit (Pharmacia). Northern blot analysis was carried out as previously described (Winters & Cederbaum, 1992a) using 2 μg of poly(A) RNA and hybridization with either NEO or CYP2E1 cDNA probes. All probes were labeled to high specific activity ($0.5–2 \times 10^9$ cpm/μg) with [α-^{32}P]dCTP according to the random primer labeling method (Feinberg & Vogelstein, 1983).

Enzyme Assays. Enzymatic activity of the transduced clones was determined in the microsomal fraction with 0.2 mM PNP, 4 mM DMN, 1 mM aniline, or 100 mM ethanol as substrate. All reactions were carried out in duplicate in 0.1 M potassium phosphate buffer (pH 7.4); reactions were initiated with NADPH (final concentration of 1 mM) at 37 °C and stopped usually after 60 min by the addition of 0.3 volume of 20% TCA (1 N HCl for the ethanol reaction). Final products of the reactions of PNP, DMN, and aniline were measured by standard colorimetric methods. Acetaldehyde produced from ethanol was measured according to a head-space gas chromatography procedure. Inhibition by antibodies was carried out by preincubation of the antibody with the microsomal system for 5 min at 37 °C before NADPH was added (Clejan et al., 1989).

For *in situ* PNP metabolism, all clones were grown in the complete MEM medium without phenol red (Gibco) until confluence, before sterile PNP (final concentration of 0.4 mM) was added into the dishes for 24 h. The production and release of *p*-nitrocatechol into the medium were assayed.

Superoxide Radical Generation Assay. Superoxide production was determined by assaying the generation of stable nitroxyl radicals formed in the reaction of hydroxylamines with $O_2^{\bullet-}$ (Rosen et al., 1982). The hydroxylamine, 1-hydroxy-2,2,6,6-tetramethyl-4-oxopiperidine, was synthesized and purified (Rashba et al., 1990) and dissolved in triply distilled, Chelex-treated water. Microsomes (usually 0.9 mg/mL) were incubated in 100 mM potassium phosphate buffer plus 2.5 mM of the hydroxylamine at room temperature. Reactions were initiated by the addition of NADPH to a final concentration in the solution of 1.2 mM. The samples were immediately transferred to a WG-812Q flat quartz cuvette (Wilmad), and spectra were recorded on a Bruker ESR 300 spectrometer equipped with a 4102ST probe. Kinetics of the reactions were monitored as a function of time by measuring the increase of the intensity of the second component of the three-line nitroxyl spectrum. A standard spectrum was recorded with a stable nitroxide radical, 4-oxo-TEMPO. Instrument settings for the spectra were as follows: sweep width 70.0 G, sweep time 20.97 s, modulation amplitude 0.5 G, microwave power 11 mW. Reactions were carried out in the absence and presence of superoxide dismutase (SOD) to calculate the rate of superoxide radical production.

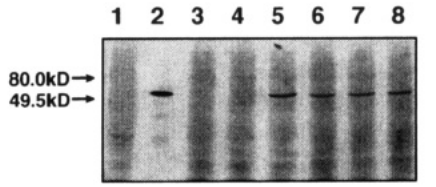

FIGURE 2: Western blot analysis using anti-human P4502E1 IgG for detection of P4502E1 expression in the transduced HepG2 clones. Forty micrograms (4 μg for human liver microsomes) of microsomal protein was loaded onto each lane. (Lane 1) HepG2 cells; (lane 2) human liver microsomes; (lanes 3 and 4) MV-3 and -5 control clones; (lanes 5–8) MV2E1-9, -15, -19, and -37, respectively.

Assay for Production of Hydrogen Peroxide. The production of hydrogen peroxide was determined by measuring the formation of formaldehyde from the oxidation of methanol by the catalase compound I complex (Hildebrandt et al., 1978). Reactions were carried out in 200 mM KCl, 50 mM Tris (pH 7.4), 100 mM methanol, 300 units of catalase (or 1 mM sodium azide), and about 0.25 mg of microsomal protein in a final volume of 100 μL. Reactions were initiated by the addition of NADPH (1.2 mM final concentration) and terminated after 60 min by the addition of 30 μL of 20% TCA. The generation of formaldehyde was determined by the Nash reaction (Nash, 1953). Rates of H_2O_2 production were calculated from the net difference in formaldehyde production between samples incubated in the presence of azide but without catalase (to evaluate formaldehyde arising from $^{\bullet}$OH-dependent or cytochrome P450-dependent oxidation of methanol) from the samples incubated in the absence of azide but in the presence of catalase.

Measurement of Lipid Peroxidation. Lipid peroxidation was assayed by measuring production of TBA-reactive metabolites (Buege & Aust, 1978). Experiments were performed in Eppendorf tubes containing 50 mM Tris-HCl, pH 7.4, varying amounts of Fe-ATP, 1 mM NADPH, and about 2.5 mg/mL microsomes in a final volume of 0.1 mL. Ferric-ATP was used at a 1:20 ferric:ATP ratio. Reactions were initiated by adding NADPH and terminated after incubation at 37 °C for 30 min by addition of 0.3 volume of 30% ice-cold TCA. After centrifugation, aliquots of the supernatant were added to an equal volume of TBA (7.3 mg/mL) and heated at 100 °C for 10 min. After the mixture cooled on ice for 2 min, absorbance was determined at 535 nm and the concentration of TBA-reactive product was calculated using an extinction coefficient of 156 mM^{-1} cm^{-1} (Buege & Aust, 1978).

RESULTS

Analysis of P4502E1 Protein in Transduced Clones. After infection of HepG2 cells with the recombinant retrovirus MV2E1 and subsequent G418 selection, 38 clones were isolated and grown separately. Western blot analysis was carried out to measure the expression level of P4502E1 protein in the microsomal fraction of these clones. Twenty of these clones expressed varying amounts of P4502E1 protein, and four of them (MV2E1-9, -15, -19, and -37) appeared to express the most. All four clones produced a protein band at 54 kDa (Figure 2, lanes 5–8) that migrated at the same position as the P4502E1 band from human liver microsomes (lane 2, Figure 2). All control HepG2 clones (infected with virus lacking the 2E1 insert) tested (two are shown in Figure 2, lanes 3 and 4) as well as uninfected HepG2 cells (lane 1) did not produce this band.

Analysis of the Integrated CYP2E1 DNA in the Transduced Clones. To demonstrate that the CYP2E1 cDNA was

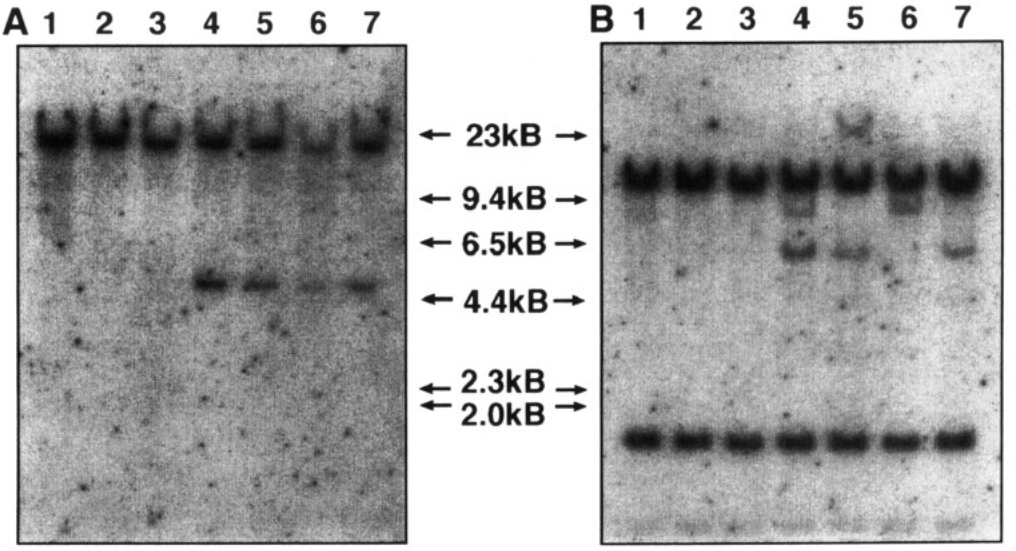

FIGURE 3: Southern blot analyses of CYP2E1 transduced clones. Ten micrograms of genomic DNA which was extracted from the transduced clones and HepG2 cells was digested with *Kpn*I (A) or *Bam*HI (B) and loaded onto each lane. The integrated viral DNA was identified by hybridization with a ^{32}P-labeled human CYP2E1 cDNA probe. For both (A) and (B): (lane 1) HepG2 cells; (lanes 2 and 3) clones MV-3 and -5, respectively; (lanes 4–7) clones MV2E1-9, -15, -19, and -37, respectively.

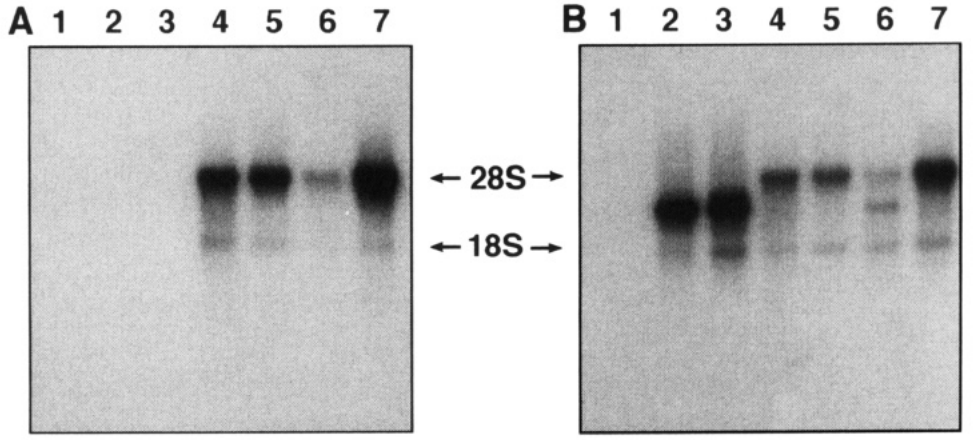

FIGURE 4: Northern blot analyses of CYP2E1 transduced clones. Two micrograms of poly(A) RNA isolated from the transduced clones and HepG2 cells was run on a 1% denaturing agarose gel. Viral RNA transcripts were identified by hybridization with either a ^{32}P-labeled human CYP2E1 cDNA probe (A) or bacterial NEO DNA probe (B). (Lane 1) HepG2 cells; (lanes 2 and 3) control clones MV-3 and -5, respectively; (lanes 4–7) clones MV2E1-9, -15, -19, and -37, respectively.

successfully integrated into the transduced clones, Southern blot analysis was carried out. DNA isolated from different clones and HepG2 cells was digested with *Kpn*I, which cuts once within each LTR (Kieschmeier et al., 1988) (Figure 1), and hybridized with a human CYP2E1 cDNA probe. The result showed one band at about 4.8 kb (Figure 3A), which was present in all four MV2E1 (lanes 4–7) clones but absent in the control MV clones (lanes 2 and 3) or HepG2 cells (lane 1), indicating no gross rearrangement or truncation of the integrated DNA copy. Southern blot analysis of the same set of DNA digested with *Bam*HI, which cuts once in the CYP2E1 cDNA (Umeno et al., 1988) but not anywhere else in the integrating (LTR to LTR) region of the vector (Kieschmeier et al., 1988) (Figure 1), showed no more than two exogenous bands in the MV2E1 clones (lanes 4–7, Figure 3B), indicating only one copy of the CYP2E1 cDNA was introduced into the genome in all four MV2E1 clones. The different banding patterns for the four clones probably reflects the random insertion of the integrating region into the host chromosome. The other bands present in all lanes in Figure 3 result from the endogenous copy of the CYP2E1 gene and appear to be consistent with the restriction map of the CYP2E1 genomic sequence (Umeno et al., 1988).

Northern Blot Analysis. To evaluate for the presence of P4502E1 RNA, Northern blot analysis was carried out using transduced MV2E1 clones and control MV clones as well as HepG2 cells. Only the transduced MV2E1 clones showed a band as detected by the CYP2E1 cDNA probe (lanes 4–7, Figure 4A). Neither control clones (lanes 2 and 3) nor HepG2 cells (lane 1, Figure 4A) showed detectable P4502E1 RNA. The lower bands on this blot may be due to a splicing event from the cryptic termination or polyadenylation sites at the 3′ end of the CYP2E1 cDNA insert (Kieschmeier et al., 1988; Song et al., 1986). Northern blot analysis with the same set of RNA but hybridized with a NEO gene probe detected bands in both MV2E1 transduced clones and control MV clones but not in HepG2 cells (Figure 4B). The difference in the transcripts found with the MV2E1 clones (lanes 4–7, Figure 4B) compared to that with control MV clones (lanes 2 and 3, Figure 4B) is in accord with the 1.7-kb CYP2E1 cDNA insert. Initiation at the TK promoter probably generates the low molecular weight RNA species (migrating at the approximate 18S region) detected by the NEO probe. None of the clones nor HepG2 cells expressed detectable P4502E1 mRNA from the endogenous copy of CYP2E1 gene.

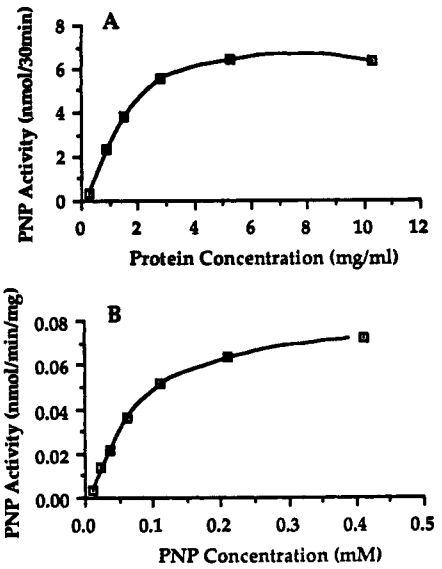

FIGURE 5: *In vitro* metabolism of PNP by microsomes from the CYP2E1 transduced clone MV2E1-9. Experiments were carried out to characterize the protein (A) and substrate dependence (B) of *p*-nitrophenol hydroxylase activity. The PNP concentration was 0.2 mM for (A), while microsomal protein was 2.5 mg/mL for (B). Reaction times were either 30 (A) or 45 (B) min.

Quantitation of P450 in MV2E1-9 Clone. CO-reduced difference spectroscopy was used to attempt to measure the P450 level in the transduced clones. Very low levels of total P450 appeared to be present, necessitating the use of high amounts of protein. Turbidity interference, however, made the spectral quantitation inaccurate. Therefore, P4502E1 apoprotein was quantitated by Western blot analysis after a standard curve was prepared with purified human P4502E1 (kindly provided by Dr. J. Lasker, Bronx VA). The final P4502E1 content in clone MV2E1-9 was estimated to be 10 pmol/mg of microsomal protein.

Enzyme Activity of the Transduced Clone. A complete *in vitro* system with microsomes from the MV2E1-9 clone, PNP, and NADPH catalyzed the formation of *p*-nitrocatechol in a linear reaction for at least 30 min (data not shown). PNP oxidation was dependent on the amount of microsomal protein up to about 3 mg of protein/mL (Figure 5A) and substrate concentration (Figure 5B). A Lineweaver–Burk plot of the substrate dependence was linear with an apparent K_m for PNP of about 0.11 mM and an apparent V_{max} of about 0.1 nmol min^{-1} (mg of microsomal protein)$^{-1}$. This would be equivalent to a V_{max} of about 10 nmol min^{-1} (nmol of P4502E1)$^{-1}$. Previous results on oxidation of PNP by rat or rabbit P4502E1 reported V_{max} values of 5.7–10 nmol min^{-1} (nmol of P4502E1)$^{-1}$ and K_m values ranging from 0.015 to 0.060 mM (Koop, 1986; Kim et al., 1988).

Oxidation of PNP was weakly inhibited by the anti-rat P4502E1 IgG but was very sensitive to inhibition by anti-human P4502E1 IgG (Table I). Preimmune IgG had no effect. The potent inhibition by the anti-human IgG validates that the PNP catalytic activity was due to the expressed P4502E1. Inhibition was found in the presence of miconazole, a general inhibitor of P450-catalyzed activity and DDC, which at a concentration of 0.1 mM has been shown to be relatively specific for inhibition of P4502E1-dependent activity (Guengerich et al., 1991; Brady et al., 1991). Inhibition was also found in the presence of ethanol, added as a competitive substrate, and 4-MP, a good ligand for P4502E1 (Table I).

Besides PNP, microsomes from MV2E1-9 were also capable of oxidizing aniline to *p*-aminophenol, DMN to formaldehyde, and ethanol to acetaldehyde (Table II). These are effective substrates for rodent and human P4502E1 (Koop, 1992; Yang et al., 1991; Lasker et al., 1987). Oxidation of DMN by MV2E1-9 (Table II) and by human liver microsomes [0.25 nmol min^{-1} (mg of microsomal protein)$^{-1}$] was about 3-fold lower than rates of PNP oxidation by the clone (Table II) and human liver microsomes [0.87 nmol min^{-1} (mg of microsomal protein)$^{-1}$]. These results differ from the comparable PNP and DMN oxidizing activities found with liver microsomes from pyrazole-induced rats (data not shown), suggesting that the human 2E1 may be a better catalyst of PNP oxidation relative to DMN oxidation as compared to the rat 2E1.

The three other clones which were shown to express P4502E1 protein (Figure 2) and mRNA (Figure 4) were also catalytically active with PNP (Table II). However, the control clones which were infected with virus lacking the 2E1 insert were essentially inactive in oxidizing PNP (Table II). Control MV-5 was also ineffective in oxidizing DMN, ethanol, and aniline (Table II).

The ability of intact HepG2 clones to oxidize PNP was determined. Transduced clones 9 and 15 and control clones 3 and 5 were incubated with PNP for 24 h, and the medium was assayed for secreted *p*-nitrocatechol. Experiments were also carried out in the presence of 4-MP to inhibit any observable activity and thus link the activity to P4502E1. 4-MP-sensitive PNP oxidation activity was at least an order of magnitude higher with the transduced clones; rates [nmol min^{-1} (mg of protein)$^{-1}$] were less than 0.2 for clones MV-3 and MV-5 and 2.1 for clones MV2E1-9 and -15, respectively.

Table I: Effect of Various Additions on the Oxidation of PNP by Microsomes from Clone MV2E1-9[a]

addition	concn (mg/nmol of P450) or (mM)	oxidation of PNP [nmol min^{-1} (mg of protein)$^{-1}$] preimmune	anti-rat 2E1	anti-human 2E1
A. control antibody	0	0.059 (100)	0.059 (100)	0.048 (100)
	1	0.060 (102)	0.048 (81)	0.021 (44)
	2	0.059 (100)	0.041 (69)	0.013 (27)
	4	0.058 (98)	0.039 (66)	0.008 (17)
	8	0.059 (100)	0.036 (61)	0.004 (8)
B. control		0.060 (100)		
miconazole	0.2	0.032 (7)		
DDC	0.1	0.004 (7)		
4-MP	1.0	0.005 (8)		
ethanol	100.0	0.012 (20)		

[a] The oxidation of PNP by microsomes from clone MV2E1-9 was determined as described under Experimental Methods in the presence of (A) the indicated amounts of antibody (preimmune, anti-rat P4502E1 IgG, or anti-human P4502E1 IgG) or (B) the millimolar concentrations of the indicated compounds. Numbers in parentheses refer to percent control activity. All reactions were carried out in duplicate.

Table II: Oxidation of Substrates by Microsomes from CYP2E1 Transduced and Control Clones[a]

clone	substrate oxidation [nmol min^{-1} (mg of protein)$^{-1}$] PNP	DMN	aniline	ethanol
MV-3	0.002			
MV-5	0.004	<0.002	<0.002	<0.005
MV2E1-9	0.059	0.018	0.019	0.085
MV2E1-15	0.025			
MV2E1-19	0.032			
MV2E1-37	0.066			

[a] The oxidation of 0.2 mM PNP, 4 mM DMN, 1 mM aniline, or 100 mM ethanol was carried out as described under Experimental Methods using microsomes from clones infected with virus lacking the P4502E1 insert (MV-3, MV-5) or containing the P4502E1 insert (MV2E1-9, -15, -19, and -37). All reactions were carried out in duplicate.

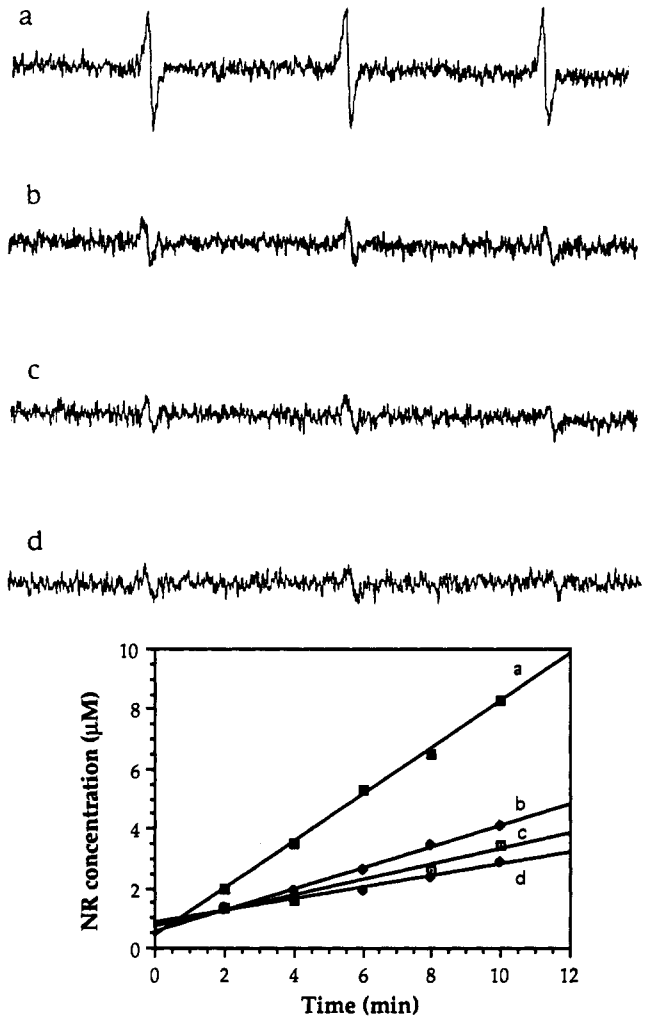

FIGURE 6: Nitroxyl radical (NR) production from the interaction of $O_2^{\bullet-}$ with 1-hydroxy-2,2,6,6-tetramethyl-4-oxopiperidine. (A, Top) ESR spectra with microsomes from MV2E1-9 in the absence (a) and presence (b) of superoxide dismutase or with microsomes from MV-5 control clone in the absence (c) and presence (d) of superoxide dismutase. Results show the spectra obtained after 10 min of reaction. (B, Bottom) Time course for the production of nitroxyl radical.

Superoxide and H_2O_2 Production. Hydroxylamines react with $O_2^{\bullet-}$ to produce a relatively stable nitroxyl radical which can readily be detected by ESR (Rosen et al., 1982). Incubation of microsomes from clone MV2E1-9 with NADPH plus the hydroxylamine detector resulted in a three-line signal with splitting characteristics identical to those of the 4-oxo-TEMPO standard (Figure 6A-a); the resulting triplet displayed the following constants: $A_N = 16.0$ G and $g = 2.0050$. This signal was strongly inhibited (60–65%) by added SOD (Figure 6A-b), indicating that it was largely $O_2^{\bullet-}$-dependent. The control MV-5 clone displayed a weak signal, barely discernible above background levels (Figure 6A-c). By following the kinetics of the intensity of the middle peak as a function of time, and from the signal intensity of the standard 4-oxo-TEMPO, rates of $O_2^{\bullet-}$ production were linear over a 10-min time period (Figure 6B) and were calculated (SOD-sensitive portion) to be about 0.03 and 0.30 nmol min^{-1} (mg of protein)$^{-1}$ for microsomes from MV-5 and MV2E1-9, respectively. In microsomes from MV2E1-9, the addition of 100 mM ethanol, 0.2 mM PNP, or 2 mM 4-MP did not alter the control rate of $O_2^{\bullet-}$ production (data not shown).

The activity of NADPH–cytochrome P450 reductase, assayed by following reduction of cytochrome *c*, was similar for microsomes from clones MV-5 [123 nmol min^{-1} (mg of protein)$^{-1}$] and MV 2E1-9 [99 nmol min^{-1} (mg of protein)$^{-1}$], suggesting that the lower rates of substrate oxidation or $O_2^{\bullet-}$ generation in control clones compared to transduced 2E1 clones was due to lack of cytochrome P450, not reductase. To demonstrate that control clones can produce $O_2^{\bullet-}$ under certain circumstances, the effect of paraquat was evaluated. This redox cycling agent interacts with reductase, not P450, to produce $O_2^{\bullet-}$, and it was expected that, in view of similar reductase activities (but different P450 levels), paraquat would be equally effective in stimulating $O_2^{\bullet-}$ production with both clones. Indeed, the addition of 2 mM paraquat increased the rates of $O_2^{\bullet-}$ production by both microsomal preparations, and rates of $O_2^{\bullet-}$ production in the presence of paraquat were similar for the two microsomal preparations (Figure 7).

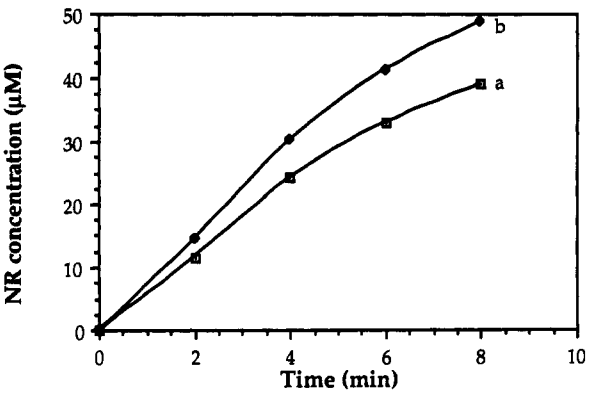

FIGURE 7: Stimulation of nitroxyl radical (NR) production upon the addition of 2 mM paraquat to microsomes from control clone MV-5 (a) and clone MV2E1-9 (b). Note the scale difference from that in Figure 6B.

Table III: Production of H_2O_2 by Microsomes from HepG2 Clones[a]

addition	concn (mM)	H_2O_2 production [nmol min^{-1} (mg of protein)$^{-1}$] MV-5	MV2E1-9
control		0.127 (100)	0.253 (100)
PNP	0.2	0.136 (107)	0.271 (107)
4-MP	1.0	0.123 (97)	0.225 (89)
aniline	1.0	0.201 (158)	0.266 (105)
ethanol	100.0	0.046 (36)	0.101 (40)

[a] H_2O_2 production was determined as described under Experimental Methods using 2.5 mg of microsomal protein/mL and a 1-h time course. Experiments were carried out in duplicate, in the absence and presence of the indicated additions. All results refer to the net activity found in the presence of catalase (3000 units/mL) minus that found in the presence of 1 mM sodium azide.

H_2O_2 can be produced in microsomes from the dismutation of $O_2^{\bullet-}$ and from the breakdown of the peroxygenated P450 complex (Kuthan et al., 1978; Kuthan & Ullrich, 1982). Microsomes from clone MV2E1-9 generated H_2O_2 in the presence of NADPH (Table III). The rate of H_2O_2 production was about 0.25 nmol min^{-1} (mg of protein)$^{-1}$ (Table III). Surprisingly, microsomes from clone MV-5 also produced H_2O_2 at a rate of about 0.12 nmol min^{-1} (mg of protein)$^{-1}$ (Table III). Since the control clone did not produce significant amounts of $O_2^{\bullet-}$ (Figure 6), most of the H_2O_2 produced is likely to arise from two-electron reduction of oxygen rather than from dismutation of $O_2^{\bullet-}$. Additional studies will be required to determine the enzymatic locus for this H_2O_2 production in control microsomes. Nevertheless, the rate of H_2O_2 production was 2-fold higher with microsomes from clone MV2E1-9. The addition of substrates or ligands of P4502E1 such as aniline, PNP, or 4-MP was without effect on H_2O_2 production (as expected) not only with the control clone but also with the MV2E1-9 clone (Table III). Ethanol,

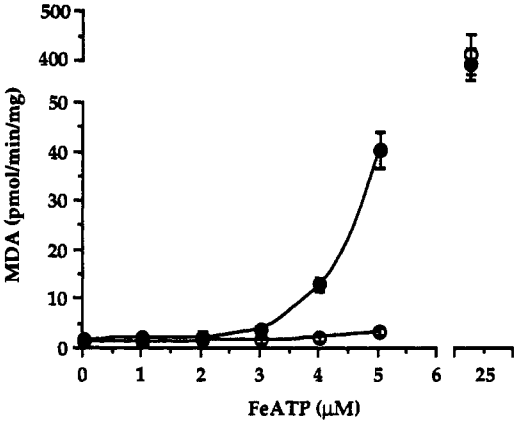

FIGURE 8: Concentration curve for the stimulation of NADPH-dependent lipid peroxidation by ferric-ATP. Production of TBA-reactive material [malondialdehyde (MDA)] was determined using microsomes from MV-5 (○) or MV2E1-9 (●).

which is a peroxidatic substrate for catalase, produced inhibition with both clones.

Lipid Peroxidation. Microsomal lipid peroxidation was evaluated by the production of TBA-reactive components. Ferric-ATP, which is very reactive in promoting microsomal lipid peroxidation (Aust et al., 1985), was used as the iron catalyst. When the ferric concentration was high (25 μM iron), TBA production was identical in microsomes from MV2E1-9 and MV-5 (Figure 8). Since chelated iron could interact with reductase as well as P450 (Aust et al., 1985; Morehouse et al., 1984; Winston et al., 1984), the comparable rates of production of TBA-reactive material with both microsomal preparations was interpreted to reflect primarily interaction of iron with the reductase when the concentration of iron was high. An iron titration curve was therefore carried out. As shown in Figure 8, little or no lipid peroxidation could be detected with ferric ATP concentrations up to 3 μM. Lipid peroxidation remained very low with the control MV-5 clone when the concentration of ferric-ATP was raised to 4 or 5 μM iron (Figure 8). However, a large increase in the production of TBA-reactive material was observed with microsomes from transduced clone MV2E1-9 at ferric-ATP concentrations of 4 and 5 μM (Figure 8). Thus, clear differences in lipid peroxidation between the two microsomal preparations can be found at "low" but not "high" concentrations of iron.

To attempt to evaluate the nature of the reactive oxygen species responsible for the TBA production in clone MV2E1-9 and to prove that the elevated activity at 4 or 5 μM ferric-ATP indeed reflected peroxidation, the effect of antioxidants was determined. Production of TBA-reactive material (in the presence of 5 μM ferric-ATP) was completely inhibited by the antioxidants trolox and propyl gallate and strongly prevented by GSH (Table IV). There was no effect by SOD, catalase, or ethanol, suggesting that $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$ did not play a major role in the overall mechanism of lipid peroxidation in the microsomes from MV2E1-9.

Comparison with Human Liver Microsomes. We compared oxidation of substrates and generation of reactive oxygen intermediates in microsomes from MV2E1-9 and from human liver (Table V). Activities were higher with the human liver microsomes when results were expressed on a per milligram of protein basis, since the total content of P450 was much higher in the human liver microsomes (about 0.34 nmol/mg of protein) than in the MV2E1-9 microsomes (about 0.01 nmol/mg of protein). However, activities were higher with MV2E1-9 microsomes when results were expressed on a total P450 basis. Oxidation of PNP or DMN was about 2.3–2.4-fold greater with MV2E1-9 microsomes than with human liver microsomes because all or most of the P450 in the clone is P4502E1, which is the primary or sole catalyst of PNP or DMN oxidation, whereas several forms of P450 are likely to be present in the human liver microsomes, some of which may not be effective catalysts for oxidation of PNP and DMN. Rates of $O_2^{\bullet-}$ and H_2O_2 production, and of lipid peroxidation, were 7–20-fold higher with MV2E1-9 microsomes than with human liver microsomes, on a per nanomole of P450 basis.

Table IV: Effect of Antioxidants on the Production of TBA-Reactive Material by Microsomes from MV2E1-9[a]

addition	concn	TBA reactivity [pmol min^{-1} (mg of protein)$^{-1}$]	effect of addition (%)
control		58.5	
trolox	0.1 mM	0	−100
propyl gallate	0.05 mM	0	−100
GSH	5.0 mM	16.4	−72
SOD	100 units/mL	64.3	+10
catalase	2600 units/mL	44.8	−23
ethanol	50 mM	50.7	−13

[a] The NADPH-dependent production of TBA-reactive material by microsomes from clone MV2E1-9 was determined in the presence of 5 μM ferric-ATP and in the absence or presence of the indicated additives. All experiments were carried out in duplicate.

DISCUSSION

The human cytochrome P4502E1 has been transiently expressed in two culture systems, COS cells through transfection (Umeno et al., 1988) and HepG2 cells via recombinant *Vaccinia* virus infection (Waxman et al., 1991; Patten et al., 1992), as well as in *Escherichia coli* (Winters & Cederbaum, 1992b) in a catalytic active form. A stable cell line constitutively expressing P4502E1 was reported by Nouso et al. (1992) in which human P4502E1 was successfully transduced into rat liver epithelial cells and mouse NIH3T3 cells using recombinant retroviral infection. The expressed P4502E1 oxidized DMN, producing covalent adducts of the carcinogen with cellular DNA (Nouso et al., 1992). HepG2 cells were selected as the target cell line in this study to stably express the human CYP2E1. This replicating cell line originates from human neoplastic liver tissue (Aden et al., 1979), expresses several liver-specific cell functions (Javitt, 1990), has been shown to catalyze oxidation of certain drug substrates (Sassa et al., 1987), contains NADPH–cytochrome P450 reductase and cytochrome b_5 (Waxman et al., 1991; Patten et al., 1992; Aoyama et al., 1990), and has a very low or nondetectable level of endogenous P4502E1 (Waxman et al., 1991; Patten et al., 1992). The current paper describes the use of retroviral-mediated gene transfer to develop a system which constitutively expresses human P4502E1 in HepG2 cells. The expressed P4502E1 resembles its authentic human liver microsomal counterpart in molecular weight and oxidation of a variety of substrates. That the expressed catalytic activity was due to P4502E1 was evident from the strong inhibition of PNP oxidation by anti-human P4502E1 IgG, as well as inhibitors (DDC), ligands (4-MP), and substrates (ethanol) for P4502E1. The level of expression which we obtain, however, is considerably lower than that reported for the *Vaccinia* virus system, e.g., about 10 pmol of P4502E1/mg of microsomal protein vs 35–45 pmol/mg of protein. In HepG2 cells and the control clones infected with virus lacking the P4502E1 insert, we could not detect P4502E1 mRNA, P4502E1 protein, or significant catalytic activity with P4502E1 substrates.

Table V: Comparison of Catalytic Activities by Microsomes from MV2E1-9 and Human Liver Microsomes[a]

reaction	activity by MV2E1-9		activity by human liver		MV2E1-9 activity/human liver activity (nmol of P450)$^{-1}$
	nmol min^{-1} (mg of protein)$^{-1}$	nmol min^{-1} (nmol of P450)$^{-1}$	nmol min^{-1} (mg of protein)$^{-1}$	nmol min^{-1} (nmol of P450)$^{-1}$	
PNP oxidation	0.059	5.90	0.87	2.56	2.3
DMN oxidation	0.018	1.80	0.25	0.74	2.4
$O_2^{\bullet-}$ generation	0.30	30.0	0.49	1.44	20.8
H_2O_2 production	0.25	25.0	0.81	2.38	10.0
lipid peroxidation	0.04	4.2	0.21	0.59	7.1

[a] The indicated reactions were carried out using microsomes from clone MV2E1-9 (P4502E1 content of 0.01 nmol/mg of protein) or from human liver (P450 content of 0.34 nmol/mg of protein). All reactions were carried out in duplicate. Results with the human liver microsomes are the average from three different samples. Lipid peroxidation was carried out with 0.005 mM ferric-ATP as the iron catalyst.

Isolated microsomes generate $O_2^{\bullet-}$ and H_2O_2 during NADPH-dependent electron transfer (Kuthan et al., 1978; Kuthan & Ullrich, 1982; Aust et al., 1972; Prough & Masters, 1973; Dybing et al., 1976; White & Coon, 1980). These reactive oxygen intermediates are produced primarily via decay of oxygenated and peroxygenated P450 complexes (Kuthan et al., 1978; Kuthan & Ullrich, 1982; White & Coon, 1980). The reduction of oxygen, while occurring with many forms of P450, appears to be especially significant for P4502E1 which has a high rate of NADPH oxidase activity even in the absence of added substrate (Gorsky et al., 1984; Ekstrom & Ingelman-Sundberg, 1989). This elevated oxidase activity may be of toxicological significance since several studies have shown that, in the presence of chelated iron, microsomes from rats treated with inducers of P4502E1, e.g., ethanol, display increased production of $^{\bullet}OH$, chemiluminescence, and lipid peroxidation (Ekstrom & Ingelman-Sundberg, 1989; Klein et al., 1983; Dicker & Cederbaum, 1987, 1988; Rashba-Step et al., 1993; Ekstrom et al., 1986; Krikun & Cederbaum, 1986; Puntarulo & Cederbaum, 1988a). Microsomes from clone MV2E1-9 generated $O_2^{\bullet-}$ at rates about 1 order of magnitude higher than microsomes from the control clone MV-5. Since activities of the reductase were similar between the two microsomal preparations, and in view of the comparable rates of $O_2^{\bullet-}$ generation in the presence of paraquat, the 10-fold higher rate of $O_2^{\bullet-}$ production in clone MV2E1-9 is due to the presence of P4502E1. In contrast to $O_2^{\bullet-}$ production, H_2O_2 was only 2-fold higher in microsomes from MV2E1-9 compared to a control clone. Interestingly, the net difference in H_2O_2 production between the two microsomal preparations [about 0.13 nmol min^{-1} (mg of protein)$^{-1}$] is about half the net difference in $O_2^{\bullet-}$ production between the two microsomal preparation [about 0.27 nmol min^{-1} (mg of protein)$^{-1}$]. This suggests that most of the increase in H_2O_2 production in MV2E1-9 microsomes is due to the increase in $O_2^{\bullet-}$ production; i.e., most of the elevated production of H_2O_2 is derived from dismutation of $O_2^{\bullet-}$ ($O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$).

The production of $O_2^{\bullet-}$ and H_2O_2 in MV2E1-9 microsomes was not affected by the addition of effective substrates for the catalytic activity of P4502E1. With cytochrome $P450_{cam}$ from *Pseudomonas putida*, the redox potential of the heme iron becomes more positive when substrate binds (Poulos & Raag, 1992; Sligar & Gunsalus, 1976); reduction of the P450 becomes thermodynamically more favorable when substrate is bound, which ensures that consumption of reducing equivalents and oxygen activation occur only in the presence of substrate (Poulos & Raag, 1992; Sligar & Gunsalus, 1976). Liver microsomal P450's do not appear to be as tightly coupled as the camphor P450 (Gorsky et al., 1984; White & Coon, 1980; Porter & Coon, 1991; White, 1991). The lack of effect of substrates on $O_2^{\bullet-}$ and H_2O_2 production by microsomes containing the expressed P4502E1 could be a reflection of the spin state of the P4502E1; when isolated, P4502E1 is in high spin state (Koop et al., 1982), which apparently results in transfer of electrons from the reductase even in the absence of substrates. We have not yet been able to assess the spin state of the expressed P4502E1 in MV2E1-9 microsomes because of its very low content.

Microsomal lipid peroxidation was first characterized by Hochstein et al. (1964), who showed a requirement for NADPH and for iron. A clear requirement that NADPH–cytochrome P450 reductase was the enzyme linking NADPH, iron, and lipid peroxidation was shown by Pederson and Aust (1972). The necessity for cytochrome P450 in microsomal lipid peroxidation is not clear. Ekstrom and Ingelman-Sundberg (1984) demonstrated that phospholipid vesicles containing both reductase and P450 catalyzed elevated rates of lipid peroxidation in the presence of NADPH compared to vesicles containing reductase only. Since iron was not added, either lipid peroxidation was catalyzed by iron present in the reagents or water or the P450 was functioning as a heme catalyst for the degradation of preformed lipid hydroperoxides (Morehouse & Aust, 1988). Pederson and Aust (1972) showed that a reconstituted system containing phospholipids, reductase, NADPH, and ferric-ADP could catalyze lipid peroxidation in the absence of P450 if ferric-EDTA was also added. The addition of P450 could substitute for the ferric-EDTA (Pederson & Aust, 1972; Morehouse & Aust, 1988). Since the reductase alone did not significantly reduce ferric-ADP, whereas microsomes or reductase plus P450 did reduce ferric-ADP, it was concluded that a major role for P450 in microsomal lipid peroxidation was to reduce the ferric-ADP catalyst (Morehouse & Aust, 1988; Vegh et al., 1988). In contrast to ferric-EDTA, which is readily reduced by the reductase (Morehouse et al., 1984; Winston et al., 1984), elevated rates of ferric-ADP reduction require reductase plus P450 (Morehouse & Aust, 1988; Vegh et al., 1988).

At a low concentration of ferric-ATP, microsomes from clone MV2E1-9 but not the control MV-5 catalyzed the production of TBA-reactive material. This production was sensitive to typical antioxidants such as trolox, propyl gallate, and GSH, linking TBA reactivity to the lipid peroxidation process. It appears that the presence of P4502E1 in MV2E1-9 is responsible for the lipid peroxidation catalyzed by low concentrations of ferric-ATP, since reductase is present in both clones. In the absence of ferric-ATP, lipid peroxidation was not observed, suggesting that the P4502E1 was not functioning, at least to a major extent, to degrade preformed lipid hydroperoxides. Rather, the requirement for NADPH and ferric-ATP suggests that the presence of P4502E1 allows the formation of an oxidizing species capable of initiating lipid peroxidation. One critical function of the P450 may be to reduce the ferric-ATP (Morehouse & Aust, 1988; Vegh et al., 1988). Future studies on reduction of ferric complexes and ferritin will address this possibility. While it is possible

that $O_2^{\bullet-}$ produced from oxygenated P450 may cause the reduction of the ferric-ATP, lipid peroxidation was not sensitive to added SOD, suggesting direct reduction of the ferric chelate by P450 rather than P450-derived $O_2^{\bullet-}$. Morehouse and Aust (1988) reported that ferric-ATP reduction by reconstituted systems was not sensitive to SOD and suggested direct reduction by the P450. The possibility that $O_2^{\bullet-}$ not accessible to added SOD catalyzes reduction of ferric-ATP, however, cannot be ruled out.

Lipid peroxidation increased when the concentration of ferric-ATP was elevated from 0.005 to 0.025 mM. At this higher concentration of ferric-ATP, production of TBA-reactive material was identical by microsomes from the two clones. This raises the possibility that at the higher concentration the ferric-ATP may now be reduced by reductase, thereby obviating the need for P450. Alternatively, the higher (but not lower) concentrations of ferric-ATP may be reduced by constitutive P450 enzymes present at low levels in the HepG2 cells or by other NADPH-dependent pathways. This raises the possibility that P4502E1 may be especially effective in reducing low concentrations of iron. Experiments to test this possibility are planned in future studies.

The oxidizing species responsible for initiation of lipid peroxidation in microsomal systems has been suggested to be $^{\bullet}OH$, ferryl, or perferryl types of oxidants, or ferrous–ferric oxygenated complexes with maximal effectiveness when the ferrous/ferric redox states are equivalent (Aust et al., 1985; Minotti & Aust, 1987; Goddard & Sweeney, 1987; Braughler et al., 1986). Microsomal lipid peroxidation in clone MV2E1-9 was not sensitive to SOD, catalase, or ethanol, suggesting little or no role for "free" $O_2^{\bullet-}$, H_2O_2, or $^{\bullet}OH$ in the overall pathway. These results are identical to previous experiments with rat liver microsomes (Aust et al., 1985; Puntarulo & Cederbaum, 1988b) and to preliminary experiments with human liver microsomes (Rashba-Step and Cederbaum, unpublished observations).

Oxygen radical production by the human P4502E1 has not been reported, although considerable research has been carried out with the rat and rabbit 2E1 (Gorsky et al., 1984; Ekstrom & Ingelman-Sundberg, 1989). The experiments concerning production of $O_2^{\bullet-}$ and H_2O_2 and lipid peroxidation catalyzed by low concentrations of ferric-ATP indicate increased production of reactive oxygen intermediates when P4502E1 is expressed. The comparisons shown in Table V reveal that rates of $O_2^{\bullet-}$ and H_2O_2 production and of lipid peroxidation were considerably greater (7–20-fold higher) with MV2E1-9 microsomes than with human liver microsomes on a per nanomole of P450 basis than were rates of substrate oxidation (2.3- or 2.4-fold higher with PNP and DMN, respectively). These differences strongly suggest that human P4502E1 is especially reactive in oxygen radical production and catalysis of lipid peroxidation compared to a mixed population of human P450 forms.

Future studies are planned to analyze the properties of the human P4502E1 constitutively expressed in HepG2 cells, with emphasis on production of reactive oxygen intermediates, interaction with iron, and consequences of exposing the cells to various toxins including ethanol.

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