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Stable Expression of Rat Cytochrome P-450IA1 cDNA in V79 Chinese Hamster Cells and Their Use in Mutagenicity Testing

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SUMMARY

V79 Chinese hamster cells genetically engineered to express cytochrome P-450IA1 are reported. A full length cDNA encoding rat cytochrome P-450IA1 was obtained from a cDNA library prepared from rat liver mRNA. The cDNA was recombined with the SV40 early promoter and expressed in V79 cells. Three V79-derived P-450IA1-expressing cell lines (XEM1, XEM2, and XEM3) were established. The presence of the rat cytochrome P-450IA1 cDNA in these hamster cells was confirmed by Southern blotting. The transcription of the cDNA into mRNA and translation into the desired cytochrome P-450 protein was detected by Northern and Western blotting. The enzymatic activity was determined by the cytochrome P-450IA1-dependent oxidation of benzo[*a*]pyrene and 7-ethoxycoumarin. After exposure to benzo[*a*]pyrene, the mutant frequency increased in XEM1 and XEM2 cells and was higher than in V79 cells in the presence of an exogenous activating system. The mutant frequency was even more increased when XEM1 and XEM2 cells were exposed to the proximate mutagen (*trans*)-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene.

Many promutagens and procarcinogens are metabolically converted by monooxygenases and other xenobiotic-metabolizing enzymes into their ultimate mutagenic or carcinogenic form. Any toxicological test system aiming at the detection of mutagens or carcinogens must, therefore, be metabolically competent ("bioactivation") and reveal the mutagenic or carcinogenic activity ("toxicological endpoint"). Toxicological test systems based on prokaryotic or eukaryotic cells have been developed (1–3). Prokaryotic as well as most of the eukaryotic cells have the shortcoming of having little or no metabolic activity to generate ultimate mutagens or carcinogens. Eukaryotic cells like the V79 Chinese hamster cell line are being widely used in toxicological investigations but they also have the serious limitation that they do not express P-450s which are needed for the activation of many promutagens. In order to compensate for this metabolic deficiency, Langenbach *et al.* (4) introduced the cocultivation of V79 cells with metabolically competent primary liver cells. However, exogenous metabolizing systems present the risk of false negative results, because metabolites might not be able to cross the cellular membrane of the test cell due to their polarity, they might bind to proteins and DNA contained in the liver cells, or they might be processed to their nonreactive end products before they have a chance to enter the test cell. The mutagenic potency of those metabolites would remain undetected. Hence, a toxicological test system based on mammalian cell lines would be improved if the test cell not only provides a toxicological endpoint but also is itself metabolically competent.

Therefore, we have started a program to improve V79 Chinese hamster cells for toxicological investigations by genetechnological means (5). Here, we report on the cloning of rat P-450IA1 cDNA, the establishment of V79-derived cell lines expressing the P-450IA1 cDNA, and their application in the mutagenicity testing of B[*a*]P and its metabolite (*trans*)-B[*a*]P-7,8-diol.

Materials and Methods

Preparation and screening of oligo(dT)-primed cDNA library. Total RNA was prepared, by the guanidine-HCl/CsCl procedure (6), from the liver of a male rat (Sprague-Dawley) pretreated with Aroclor-1254 (500 mg/kg of body weight). Polyadenylated RNA was enriched by affinity chromatography on an oligo(dT)-Sepharose column (Pharmacia). Synthesis of cDNA was essentially carried out as described by Gubler and Hoffmann (7). The cDNA was inserted into the *Bam*HI site of pUC19 using a *Bam*HI/blunt end adaptor (Boehringer Mannheim), according to a procedure developed by Haymerle *et al.* (8). Recombinant plasmid DNA was transferred into *Escherichia coli* strain C600, following the procedure developed by Hanahan (9). Bac-

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ABBREVIATIONS: P-450, cytochrome P-450; B[*a*]P, benzo[*a*]pyrene; (*trans*)-B[*a*]P-7,8-diol, (*trans*)-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kbp, kilobase pair; AHH, aryl hydrocarbon hydroxylase; SSC, saline sodium citrate; PAGE, polyacrylamide gel electrophoresis; HPRT, hypoxanthine phosphoribosyl-transferase.

terial colonies were screened with a radioactively labeled probe derived from a mouse P_1-450 cDNA (10), which is known to be an orthologue of rat P-450IA1 (11). In order to discriminate between cDNA encoding P-450IA1 or P-450IA2, a probe prepared from the 0.409-kbp *Nde*I/*Avr*II fragment of the P_1-450 cDNA, which shares only 60% homology with the corresponding sequence in the rat P-450IA2 gene, was used to screen for positive colonies from the first screening round. After hybridization with this probe, nitrocellulose filters were washed at 60° in 0.1 × SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS, to ensure sufficient stringency for detection of cDNA encoding P-450IA1 only. Under these conditions P-450IA2 cDNA was not detected. From bacterial colonies, detected with this probe, plasmid DNA was prepared and digested with *Bam*HI, and the size of the cDNA insert was analyzed by electrophoretical separation on a 0.7% agarose gel. The two largest cDNA inserts obtained were characterized by restriction enzyme mapping and partial DNA sequencing at the 5′ end.

Maxam-Gilbert sequencing. The 0.3-kbp *Bam*HI/*Pst*I fragment representing the 5′ end of the cloned P-450IA1 cDNA was sequenced according to the chemical method (12), with DNA bound to DEAE-membranes (Schleicher and Schüll) (13). About 0.75 μg of the purified fragment was radioactively end-labeled at the *Bam*HI end, using the Klenow fragment of DNA polymerase I. After heating to 70° for 15 min and freezing in dry ice to inactivate the enzyme, 8 μg of sheared carrier salmon sperm DNA and 5 μg of yeast tRNA were added, the whole mixture was ethanol precipitated, and the pellet was washed with 70% ethanol. The DNA was incubated in 66% formate for purine-specific DNA modification. Reactions were stopped by filling the wells containing the DEAE-membranes with 10 ml of 100% ethanol at room temperature. Final pellets were washed twice using 70% ethanol. Cleavage products were analyzed on polyacrylamide gradient thin gels.

Cloning of P-450IA1 cDNA into the pSV2 vector. The 2.2-kbp cDNA insert was separated from the plasmid pUC19 by cleavage with *Bam*HI and 0.7% agarose gel electrophoresis. The *Bam*HI insert cDNA was recovered from the agarose gel by electroelution. The *Bam*HI insert cDNA was partially digested by *Nco*I, making use of the *Nco*I sites contained in the *Bam*HI/blunt end linker at the 5′ end and the 3′ end of the cDNA, in order to generate a *Nco*I/*Bam*HI cDNA insert fragment whose 5′ end linker was cut with *Nco*I and 3′ end linker with *Bam*HI. The plasmid pSV450 containing the P-450IIB1 cDNA (5) was prepared to take up the *Nco*I/*Bam*HI P-450IA1 cDNA by digestion with *Nco*I and *Bgl*II and separation from the P-450IIB1 cDNA. The *Nco*I/*Bgl*II fragment of the plasmid pSV450 containing the SV40 promoter and the SV40 polyadenylation site was linked to the *Nco*I/*Bam*HI P-450IA1 cDNA fragment. The correct orientation of the *Nco*I/*Bgl*II P-450IA1 cDNA fragment with respect to the SV40 promoter and to the SV40 polyadenylation site was verified by restriction enzyme analysis. This construct, pSV450IA1, was used for gene transfer into V79 cells.

Cell culture. V79 Chinese hamster cells were maintained in Dulbecco-Vogt's Eagle's medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were free of *Mycoplasma* contamination (14).

Transfection of cells. Transfection was carried out according to the method of Graham and van der Eb (15). Exponentially growing V79 cells were trypsinized, seeded at a density of 5×10^5 cells/60-mm Petri dish, and incubated overnight in 10 ml of growth medium. *Eco*RI-linearized plasmid DNA pSV450IA1 (20 μg) (see Fig. 2) and pMTneo 342-2 (1 μg) (5) were mixed in 1.0 ml of HEPES-buffered saline (137 mM NaCl, 6 mM dextrose, 5 mM KCl, 0.7 mM Na_2HPO_4, 20 mM HEPES, adjusted to pH 6.95–7.05 with 0.5 M NaOH). The DNA was precipitated by the addition of 52.6 μl of 2.5 M $CaCl_2$ (final concentration, 125 mM), and the mixture was left at room temperature for 25 min and then added to 1×10^6 V79 cells in a 60-mm Petri dish. After a 4-hr incubation, cells were subjected to 15% (v/v) glycerol shock for 2 min, washed twice with fresh medium, and refed with 10 ml of growth medium. After overnight incubation, cells were split 1:5 in 60-mm Petri dishes. G-418 for selection was added on the third day after transfection, at a concentration of 800 μg/ml of growth medium. G-418-resistant colonies appeared 10 days after transfection, were picked 3 weeks later by cloning cylinders, and were grown in mass culture for further studies.

Preparation of chromosomal DNA. Genomic DNA was obtained by differential lysis of cells and nuclei. Cells were trypsinized and collected by centrifugation at 100 × *g*. Pelleted cells were resuspended in 10 mM Tris·HCl, pH 7.3, 1.5 mM $MgCl_2$, 0.15 M NaCl, and Nonidet P-40 detergent was added at 10% final concentration. Cellular membranes were disrupted by vigorous shaking on a Vortex apparatus for 5 sec. Intact cell nuclei were harvested at 800 × *g*. The supernatant was discarded, and nuclei were treated with 5% SDS in 10 mM EDTA, 0.1 M NaCl. This solution was incubated at 37° in the presence of 200 μg/ml proteinase K (Merck). DNA was purified and isolated by two phenol/chloroform (1:1) extraction cycles, during a slow rotation of the tubes for 15 min. Phases were separated at 800 × *g* for 15 min. The supernatant was transferred to a new tube. DNA was precipitated with 2.5 volumes of ethanol and resuspended in 10 mM Tris·HCl, pH 7.5.

Preparation of total RNA. Total RNA from cells and from livers of rats pretreated with Aroclor 1254 (gift from Bayer AG, Leverkusen, FRG), was prepared by the guanidium HCl/CsCl procedure (6).

Nucleic acid analysis. Genomic DNA was digested to completion with 5 units of *Eco*RI/μg of DNA, at 37° overnight. DNA fragments were separated electrophoretically on 0.7% agarose and transferred to nitrocellulose filters by the method of Southern (16). For hybridization, the ^{32}P-labeled 1.2-kbp internal *Pst*I fragment of the P-450IA1 cDNA was used. Hybridizations were performed in 6× SSC, 50% formamide, 1× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone) 100 μg of salmon sperm DNA/ml, at 45° for 18 hr. Filters were washed in 0.1× SSC and 0.1% SDS for 15 min at 60°, with two changes. Fluorography was carried out by exposure of Kodak X-Omat film to dried filters at −70°, in conjunction with intensifying screens.

Total RNA was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde and was blotted onto nitrocellulose filters (Schleicher and Schüll). Hybridization conditions were as described for Southern blotting.

Protein analysis. The presence of P-450IA1 protein in the cells was detected by electrophoretic separation of cellular homogenate (SDS-PAGE) and immunological detection (immunoblotting) as follows. SDS-PAGE was performed according to the method of Laemmli (17), with the following modifications. The stacking gel was 5% acrylamide and 0.1% bisacrylamide in 0.38 M Tris phosphate, pH 6.7, 0.1% SDS. The separating gel was 10% acrylatmide and 0.2% bisacrylamide in 0.38 M Tris·HCl, pH 8.9, 0.1% SDS. Transfer of proteins to nitrocellulose sheets and immunological detection of proteins on nitrocellulose was carried out as described (5). Anti-P-450IA1 IgG from rabbit was used as first antibody. Immunoblots were then incubated with pig anti-rabbit IgG conjugated to peroxidase. Peroxidase staining was done with diaminobenzidine and H_2O_2 as substrates.

Enzyme assays. AHH and 7-ethoxycoumarin *O*-deethylase activities were measured in cell lysates obtained from 2×10^7 cells (two confluent 75-cm^2 flasks). Cells were harvested by trypsin, washed with phosphate-buffered saline, and resuspended in 1 ml of 50 mM Tris·HCl, pH 7.5. Cells were then disrupted with a Branson-B15 sonifier to yield cell lysate. AHH activity was determined spectrofluorometrically, with excitation at 396 nm and emission at 522 nm (18). The reaction volume was 1 ml and contained 50 μmol of Tris·HCl (pH 7.5), 3 μmol of $MgCl_2$, 0.6 μmol of NADPH, 100 nmol of B[*a*]P, and between 200 and 800 μl (0.5 to 1.5 mg of total protein) of cell lysate. The fluorescence readings were quantitated by comparison with the standard curve of 3-OH-B[*a*]P and are expressed as pmol of 3-OH-B[*a*]P formed/min/mg of protein. 7-Ethoxycoumarin *O*-dealkylation activity was also measured spectrofluorometrically, with excitation at 368 nm and emission at 456 nm, essentially as described (19). 7-OH-coumarin was used as reference. Activity is expressed as pmol/min/mg of protein. Protein content was determined with a Bio-Rad protein assay kit (Bio-Rad), using serum albumin as a standard.

Mutagenicity test. B[*a*]P (Sigma) and its metabolite (*trans*)-B[*a*]P-7,8-diol were tested for mutagenicity in V79, SD1, XEM1, and XEM2 cells. (*trans*)-B[*a*]P-7,8-diol was prepared by reduction of benzo[*a*]pyrene-7,8-quinone with sodium boron, as described (20). On day 1, 1.5 × 10^6 cells were seeded with 30 ml of medium in 15-cm Petri dishes. After 18 hr, the test compound or solvent only (60 μl) was added. Twenty-four hours later, the exposure was terminated by a change of the medium. On day 4, the cells were detached by treatment with trypsin and subcultured at a density of 3 × 10^6 cells/15-cm Petri dish. On day 8, they were subcultured again at a density of 10^6 cells/15-cm Petri dish in medium containing 6-thioguanine (7 μg/ml), to determine the number of mutants (six replicate plates). The cloning efficiency was determined by plating 100 cells, with 5 ml of 6-thioguanine-free medium, in 6-cm Petri dishes (three replicate plates). The colonies were counted after incubation for 9 days (cloning efficiency plates) or 10 days (selection plates).

Results

Full length P-450IA1 cDNA. About 100,000 colonies of the cDNA library were screened. The first screen yielded 30 positive colonies. After rescreening, the number of positive clones decreased to 22. These colonies were then hybridized to the 0.409-kbp *Nde*I/*Avr*II fragment of the P_1-450 cDNA, in order to discriminate between P-450IA1 and P-450IA2. From the 22 positive colonies, 9 were detected to hybridize to the P-450IA1-specific *Nde*I/*Avr*II fragment. The recombinant plasmid DNA of these 9 colonies was prepared, digested with *Bam*HI, and analyzed by agarose gel electrophoresis. Two recombinant plasmid DNAs contained a *Bam*HI insert of about 2.2 kbp (Fig. 1). The 5′ end sequence of these inserts was GGTCCTAGAGAACACTCTTCAGTTCAGTCC and was found to be identical to the sequence of rat P-450IA1 cDNA cloned by Yabusaki *et al.* (21). The rat P-450 cDNA described here contains a 5′ untranslated region and starts at position −60. Based on the 2.2-kbp size and restriction enzyme analysis of the cDNA and comparison with the published cDNA sequence (21), it was concluded that the second polyadenylation site at position 2628 is certainly missing in the cDNA cloned by us, but the first polyadenylation site at position 2144 may be present.

Insertion of P-450IA1 cDNA into the expression vector pSV2. The full length P-450IA1 cDNA was recombined with the pSV2 vector, placed under the control of the SV40 early promoter and SV40 polyadenylation site by insertion of the *Nco*I/*Bam*HI P-450IA1 cDNA fragment of pUC19/450IA1 into the *Nco*I and *Bgl*II sites of pSV450. The resulting plasmid pSV450IA1 is shown in Fig. 2.

Construction of V79 cells with recombinant plasmid pSV450IA1. The plasmid pSV450IA1 (Fig. 2) was co-transfected with the plasmid pMTneo342-2 (5) into V79 Chinese hamster cells, as recently described for rat P-450IIB1 (5). G-418-resistant cell colonies were propagated. Chromosomal DNA was isolated for analysis in a Southern blot. Chromosomal DNA was digested with *Eco*RI and hybridized to the internal 1.2-kbp *Pst*I/*Pst*I fragment of the P-450IIA1 cDNA shown as a dark line in Fig. 2. Two hybridizing fragments of about 10 and 2.3 kbp (Fig. 3A, *lane a*) were detected in V79 chromosomal DNA and represent the hamster P-450IA1 or P-450IA2 gene sharing homology with rat P-450IA1 or P-450IA2 genes. Three cell lines, XEM1, XEM2, and XEM3, were identified that contain a hybridizing fragment of about 20 kbp (Fig. 3A, *lanes b*, *c*, and *d*), which appeared to be identical in all three independently isolated cell clones, in addition to the V79-specific fragments. XEM3 contained, in addition to the 20-kbp fragment and the hamster fragments, extremely strongly hybridizing fragments of 4.5 and 9 kbp (Fig. 3A and 3B, *lane d*). The linearized plasmid pSV450IA1 that was used for transfection is shown in Fig. 3A (*lane e*). The result of the Southern blot was unusual for two reasons. First, all three cell lines showed exactly the same chromosomal DNA fragment of 20 kbp after *Eco*RI digestion, containing the newly acquired rat P-450IA1 gene, even though all three cell lines were originally isolated as independent colonies in two different transfection experiments. This might be explained by site-specific integration of P-450IA1 cDNA. Second, the strong signal obtained for the 20-kbp fragment in XEM3 cells as well as additional strongly hybridizing fragments indicated gene amplification occurring during propagation of this clone to mass culture. This cell line is currently being further investigated on the mRNA and protein levels.

Expression of the P-450IA1 gene. To test whether the integrated *P*-450IA1 cDNA is expressed in the cell lines XEM1, XEM2, and XEM3, total RNA was isolated, electrophoretically separated on 1% agarose gels under denaturing conditions, and compared with total RNA obtained from V79 cells as well as

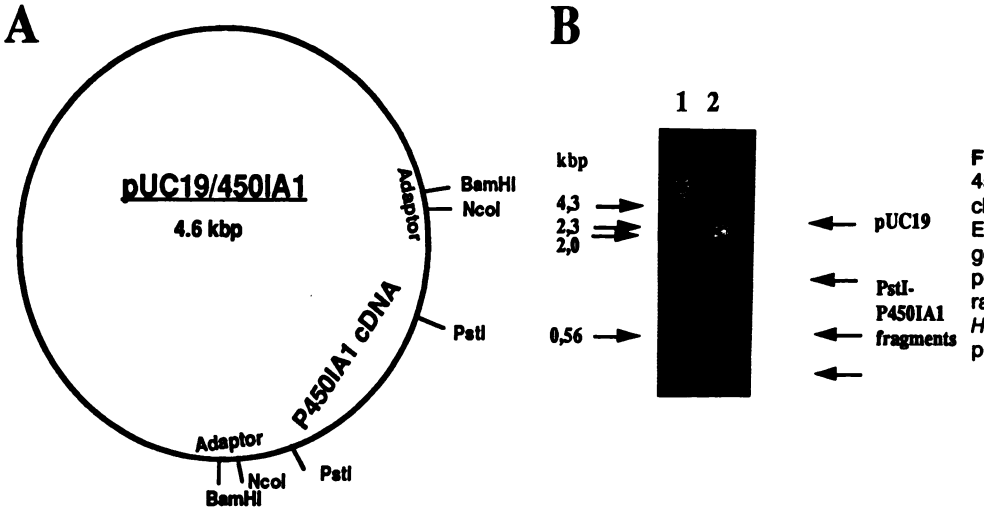

Fig. 1. A, Recombinant plasmid pUC19/450IA1 containing full length P-450IA1 cDNA, as obtained from the cDNA library. B, Ethidium bromide-stained DNA fragments generated by *Bam*HI/*Pst*I digestion of pUC19/450IA1, electrophoretically separated on 0.7% agarose. *Lane 1*, λ DNA *Hin*dIII fragments as size markers; *lane 2*, pUC19/450IA1 *Bam*HI/*Pst*I fragments.

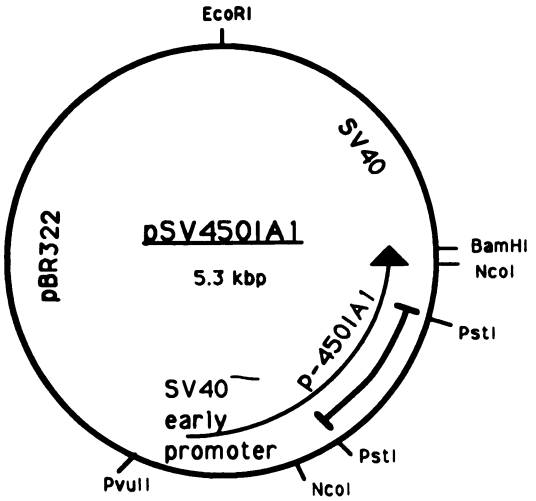

Fig. 2. Recombinant plasmid pSV450IA1 as used for gene transfer into V79 cells.

RNA obtained from livers of Aroclor 1254-treated rats (Fig. 4). Two reacting mRNA were detected in total RNA from rat liver, representing P-450IA1 mRNA (upper band) and P-450IA2 mRNA (lower band) (Fig. 4, *lane a*). V79 did not contain hybridizing mRNA (Fig. 4, *lane b*), whereas cell lines XEM1, XEM2, and XEM3 contained hybridizing mRNA (Fig. 4, *lanes c*, *d*, and *e*). The size of the mRNA detected in these cell lines was smaller, by about 500 bases, than the P-450IA1 mRNA in rat liver. The most likely reason is that our cDNA isolate was shorter, by 500 bp, than the cDNA described by Yabusaki *et al.* (21) and, thus, was missing the second polyadenylation site. Most of the P-450IA1 mRNA was detected in XEM2 cells, and less P-450IA1 mRNA was detected in XEM1 and XEM3 cells. Cell lines XEM1 and XEM3 appear to be unstable, for several reasons that are currently being further investigated. XEM1 is losing activity; XEM3 is undergoing amplification. XEM2 cells are still stable after more than 100 generations.

Cell lysates were separated by SDS-PAGE, transferred to a nitrocellulose filter, and probed with a polyclonal antibody directed against P-450IA1 (immunoblotting). Cell lines XEM2 and XEM3 were found to contain immunoreactive protein (Fig. 5, *lanes e* and *f*) that comigrated with P-450IA1 in liver homogenate (Fig. 5, *lanes a* and *g*). XEM1 cells contained very little immunoreactive protein by the time homogenate was prepared for immunoblotting (Fig. 5, *lane d*), because this cell line turned out to be unstable. The parental cell line V79 did not contain immunoreactive material (Fig. 5, *lane c*).

Enzymatic characterization of the P-450IA1 cDNA product. AHH and 7-ethoxycoumarin *O*-deethylase activities were detected in total protein of XEM1, XEM2, and XEM3 cells and compared favorably with the overall activities measured in total protein of hepatocytes of untreated rats (Table 1). Even though B[*a*]P and 7-ethoxycoumarin are primarily metabolized by P-450IA1 (22), P-450 forms other than P-450IA1 may substantially contribute to the metabolism of both compounds in a hepatocyte homogenate. Therefore, the activity in hepatocytes is not necessarily comparable to that in the newly established cell lines specifically expressing P-450IA1. No activity was detected in V79 cells, whereas XEM1 and XEM2 cells were capable of efficient metabolism of B[*a*]P and 7-ethoxycoumarin. XEM2 cells had an activity 5 times higher than XEM1 cells.

Mutagenicity test with B[*a*]P and (*trans*)-B[*a*]P-7,8-diol. B[*a*]P and its metabolite (*trans*)-B[*a*]P-7,8-diol need metabolic activation to be mutagenic (23). XEM1 and XEM2 cells were exposed to 1, 3, and 10 μM B[*a*]P and to 0.03, 0.10, 1.00, 10.00, and 30.00 μM (*trans*)-B[*a*]P-7,8-diol for 24 hr. After an expression period of 6 days, cells were cultivated in the presence of 6-thioguanine. Colonies of $HPRT^-$ cells were scored after 10 days (Table 2). Spontaneous mutant frequencies varied between 1 and 9 colonies/10^6 cells in all cell lines. In V79 and SD1 cells, (*trans*)-B[*a*]P-7,8-diol was effective only at concentrations above 3 μM and caused 45 $HPRT^-$ V79 colonies and 32 $HPRT^-$ SD1 colonies per 10^6 cells. B[*a*]P was effective in XEM1 and XEM2 cells (Table 2). In XEM1 cells, B[*a*]P was less effective than in XEM2 cells, which have a 5 times higher P-450IA1 activity than XEM1 cells. A very high mutant frequency, ranging between 85 $HPRT^-$ colonies/10^6 cells at 0.03 μM (*trans*)-B[*a*]P-7,8-diol and 1798 $HPRT^-$ colonies/10^6 cells at 30 μM (*trans*)-B[*a*]P-7,8-diol, was observed in XEM1 and XEM2. In XEM2 cells, a concentration of (*trans*)-B[*a*]P-7,8-diol of more than 3 μM was cytotoxic (Table 2).

Discussion

A new set of V79-derived cell lines genetically engineered for expression of rat P-450IA1 is presented. These cell lines, XEM1, XEM2, and XEM3, exhibit P-450IA1-specific enzyme activities, as determined by the metabolic conversion of B[*a*]P and 7-ethoxycoumarin. The activity in these cells was the same as or even higher than that observed in hepatocytes of untreated

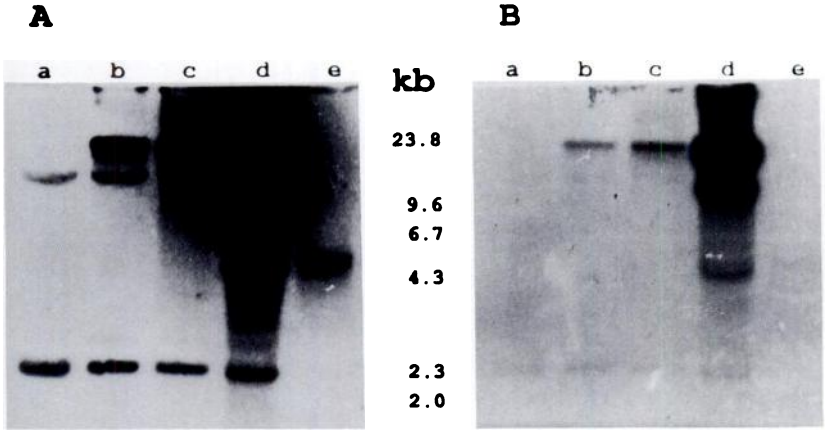

Fig. 3. Southern blot of *Eco*RI-digested chromosomal DNA. DNA was hybridized to the internal *Pst*I fragment of P450IA1 cDNA. Each lane contained 15 μg of digested chromosomal DNA. *Lane a*, cell line V79; *lane b*, cell line XEM1; *lane c*, XEM2; *lane d*, XEM3; *lane e*, *Eco*RI-linearized plasmid pSV450IA1 (200 pg). *Hin*dIII-digested λ DNA served as size markers. A, Filter was autoradiographed for 7 days. B, Same filter as in A, except filter was autoradiographed for only 2 days in order to make the amplified DNA in XEM3 cells (*lane d*) visible.

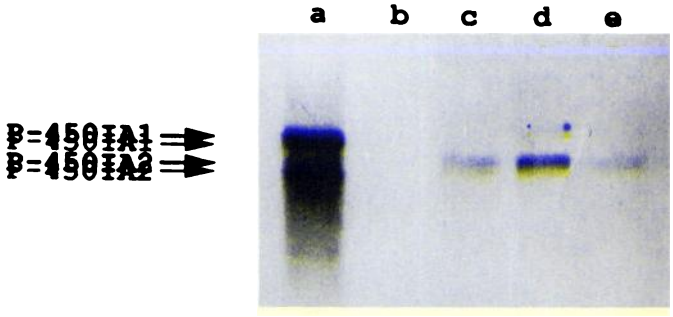

Fig. 4. Northern blot of P-450IA1 mRNA. Total RNA was hybridized to the internal *Pst*I fragment of P450IA1 cDNA. Each lane contains 15 μg of total RNA. *Lane a*, hepatocytes of Aroclor 1254-treated liver; *lane b*, V79 cells; *lane c*, XEM1 cells; *lane d*, XEM2 cells; *lane e*, XEM3 cells.

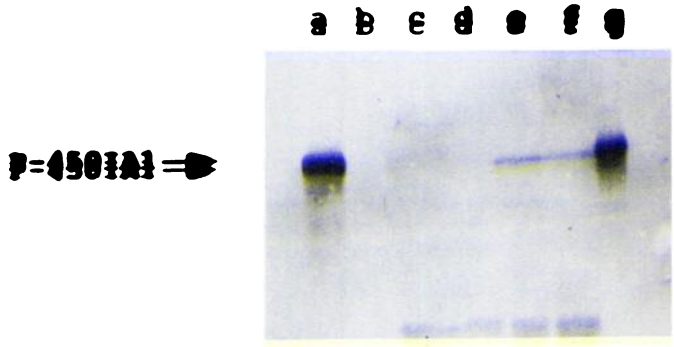

Fig. 5. Immunoblot of P-450IA1 protein. Samples are sonicated cell lysates from hepatocytes of Aroclor 1254-treated rats in *lane a* (100 μg of total protein) and *lane g* (50 μg of total protein) (*lane b* was void) and sonicated cell lysates (100 μg of total protein) of V79 cells in *lane c*, of XEM1 cells in *lane d*, of XEM2 cells in *lane e*, and of XEM3 cells in *lane f*.

TABLE 1

Enzymatic activity in V79, XEM1, XEM2, and XEM3 cells and hepatocytes of untreated Sprague-Dawley rats

Cells	Specific activity	
	7-Ethoxycoumarin	AHH
	pmol · mg^{-1} *· min*$^{-1}$	
V79	≤0.2	≤0.2
XEM1	15.2	9.5
XEM2	84.2	50.2
XEM3	43.4	21.3
Hepatocytes from untreated rats	39.3	45.0

rats. Activity remains stable in cell line XEM2 after more than 100 generations, whereas some stability problems were encountered in cell lines XEM1 and XEM3.

So far, no P-450IA1 or P-450IA2 mRNA (Fig. 4) and protein (Fig. 5) and no P-450IIB1 mRNA and protein were detected, and no AHH activity could be measured in V79 cells, with a detection limit of 0.2 pmol/min/mg of protein (5). Others recently reported an AHH activity for V79 cells of 0.03 pmol/min/mg of protein (24). The V79 cells that were used by us for gene transfer were measured by these authors independently and were found to have an AHH activity of 0.003 pmol/min/mg of protein.[1] Due to their apparent lack of P-450 activity,

[1] F. J. Wiebel, personal communication.

TABLE 2

Mutagenicity test with B[*a*]P and its metabolite (*trans*)-B[*a*]P-7,8-diol

Substrate	Frequency of 6-thioguanine-resistant cells × 10^6			
	V79	SD1	XEM1	XEM2
μM				
B[*a*]P				
1.00	ND[a,b]	ND	8	91
3.00	ND	ND	11	185
10.00	ND	ND	17	257
(*trans*)-B[*a*]P-7,8-diol				
0.01	ND	ND	ND	29
0.03	5	3	85	225
0.10	8	6	360	618
0.30	14	6	ND	1154
1.00	9	2	1208	Toxic[c]
3.00	15	14	1401	Toxic[c]
10.00	45	32	1798	Toxic[c]
30.00	Toxic[c]	Toxic[c]	Toxic[c]	Toxic[c]
None	9	3	2	4
	5	5	3	9

[a] ND, not done.
[b] B[*a*]P has been repeatedly reported to be not mutagenic in V79 cells in the absence of an extracellular metabolizing system (23, 28, 31).
[c] The number of surviving cells was so low that an adequate determination of the mutant frequency was not possible.

V79 cells appear to be the best choice as recipient cells for gene transfer of any P-450-encoding DNA. Thus, cells are created that exhibit one specific P-450 form only. A panel of genetically engineered V79-derived cell lines could serve as an analytical tool in drug metabolism studies for the identification of the metabolically competent P-450 form and the metabolite profile generated by a particular P-450. This work is being done in collaboration with Drs. Maurer and Fischer (SANDOZ, Basel) and will be published soon.

The type of recipient cell for P-450 gene transfer should also be chosen depending on the toxicological end point to be investigated. V79-derived cell lines are best suited for mutagenicity studies for several reasons. They grow quickly, with a generation time of 12 hr. Results are of high reproducibility, because the cloning efficiency is more than 80%. V79 cells also maintain a stable karyotype with just one X chromosome, on which the HPRT test gene is located. Other cell lines genetically engineered for the expression of P-450s may have other advantages (25–27). The mutagenicity studies presented here demonstrate the usefulness of these newly established V79-derived cell lines. It has already been shown repeatedly by us and others that B[*a*]P is not mutagenic in the V79 parental cell line in the absence of an extracellular metabolizating system (23, 28, 29). However, in the V79-derived P-450IA1-expressing cell line XEM1, B[*a*]P was slightly mutagenic and it was more mutagenic in XEM2 cells, which have a 5 times higher P-450IA1 activity compared with the XEM1 cells. Usually, epoxides of B[*a*]P formed by P450IA1 are further processed to less reactive diols by epoxide hydrolase (23, 30). However, V79 cells were found to have an epoxide hydrolase activity of 118 pmol/min/mg of total protein (32), which is similar to the levels observed in many extrahepatic tissues of rodents (31), whereas freshly prepared hepatocytes showed an epoxide hydrolase activity of 5300 pmol/min/mg of total protein (32). Because even in liver enzyme-mediated assays the addition of purified epoxide hydrolase increased the mutagenicity of B[*a*]P (determined in *Salmonella typhimurium*) (33), the relatively

low epoxide hydrolase activity in V79 cells may be a limiting factor in the activation of B[*a*]P in XEM1 and XEM2 cells in a way that increases mutagenicity. This will be studied in detail as soon as a V79- and a XEM2-derived cell line genetically engineered for an increased level of epoxide hydrolase are established. The mutant frequency increased to a maximum of 1798 $HPRT^-$ colonies/10^6 XEM1 cells upon exposure to the proximal mutagen (*trans*)-B[*a*]P-7,8-diol. This is in agreement with the well known observation that P-450IA1 possesses the highest catalytical activity for the P-450-dependent monooxygenase steps involved in the metabolic activation of B[*a*]P to both diastereomeric bay-region (*trans*)-B[*a*]P-7,8-diol-9,10-oxides among the purified P-450 forms yet investigated (34, 35). In V79 and SD1 cells, a weak increase in the mutant frequency was observed at a very high substrate concentration. This effect may be due to chemical activation in the solvent (36). At a concentration of 0.1 μM (*trans*)-B[*a*]P-7,8-diol, the mutant frequency in XEM1 cells was 360 and in XEM2 cells 618 $HPRT^-$ colonies/10^6 cells (Table 2). In earlier studies (28) on the mutagenic potency of this compound in V79 cells with exogenous activation, using an S9 mixture prepared from Aroclor 1254-treated rats, a 150-fold higher concentration (5 μg/ml ≈ 15 μM) of (*trans*)-B[*a*]P-7,8-diol was needed to reach the mutation frequencies reported here for XEM1 and XEM2 cells. This clearly demonstrates that genetically engineered V79 cells capable of metabolic activation can be more sensitive than V79 cells that depend on extracellular metabolic activation.

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References

1. Malling, H. V. Dimethylnitrosamine: formation of mutagenic compounds by interaction with mouse liver microsomes. *Mutat. Res.* **13**:425–429 (1971).
2. Ames, B. N., P. Sims, and P. L. Grover. Epoxides of polycyclic hydrocarbons are frameshift mutagens. *Science (Wash. D. C.)* **176**:47–49 (1972).
3. Ames, B. N., J. McCann, and E. Yamasaki. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsomal mutagenicity test. *Mutat. Res.* **31**:347–364 (1975).
4. Langenbach, R., H. J. Freed, and E. Huberman. Liver cell-mediated mutagenesis of mammalian cells by liver carcinogens. *Proc. Natl. Acad. Sci. USA* **75**:2864–2867 (1978).
5. Doehmer, J., S. Dogra, T. Friedberg, S. Monier, M. Adesnik, H. R. Glatt, and F. Oesch. Stable expression of rat cytochrome P-450IIB1 cDNA in Chinese hamster cells (V79) and metabolic activation of aflatoxin B_1. *Proc. Natl. Acad. Sci. USA* **85**:5769–5773 (1988).
6. Chirgwin, J. M., A. E. Przybla, R. J. MacDonald, and W. J. Rutter. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294–5299 (1979).
7. Gubler, U., and B. J. Hoffman. A simple and very efficient method for generating cDNA libraries. *Gene* **25**:263–269 (1983).
8. Haymerle, H., J. Herz, M. Bressan, R. Frank, and K. Stanley. Efficient construction of cDNA libraries in plasmid expression vectors using an adaptor strategy. *Nucleic. Acids Res.* **14**:8615–8624 (1986).
9. Hanahan, D. Studies on transformation of *E. coli* with plasmids. *J. Mol. Biol.* **166**:557–580 (1983).
10. Kimura, S., F. J. Gonzalez, and D. Nebert. The murine *Ah* locus: comparison of the complete cytochrome P1-450 and P3-450 cDNA nucleotide and amino acid sequences. *J. Biol. Chem.* **259**:10705–10713 (1984).
11. Adesnik, M., and M. Atchison. Genes for cytochrome P-450 and their regulation. *Crit. Rev. Biochem.* **19**:247–305 (1985).
12. Maxam, A. M., and W. Gilbert. Sequencing end-labeled DNA with base specific chemical cleavage. *Methods Enzymol.* **65**:499–560 (1980).
13. Chuvpilo, S. A., and V. V. Kravchenko. A simple and rapid method for sequencing DNA. *FEBS Lett.* **179**:34–36 (1984).
14. Russel, W. C., C. Newman, and D. H. Williamson. A simple cytochemical technique for demonstration of DNA in cells infected with *Mycoplasma* and viruses. *Nature (Lond.)* **253**:461–462 (1975).
15. Graham, F. L., and A. van der Eb. A new technique for the assay of infectivity of human adenovirus 5 DNA. *J. Virol.* **52**:456–467 (1973).
16. Southern, E. M. Detection of specific sequences among DNA fragments separated by gel electrohoresis. *J. Mol. Biol.* **98**:503–517 (1975).
17. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.)* **227**:680–685 (1970).
18. Nebert, D. W., and H. V. Gelboin. Substrate inducible microsomal arylhydrocarbon hydroxylase in mammalian cell culture. I. Assay and properties of induced enzyme. *J. Biol. Chem.* **243**:6242–6249 (1968).
19. Greenlee, W. F., and A. Poland. An improved assay of 7-ethoxycoumarin *O*-deethylase activity: Induction of hepatic enzyme activity in C57BL/6J and DBA/2J mice by phenobarbital, 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J. Pharmacol. Exp. Ther.* **205**:596–605 (1978).
20. Platt, K. L., and F. Oesch. Efficient synthesis of non-K-region dihydrodiols of polycyclic aromatic hydrocarbons from *O*-quinones and catechols. *J. Org. Chem.* **48**:265–268 (1983).
21. Yabusaki, Y., M. Shimizu, H. Murakami, K. Nakamura, K. Oeda, and H. Ohkawa. Nucleotide sequence of a rat full-length cDNA coding for 3-methylcholanthrene-induced rat liver cytochrome P-450MC. *Nucleic Acids Res.* **12**:2929–2938 (1984).
22. Conney, A. H. Induction of microsomal cytochrome P-450 enzymes: the first Bernard B. Brodie lecture at Pennsylvania State University. *Life Sci.* **39**:2493–2518 (1986).
23. Glatt, H. R., and F. Oesch. Structural and metabolic parameters governing the mutagenicity of polycyclic aromatic hydrocarbons, in *Chemical Mutagens: Principles and Methods for their Detection* (F. J. de Serres, ed.), Vol. 10. Plenum Publishing Corporation, New York, 73–127 (1986).
24. Kiefer, F., and F. J. Wiebel. V79 Chinese hamster cells express cytochrome P-450 activity after simultaneous exposure to polycyclic aromatic hydrocarbons and aminophylline. *Toxicol. Lett.* **48**:265–273 (1989).
25. Crespi, C. L., R. Langenbach, K. Rudo, Y.-T. Chen, and R. L. Davies. Transfection of a human cytochrome P-450 gene into the human lymphoblastoid cell line AHH1 and use of the recombinant cell line in gene mutation assays. *Carcinogenesis (Lond.)* **10**:295–301 (1989).
26. Davies, R. L., C. L. Crespi, K. Rudo, T. R. Turner, and R. Langenbach. Development of a human cell line by selection and drug-metabolizing gene transfection with increased capacity to activate promutagens. *Carcinogenesis (Lond.)* **10**:885–891 (1989).
27. Battula, N. Transduction of cytochrome P3-450 by retroviruses: constitutive expression of enzymatically active microsomal hemoprotein in animal cells. *J. Biol. Chem.* **264**:2991–2996 (1989).
28. Glatt, H. R., A. Seidel, O. Ribeiro, C. Kirkby, P. Hirom, and F. Oesch. Metabolic activation to a mutagen of 3-hydroxy-*trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene, a secondary metabolite of benzo[*a*]pyrene. *Carcinogenesis (Lond.)* **8**:1621–1627 (1987).
29. Bradley, M. O., B. Bhuyan, M. C. Francis, R. Langenbach, A. Peterson, and E. Huberman. Mutagenesis by chemical agents in V79 Chinese hamster cells: a review and analysis of the literature. *Mutat. Res.* **87**:81–142 (1981).
30. Levin, W., A. W. Wood, A. Y. H. Lu, D. Ryan, S. West, A. H. Conney, D. R. Thakker, H. Yagi, and D. M. Jerina. Role of purified cytochrome P-448 and epoxide hydrase in the activation and detoxification of benzo[*a*]pyrene, in *Drug Metabolism Concepts* (D. M. Jerina, ed.), *American Chemical Society ARC Symposium Series* No. 44. American Chemical Society, Washington, DC, 99–125 (1977).
31. Oesch, F., H. R. Glatt, and H. U. Schmassmann. The apparent ubiquity of epoxide hydratase in rat organs. *Biochem. Pharmacol.* **26**:603–607 (1977).
32. Turchi, G., M. A. Carlucio, F. Oesch, I. Gemperlein, and H. R. Glatt. Characterization of an epithelial, nearly diploid liver cell strain, from Chinese hamster, able to activate promutagens. *Mutagenesis* **2**:127–135 (1987).
33. Bentley, P., F. Oesch, and H. R. Glatt. Dual role of epoxide hydratase in both activation and inactivation of benzo[*a*]pyrene. *Arch. Toxicol.* **39**:65–75 (1977).
34. Deutsch, J., J. C. Leutz, S. K. Yang, H. V. Gelboin, Y. L. Chiang, K.P. Vatsis, and M. J. Coon. Regio- and stereoselectivity of various forms of purified cytochrome P-450 in the metabolism of benzo[*a*]pyrene and (−)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene as shown by product formation and binding to DNA. *Proc. Natl. Acad. Sci. USA* **75**:3123–3127 (1978).
35. Wilson, N. M., M. Christou, C. R. Turner, S. A. Wrighton, and C. R. Jefcoate. Binding and metabolism of benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene by seven purified forms of cytochrome P-450. *Carcinogenesis (Lond.)* **5**:1475–1483 (1984).
36. Glatt, H. R., R. Billings, K. L. Platt, and F. Oesch. Improvement of the correlation of bacterial mutagenicity with carcinogenicity of benz[*a*]pyrene and four of its major metabolites by activation with intact liver cells instead of cell homogenate. *Cancer Res.* **41**:270–277 (1981).

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