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Genetics

Introduction of cytochrome P450IA2 metabolic capability into cell lines genetically matched for DNA repair proficiency/deficiency

(CHO cells/mutagen sensitivity/beef mutagens/mutagenesis testing)

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ABSTRACT **We introduced into the CHO cell line the cDNA of the mouse cytochrome P_3450 (P450IA2) gene, which oxidizes aromatic amines. A cDNA clone of P_3450 was transfected into mutant UV5 cells, which is defective in nucleotide excision repair. Expression of the P_3450 cDNA was measured using 9000 × *g* supernatant (S9) fractions from CHO cells to evaluate *Salmonella* TA1538 mutagenicity with the mutagen 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ). The P_3450-expressing clone UV5P3 was reverted to repair proficiency using ethyl methanesulfonate to obtain the UV-resistant clone 5P3R2, which maintained the same level of P_3450 protein activity as UV5P3. These genetically similar cell lines were compared for toxicity and mutation induction at the *aprt* locus. With 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (the most prevalent mutagen found in fried beef) the differential sensitivity due to repair deficiency/proficiency was ≈40-fold, and with IQ there were smaller, but significant, differences in sensitivity. These genotoxic effects occurred at doses that were ≈10 times lower than those that previously gave similar effects in S9-mediated assays. Thus, these cell lines should be valuable for genotoxicity analysis as well as important for assessing DNA repair when evaluating compounds that undergo metabolic activation.**

When cells within an organism are exposed to environmental mutagens, two processes determine the magnitude of the genetic effects. (*i*) Metabolic conversions can activate compounds to electrophilic derivatives, which react with cellular DNA, and detoxify reactive species. (*ii*) The damage produced in DNA by covalent binding of metabolites is subject to removal by repair processes. Unrepaired DNA adducts often cause mutations that may initiate cancer and other deleterious effects (1). The optimal use of cultured cell lines for mutation assays requires manipulating the processes of metabolism and repair within one experimental system. We demonstrate this approach with the widely used Chinese hamster ovary (CHO) cell line.

Currently, most mammalian cell mutation assays for the short-term assessment of mutagenic/carcinogenic chemicals employ cells that are not representative of the metabolic capacity of cells in key tissues of the intact organism. Cytochrome P450 monooxygenase enzymes are needed for the metabolism of nonpolar organic chemicals (2, 3), which CHO cells generally cannot activate. Test systems have relied on metabolism by enzymes provided in a supplementary liver fraction (9000 × *g* supernatant, S9) to mimic the desired metabolism (4, 5). The metabolites produced must, therefore, penetrate the target cell to react with nuclear DNA. Moreover, S9 is a complex mixture that produces many competing reactions, making it difficult to standardize and to understand the contribution of each pathway.

A class of dietary compounds known as aminoimidazoazaarenes, which occur in fried beef (6), are bacterial mutagens and rodent carcinogens (7–10). Several of these compounds, such as 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), are among the most potent mutagens in the Ames/*Salmonella* test (6). However, IQ had surprisingly weak genotoxic effects in CHO cells when the rat-liver S9 fraction was used for activation (11). It was unclear whether this difference in potency was due to a difference in the genetic sensitivity between the two systems or due to the failure of unstable metabolites to penetrate the nucleus of CHO cells (12). However, another beef mutagen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (13) was 100 times less potent than IQ in *Salmonella*, but highly mutagenic in CHO cells (14). To understand the disparate responses between these systems, cells having efficient oxidative metabolism are needed. Therefore, for expression of such activity in CHO cells, we have initially used the mouse cytochrome P_3450 (IA2 subfamily) gene (15). The P450IA2 cytochromes are implicated in the metabolism of a variety of heterocyclic amines (16–20).

Although other laboratories have transfected cytochrome P450 cDNA to obtain responsiveness to mutagens (21–23), we present a more versatile approach. Most mammalian cell lines used to measure mutations have the drawback of DNA repair capacity. Therefore, we first introduced the mouse P_3450 cDNA into hypersensitive UV5 cells that lack nucleotide excision repair (24–26). As our goal was to isolate a pair of genetically similar cell lines differing in repair capacity while equally expressing cytochrome P_3450, we then reverted the resulting cells to repair proficiency.

MATERIALS AND METHODS

Cells and Culture Conditions. CHO parental line AA8, which is heterozygous at the *aprt* locus (27), was used to derive repair-deficient UV5 (24), which is fully deficient in nucleotide excision repair and is corrected by the cloned human gene *ERCC2* (28). Cells were grown in α-modified minimal essential medium supplemented as described (28). Doubling times measured in suspension culture were as follows: UV5, 12 hr; UV5P3, 16 hr; 5P3R2, 17 hr.

Construction of P_3450 Expression Plasmid. The full-length mouse P_3450 coding sequence, originally isolated using the pcD mammalian expression vector (29, 30), was kindly provided by Daniel Nebert (University of Cincinnati Medical Center, Cincinnati) in a plasmid designated pm$P_3$450FL (29). Digestion of this plasmid with *Bam*HI released an insert of 2.0 kilobases and two other fragments that did not match pcD (31). Therefore, we isolated the cDNA insert and introduced it into pcD after substantially shortening the leader and trailer sequences. The insert was doubly digested with *Rsa* I (par-

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Abbreviations: IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine.

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tial) and *Dra* I (total). Because the ATG start codon is closely flanked by an *Rsa* I site on either side, partial digestion with *Rsa* I resulted in 4 nucleotides of leader in some molecules. Digestion with *Dra* I left 51 nucleotides on the 3′ side of the TGA stop codon. Since the desired 1600-base-pair fragment was blunt-ended, an adapter to give *Bam*HI termini was constructed from the complementary commercial oligonucleotides d(5′-GATCCCCGGG-3′) and d(5′-pCCCGGG-3′) (New England Biolabs). A ligation reaction was performed with a 50:1 molar ratio of adapter to insert by using a procedure from the supplier. Excess adapter was removed by membrane filtration using a Centricon-30 microconcentrator (Amicon). The 2.98-kilobase pcD vector was prepared from a clonal isolate of the pcD human cDNA library (31) by digesting with *Bam*HI and isolating the vector fragment. Ligation of the vector to the modified insert was performed using a 1:5 molar ratio of vector to insert without phosphatase treatment. The ligation products were transfected into *Escherichia coli* AG1 competent cells (Stratagene) according to a protocol supplied by the manufacturer, and four of eight colonies that were examined contained plasmids with inserts. One of the four plasmids had the correct orientation and digestion pattern and was designated pcDP3.

Transfection of UV5 with P_3450 cDNA. To isolate transformants expressing the P_3450 cDNA, UV5 cells were cotransfected with pcDP3 and the dominant marker plasmid pSV2gpt, which is selectable in medium containing mycophenolic acid (28). Cells at a density of 4×10^6 cells per 100-mm dish in 10 ml of medium were exposed to 1 ml of calcium phosphate precipitate (32) containing 3 μg of pSV2gpt and 12 μg of pcDP3 DNA for 4 hr at 37°C. The medium was gently aspirated, and 2.5 ml of 20% (vol/vol) glycerol/medium at room temperature was added slowly down the side of the dish. After 60 sec, the glycerol was removed and the dish was rinsed three times with serum-free medium before adding 20 ml of complete medium. Cells were grown for 24 hr in normal medium before being replated into MAXTA selective medium (28). Colonies arose at a frequency of 8×10^{-5}, and eight were isolated for evaluation of P_3450 enzyme activity.

Detection of P_3450 Enzyme Activity by *Salmonella* Mutagenesis. Approximately 2.5×10^8 exponentially growing CHO cells were collected by centrifugation, rinsed in phosphate-buffered saline (PBS), resuspended in 1.5 ml of PBS, and disrupted using a Polytron PT1035 (Brinkmann) for 3 min at a setting of 6. The preparation was centrifuged at $400 \times g$ for 5 min and then the supernatant was centrifuged at $9000 \times g$ for 10 min. The supernatant fraction (S9) was taken and used immediately for *Salmonella* mutation assay, which was done according to standard procedures except the stock cofactor mixture additionally contained NADPH (33). Each plate received 200 μl of S9, in a total volume of 0.5 ml of cofactor mixture, and IQ (obtained from Toronto Research Chemicals, Downsview, ON).

Detection of P_3450 Protein by Immunoblotting. Microsome fractions were prepared from 3-ml S9 fractions (from 1 liter of the CHO suspension cultures) by centrifuging in a Sorvall SM24 rotor at 4°C for $9000 \times g$ for 10 min, collecting the supernatant, and then centrifuging the supernatant in a Beckman 70.1 Ti rotor at $10^5 \times g$ for 60 min. The pellet fraction was resuspended gently with a Polytron in 1 ml of 0.05 M Tris·HCl (pH 7.5) in 20% glycerol. Microsome fractions and purified rabbit cytochrome P450 forms 4 and 6 (34), provided by Michael McManus (School of Medicine, Flinders University of South Australia, Bedford Park), were subjected to SDS/polyacrylamide gel electrophoresis in 10% gels using the Hoefer SE 200 minigel apparatus and procedures. Proteins were transferred electrophoretically from the gel onto a nitrocellulose membrane (35). Nonspecific binding sites on the membrane were blocked by incubating in a solution consisting of 1.5% (wt/vol) bovine serum albumin, 0.05% Tween 20, and PBS (pH 7.4) for 60 min at 37°C. Primary goat anti-form 4 (rabbit) IgG antibody (34) (from M. McManus) was added to the same solution at a final concentration of 20.9 μg/ml and incubated further for 4 hr at 37°C using a procedure provided by M. McManus. The blot was washed for four 10-min periods before adding a secondary biotinylated anti-goat IgG made in rabbit (BA-5000, Vector Laboratories). This mixture was incubated for 30 min at room temperature with gentle shaking. After three washings, the blot was incubated with avidin and biotinylated horseradish peroxidase macromolecule complex (Vectastain ABC kit, Vector Laboratories) for 30 min at room temperature according to the procedure supplied by the manufacturer. Briefly, the blot was washed again three times in PBS without Tween-20 (all previous washings were done in PBS with 0.05% Tween-20) and transferred to the peroxidase substrate solution composed of 18 mg of 4-chloro-1-naphthol, 6 ml of methanol, 30 ml of PBS, and 150 μl of 3% (vol/vol) H_2O_2. Immunoreactive bands of purple color developed in 15 min.

Reversion of the P_3450-Expressing Cells to Repair Proficiency. To produce repair-proficient revertants of UV5P3 cells, an exponentially growing suspension culture containing 2×10^7 cells was treated with ethyl methanesulfonate (150 μg/ml) for 18 hr. Cells were subcultured for 3 days of expression and then plated into 150-mm dishes at 4.0×10^6 cells per dish. After a 4-hr incubation for attachment, dishes were UV-irradiated at 5 J/m^2 (24) and incubated at 37°C for 48 hr. After a second irradiation at 5 J/m^2, the dishes were incubated for 7 days and colonies were isolated using Pipetman tips and expanded for analysis.

Assays for Cell Killing and Mutation at the *aprt* Locus. In dose–response experiments, PhIP or IQ was added to exponentially growing cells in T75 flasks (Corning) containing 3×10^6 cells in 30 ml of medium. After 48 hr exposure, the cells were rinsed twice with PBS and removed with trypsin. Survival dishes and 200-ml suspension cultures at 2.5×10^4 cells per ml were initiated (27). After 3 days of growth, cultures were plated for cloning efficiency and mutations unless the cell concentration was below 2×10^5 per ml, in which case the cells were grown for 1–7 more days. *aprt* mutants were selected in medium containing 8-azaadenine (80 μg/ml) and 10% (vol/vol) dialyzed fetal bovine serum (27). Plating-efficiency dishes were incubated for 8–10 days, and 8-azaadenine dishes were incubated for 10–12 days. Stock solutions of PhIP and IQ were prepared at 10 mg/ml and 6.6 mg/ml, respectively, in dimethyl sulfoxide and stored at −20°C. Control cultures were exposed to dimethyl sulfoxide. PhIP was synthesized and provided by Mark Knize (36).

RESULTS

Isolation of P_3450 Transformants of UV5 Cells. Repair-deficient UV5 cells were cotransfected with plasmid pcDP3 and pSV2gpt selectable marker plasmid. Eight clones resistant to mycophenolic acid were isolated for analysis. To determine expression of the P_3450 cDNA, S9 fractions were prepared from each transformant culture and used to measure the mutagenicity produced by IQ in the Ames/*Salmonella* test strain TA1538. Five of the eight transformants gave no detectable mutagenesis above background whereas two transformants gave high, and very similar, levels of mutagenesis. Clone UV5P3-3 was subcloned to eliminate possible genetic heterogeneity accompanying the transformation process. Several subclones were tested for their sensitivity to killing by PhIP in the differential cytotoxicity assay (37) and found to be very similar, indicating phenotypic stability. Subclone UV5P3-3-3 was designated "UV5P3" and used in further studies.

Detection of P_3450 Protein by Immunoblotting. Based on S9-mediated *Salmonella* mutagenesis, it was clear that transformants expressed a cytochrome P450 cDNA. To verify that the expressed cDNA encoded the mouse P_3450 protein, an immunoblot was prepared as shown in Fig. 1. Staining of protein was performed using a primary antibody made against the rabbit cytochrome P450 form 4, which is equivalent to the mouse P_3450 protein (15). In the position of the gel marked by the arrow, no protein was present in UV5 cells (lane 1) whereas UV5P3 cells (lane 2) contained a protein of ≈54 kDa, in agreement with the size reported (38). Lanes 3 and 4 contain purified rabbit cytochrome P450 forms 4 and 6, respectively, which are both recognized by the antibody.

Reversion of the Defective Repair Gene in UV5P3. Our aim was to derive a repair-proficient line from clone UV5P3. Initially we transfected UV5P3 with the *ERCC2* repair gene, but the resulting transformant clones tested were somewhat genetically unstable. Therefore, we tried an alternative approach of reverting the repair deficiency. Since the UV5 line was originally produced by mutagenesis with ethyl methanesulfonate, we reasoned that this compound might produce phenotypic revertants. After mutagenesis and expression, cells were plated and exposed to two separate UV irradiations to select for revertants. UV-resistant colonies arose at a frequency of 2×10^{-6}.

Several of the larger colonies were isolated and expanded. The UV-survival characteristics of two of these revertants (5P3R1 and 5P3R2) are shown in Fig. 2. Both revertants had essentially the same level of UV resistance as the wild-type AA8 cells, which were ≈6-fold more resistant than the repair-deficient UV5P3 cells, based on D_{10} (the dose at which 10% of the cells survive) fluences.

Identification of a Revertant Having an Unaltered P_3450 Level. The mutagenic reversion process could have altered the expression of the P_3450 protein. Therefore, we compared the level of P_3450 protein in several revertants with that in UV5P3 cells using the same *Salmonella* TA1538 mutagenesis procedure used to identify the transformants. Fig. 3 summarizes the data from one experiment on revertants 5P3R1 and 5P3R2. Line 5P3R1 produced less mutagenic activity than the parental UV5P3 cells, but 5P3R2 had the same amount as UV5P3. In a second experiment the absolute values of activity for the CHO lines were somewhat higher, but the relative activities for UV5P3 and the two revertants were very similar. Therefore, revertant 5P3R2 was considered to be not measurably different from UV5P3 and was chosen for detailed mutagenesis studies. (A third revertant had lower activity than 5P3R1.) We also compared the average value of the P_3450 protein activity, by *Salmonella* reversion, in UV5P3 and 5P3R2 versus that in standard Aroclor-induced rat liver. In the two experiments mentioned above, the values for rat liver were ≈10-fold higher than the CHO values for equivalent amounts of protein.

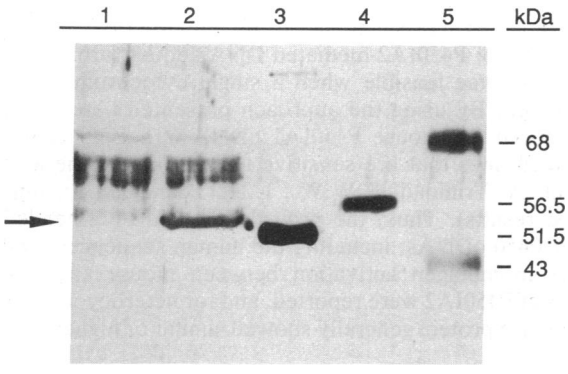

FIG. 1. Immunoblot of P450 proteins. The primary IgG antibody was made against rabbit cytochrome P450 form 4 (34) and detected with a biotinylated secondary antibody. Lanes: 1, 60 μg of microsomal fraction from UV5; 2, 54 μg of microsomal fraction from UV5P3 cells; 3, 5.8 pmol of rabbit P450 form 4; 4, 5.8 pmol of rabbit P450 form 6; 5, size standards (100 ng each of biotinylated bovine serum albumin and ovalbumin). The sizes of the rabbit P450 forms 4 and 6, marked at 51.5 and 56.5 kDa, respectively, were reported (18).

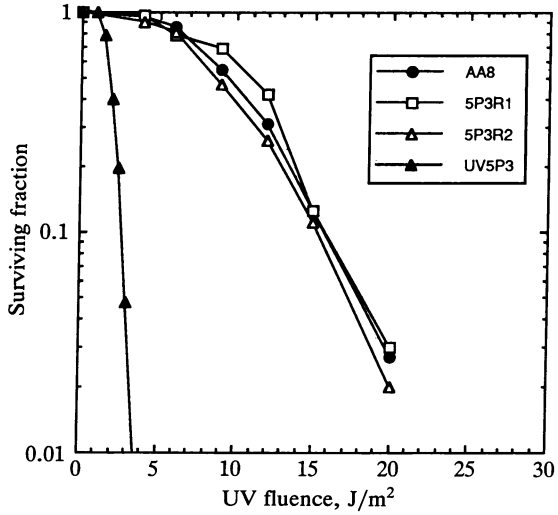

FIG. 2. UV-radiation survival curves of UV-resistant revertants versus parental lines UV5P3 and AA8.

Cell Killing and Mutagenesis Mediated by Metabolism of PhIP and IQ. Dose–response experiments were performed using the fried-beef mutagens PhIP and IQ, whose structures are shown in Figs. 4 and 5. Fig. 4 presents the results with PhIP for loss of colony-forming ability and induced mutations at the *aprt* locus. In UV5 cells there was no detectable effect on survival or mutagenesis at the highest dose tested (30 μg/ml). Line 5P3R2 showed pronounced cell killing and weak, but significant, mutation induction at 2–6 μg/ml. In comparison, the repair-deficient UV5P3 cells had a much more sensitive response. Measurable cell killing and highly significant mutagenesis occurred at the lowest dose tested (0.1 μg/ml). The response of the parental line before reclonning (UV5P3-3) was similar to that of subclone UV5P3. In summary, the differential effect of cellular repair capacity was ≈15-fold in terms of sensitivity to cell killing and ≈40-fold in terms of mutagenesis.

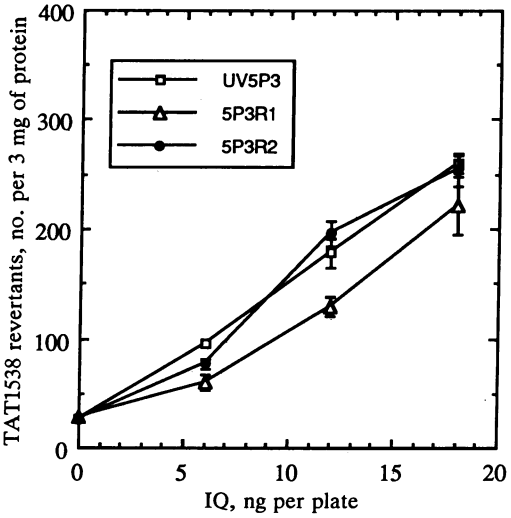

FIG. 3. *Salmonella* TA1538 reversion produced by IQ in the presence of S9 fraction. In the absence of S9, plates with 20 ng of IQ did not differ from solvent controls.

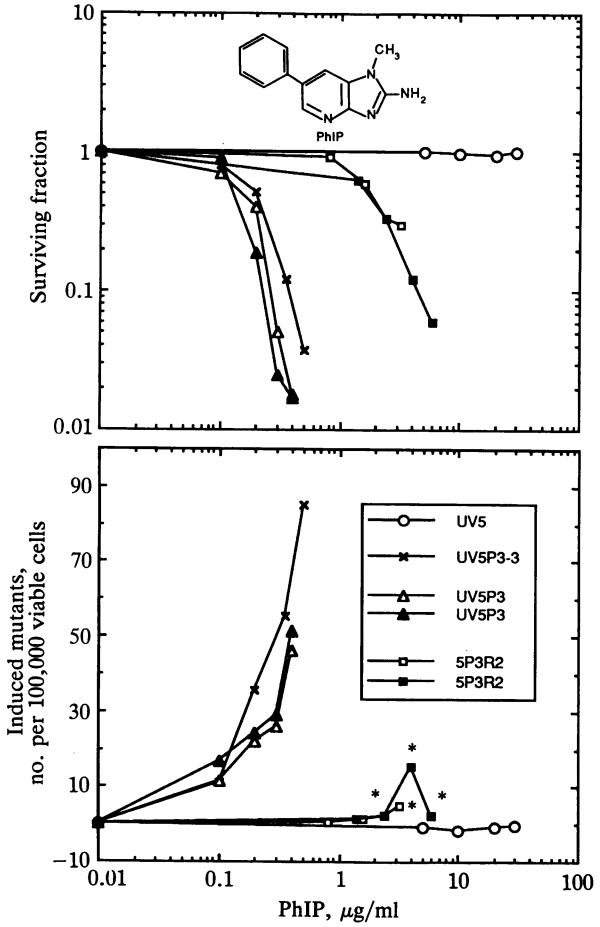

FIG. 4. Cell survival and induced mutations with PhIP. Different symbols show independent experiments. (*Upper*) Colony-forming ability (for definitions of symbols, see *Lower*). (*Lower*) Induced mutants at the *aprt* locus. The average spontaneous frequencies of duplicate control cultures were as follows: UV5, 2.9×10^{-5}; UV5P3-3, 7.5×10^{-5}; UV5P3, 7.1×10^{-5} and 8.9×10^{-5}; 5P3R2, 1.4×10^{-5} and 2.4×10^{-5}. Based on historical controls, data points marked by * were significantly elevated ($P < 0.01$, using tolerance intervals for a normal distribution).

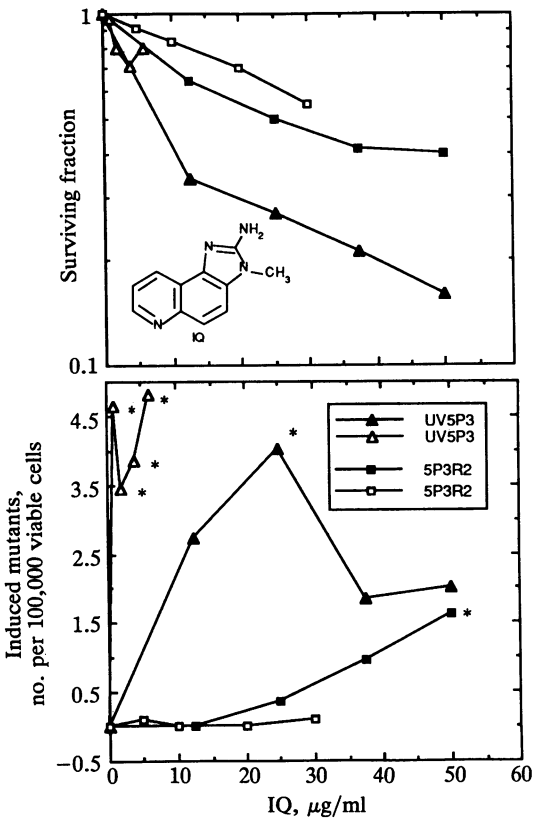

FIG. 5. Cell survival and induced mutations with IQ. The results of two experiments (open and solid symbols) are shown for lines UV5P3 and 5P3R2. (*Upper*) Colony-forming ability (for definitions of symbols, see *Lower*). (*Lower*) Induced mutants at the *aprt* locus. The average spontaneous frequencies of duplicate control cultures were as follows: UV5P3, 8.1×10^{-5} and 6.6×10^{-5}; 5P3R2, 2.1×10^{-5} and 2.1×10^{-5}. Based on historical controls, data points marked by * were significantly elevated ($P < 0.05$, using tolerance intervals for a normal distribution).

The two experiments performed with IQ were done over somewhat different dose ranges because of the atypical dose dependence seen with this compound (Fig. 5). The survival curves for both UV5P3 and 5P3R2 are unusual because of their shallow slopes; cell killing does not increase rapidly with increasing dose. Significant levels of induced mutations were seen in UV5P3 cells over the range of 1 to 50 μg/ml and in 5P3R2 cells at the highest dose (see Fig. 5). Mutant frequencies seemed to decline at higher dose in UV5P3 cells, suggesting the preferential conversion of damage to lethal lesions. Overall, the absolute numbers of induced mutants obtained with IQ were much lower than with PhIP. Thus, the relatively high background of spontaneous mutants consistently seen in UV5P3 cultures (Figs. 4 and 5) reduced the sensitivity of detecting induced mutants. In spite of the high background, significant mutation induction with IQ was detected in UV5P3 cells, even at doses resulting in 80–90% survival.

DISCUSSION

In this study we incorporated the combined features of DNA repair deficiency/proficiency and metabolic activation proficiency into the CHO cell line that is widely used for mutagenesis testing (39). The growth characteristics of the resulting cell lines (UV5P3 and 5P3R2) are very similar in terms of doubling times and plating efficiencies. Expression of the mouse P_3450 cDNA rendered both cell lines responsive to two heterocyclic amines (Figs. 4 and 5) at doses that were ≈10 times lower than those giving similar effects in S9-mediated assays (11, 14). This system has other advantages besides increased sensitivity. For compounds requiring P450IA2 activation, cytotoxicity assays can be used to efficiently detect genotoxicity, based on differential killing due to differences in repair capacity (37). Quantitative mechanistic studies of P450IA2-mediated DNA adduct formation and repair become feasible when a single cytochrome P450 is expressed. By using the approach presented here, we also introduced the mouse P450IA1 cDNA into UV5 cells and obtained lines that are sensitive to benzo[*a*]pyrene at ≤30 ng/ml (A. Trinidad, R.W.W., L.H.T., and J.S.F., unpublished results). Thus, the procedure could be extended to other P450 cDNAs, including the human sequences. Differences in mutagen activation between mouse and human forms of P450IA2 were reported, and for heterocyclic amines the mouse protein generally showed similar or higher activity (40).

An unexpected property of repair-deficient cells expressing the P_3450 cDNA is an elevated frequency of spontaneous *aprt* mutants. This elevation was seen with the primary clone UV5P3-3 as well as its subclone UV5P3. The average values of the duplicate spontaneous frequencies for UV5P3-3 (two experiments) and its subclone UV5P3 (four experiments) were 7.7×10^{-5}, compared with values of 2.0×10^{-5} for 5P3R2 (four experiments) and 2.4×10^{-5} for UV5 (two

experiments). All experiments were done with clonal stocks frozen immediately after expansion to mass culture, thus minimizing the accumulation of mutations. This 3-fold elevation in spontaneous mutant frequency, which is seen only in repair-deficient $P_3$450-expressing cells, suggests that $P_3$450 expression may be producing an endogenous mutagen, whose adducts can be removed by repair. Mutation rates determined by fluctuation experiments are needed to clarify this issue (41).

Several cytochrome P450 cDNAs have been introduced into other mammalian cell lines. For example, mouse cytochromes $P_1$450 (IA1) and $P_3$450 (IA2) were expressed in a transient system using a vaccinia virus vector, allowing the over-produced proteins to be studied (38). The cDNAs of rat cytochromes P450IIB1 and P450IA1, under the control of simian virus 40 sequences, were expressed in stably transformed V79 hamster cells (21, 42). In human lymphoblasts, the cDNAs of several genes involved in xenobiotic metabolism were introduced using episomal Epstein–Barr virus vectors (22). The expression of either human P450IA1 or P450IA2 cDNA in this system enhanced the mutagenicity of aflatoxin B_1 (22, 43). Human P450IIA2 and epoxide hydrolase cDNAs were introduced both singly and on the same vector, producing cells that were readily mutagenized by dimethylnitrosamine, had increased sensitivity to benzo[*a*]pyrene, and expressed appreciable epoxide hydrolase activity (23).

Our findings provide insights into the mutagenic potency and relevance of IQ and PhIP, which are animal carcinogens of moderate, and similar, potency (7–10). In earlier S9-mediated mutation experiments, IQ produced barely detectable mutagenicity in repair-proficient AA8 cells at concentrations >100 μg/ml whereas in repair-deficient UV5 cells mutations were detected >20 μg/ml (11). Cell survival curves plateaued and did not differ between AA8 and UV5 cells. Thus, compared with the previous study, the results presented here for introduced cytochrome P450 capability show much greater sensitivity and unequivocal genotoxicity with IQ (Fig. 5). The cause of the erratic dose dependence of mutagenicity in repair-deficient UV5P3 cells is unclear but may be partly due to the reduced experimental sensitivity caused by high background frequencies. With PhIP (Fig. 4), metabolic proficiency resulted in even greater potency than with IQ. The magnitude of the effect of repair capacity on cell killing with PhIP was ≈10-fold, more than was seen with any other tested compounds that produce bulky monoadducts (25, 26, 37). PhIP-induced mutations occurred at ≈40 times lower doses in repair-deficient cells than in repair-proficient cells. Thus, these adducts appear to be very efficiently removed by nucleotide-excision repair, a finding of potential dietary significance.

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