Mutagenicity, Unscheduled DNA Synthesis, and Metabolism of 1-Nitropyrene in the Human Hepatoma Cell Line HepG2

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ABSTRACT

The cell line HepG2 is derived from a well differentiated human hepatoblastoma, which retains many of the morphological characteristics of liver parenchymal cells. These cells at passages >95 were found to metabolically activate carcinogens to genotoxic metabolites. The addition of 6.8 μM 1-methyl-3-nitro-1-nitrosoguanidine, 5.3 μM 4-nitroquinoline-N-oxide, and 4-20.3 μM 1-nitropyrene resulted in the induction of mutations at the HGPRT locus as determined by 6-thioguanine resistance. This is the first description of the induction of mutations in these cells. Additionally, unscheduled DNA synthesis in the presence of 4 mM hydroxyurea was increased by 9% with 5.3 μM 4-nitroquinoline-N-oxide, 57% with 13.6 μM 1-methyl-3-nitro-1-nitrosoguanidine, and 380% with 8.2 μM 1-nitropyrene. High performance liquid chromatographic analysis of metabolites formed following incubation of HepG2 with either [3H]-1-nitropyrene or [14C]benzo(a)pyrene demonstrate the occurrence of aryl oxidation as well as nitroreduction.

INTRODUCTION

The polycyclic aromatic hydrocarbons, and many other environmental chemicals, are typically activated to mutagens and carcinogens through epoxidation by the cytochrome P-450 system (1, 2). Nitrated polycyclic aromatic hydrocarbons (nitroaranes) are environmental pollutants that are metabolized through the same epoxide mechanisms (3); however, nitroreduction of the exocyclic nitro group to form a reactive aryl nitrenium ion is thought to play the major role of metabolic activation of these compounds (3, 4). Certain cells used for in vitro assays are deficient in some nitroreductases, and thereby are refractory to mutagenesis by the nitroarenes (5, 6). On the other hand, bacteria including Salmonella typhimurium are mutagenic quite readily by nitroarenes as the result of the presence of a variety of nitroreductases (7, 8).

Many in vitro tests have been used in the evaluation of the mutagenicity and potential carcinogenicity of chemicals (9). Several of these tests involve the use of bacterial cells (10) or mammalian fibroblast and epithelial cell lines (11–13). Many of these assays are limited to rodent cell lines, and the enzymes responsible for most of the activation and deactivation pathways of chemical mutagens and carcinogens are not present. Therefore, in order to demonstrate the activity of these chemicals, reliance is placed upon the addition of exogenous preparations of enzymes (10, 14), cocultivation with hepatocytes (15–19), or on cell-mediated assays (20, 21). A more useful approach would be to obtain a target cell that retains, through continued passage, the enzymes necessary for the activation of many of the mutagens and carcinogens. Primary hepatocytes from either rodents or humans (18, 22–24) are viable candidates; however, the generally nonproliferating hepatocyte dedifferentiates in culture and the concentration of the enzymes that are necessary for activation of chemicals progressively declines (18). Cell lines have been developed from rodent and human hepatomas; however, many of these cells do not retain sufficient levels of the enzymes necessary for metabolic activation of chemicals (18, 25–27).

Recently, a cell line was derived from a human hepatoblastoma HepG2, which retains its parenchymal hepatocellular character (28), and contains the enzymes necessary for the activation of polycyclic aromatic hydrocarbons (29). In addition, these cells were found to be responsive to the induction of sister chromatid exchanges upon exposure to a genotoxicant (30). In this present study we use HepG2 cells and describe the induction of gene mutations at the HGPRT gene locus and DNA damage measured by unscheduled DNA synthesis by 4-NQO, MNNG, and 1-nitropyrene. This is the first demonstration of induced mutagenesis in the human cell line HepG2. In addition we report the metabolism of the environmental pollutant 1-nitropyrene in these cells, and compare it to that of benzo(a)pyrene, whose metabolism in HepG2 was reported earlier (29).

MATERIALS AND METHODS

Chemicals. [4,5,9,10-3H]-1-Nitropyrene (920 mCi/mmol) was obtained from Chemsyn Inc., Lenexa, KS, and was determined to be greater than 99% pure by gas and thin layer chromatography. 1-Nitropyrene (dinitropyrene free) was a gift from R. Mermelstein, Xerox Corp., Rochester, NY. [methyl-3H]Thymidine (80 Ci/mmol) and [G-14C]BaP (59 Ci/mol) were purchased from New England Nuclear, and the labeled BaP was purified by HPLC immediately prior to use (31). 4-NQO and MNNG were obtained from the National Cancer Institute Chemical Repository. Aryl sulfatase and β-glucuronidase were obtained from Sigma Chemical Co. All other chemicals were at least reagent grade.

Cells and Media. The HepG2 cells were obtained from L. Diamond, Wistar Institute, Philadelphia, PA, at passage 95. The cells were maintained in continuous culture in minimal essential media (Gibco) with the addition of 10% (v/v) heat-inactivated fetal bovine serum (Gibco). The cells were subcultured at 1:2–1:3 splits every 7–10 days, essentially as described by Diamond et al. (29). The medium was changed every 4–5 days, and the cells were maintained at 37°C under 5% CO2 and high humidity. The plating efficiency under the conditions used was approximately 25%. Stocks of cells were routinely frozen and stored in liquid N2.

Mutation Assay. Cells for the mutation assay were prepared by seeding 1 × 106 cells onto 100-mm dishes (13 × 105 cells/cm2). After 2 days at 37°C, when the cells were in the exponential growth phase, the medium was changed to MEM plus 2.5% HI FBS. The test chemicals dissolved in DMSO were then added to a final DMSO concentration in the medium of less than 0.5%. Following a 3-h incubation period, the medium containing 2.5% serum was removed, the cells were washed with Hank's balanced salt solution without Ca2+ and Mg2+, and then removed with 0.25% trypsin-EDTA (Gibco). The cells (0.5–1 × 106) were then seeded onto 60-mm dishes with 5 ml of regular medium containing 10% serum for survival estimation which, after one 10-day incubation

Received 12/1/86; revised 3/10/87; accepted 3/19/87.

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2 Supported by the NIH, with Grant ES 03648 to P. C. H., Grant CA 32126 to G. D. M., and Grant ES 02827 to H. S. R.

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2 The abbreviations used are: 4-NQO, 4-nitroquinoline-N-oxide; MNNG, 1-methyl-3-nitro-1-nitrosoguanidine; BaP, benzo(a)pyrene; HPLC, high performance liquid chromatography; MEM, minimal essential medium; HI FBS, heat-inactivated fetal bovine serum; DMSO, dimethyl sulfoxide; HBSS, Hank's balanced salt solution; 6TG, 6-thioguanine; 6TG', 6-thioguanine resistant; UDS, unscheduled DNA synthesis; DNP, dinitropyrene; 1-NP, 1-nitropyrene.

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at 37°C, surviving cell colonies were fixed with methanol and stained with 0.5% methylene blue in 50% methanol. The rest of the cells were plated and maintained for 10 days in culture with regular medium which included 2-3 subcultures to allow expression of the mutant phenotype.

The cells were then removed with trypsin, enumerated, and 0.5-1 × 10⁶ cells were plated in 100-mm plates (6.13 × 10⁵ cells/cm²) and grown in regular medium containing 30 μM 6 TG for the selection of 6 TG' colonies. Plating efficiency was determined in the absence of 6 TG by seeding 0.5-1 × 10⁶ cells onto 60-mm dishes with 5 ml of regular medium. After 10 days of incubation at 37°C under 5% CO₂ in air and high humidity, the colonies were stained with 0.5% methylene blue in 50% methanol and counted. The cells used for the detection of HGPRT mutant colonies were incubated with 6 TG for 20-25 days with medium changes every 4-5 days; the 6 TG' colonies were washed with HBSS, fixed with methanol, and stained with 0.5% methylene blue in 50% aqueous methanol. The colonies were counted, and the mutation frequencies were determined by dividing the number of colonies by the number of cells plated, times the plating efficiency, and expressed as the number of 6 TG' clones/10⁵ clonable cells. For each level of chemical tested 3-4 replicate plates were used throughout the assay.

Unscheduled DNA Synthesis. HepG2 cells at confluence in 60-mm plates (4-5 × 10⁶ cells), were exposed to various concentrations of the chemicals 1 h following a medium change to 5 ml of MEM plus 2.5% HI FBS containing 4 mM hydroxyurea. [methyl-³H]Thymidine (3 μCi/ml) and the test chemicals (in DMSO) were added and incubated for 2 h. The monolayer was washed with HBSS, removed with a rubber policeman, centrifuged at 100 × g, and stored overnight at −20°C. The DNA was precipitated and collected by the trichloroacetic acid method (32). The presence of radiolabel in the DNA was determined by liquid scintillation counting, using Scintisol (Isolab, Akron, OH) as the scintillant. DNA was quantitated spectrophotometrically (33).

Metabolism of 1-Nitropyrene and Benzo(a)pyrene. The medium of a confluent layer of cells on a 60-mm plate (4-5 × 10⁶ cells) was changed to MEM plus 2% HI FBS and the cells were incubated for 15 min at 37°C. To these cells were added either [³H]-1-nitropyrene or [³H]BaP to final concentrations of 4 μM and 1.3 μM, respectively, and the cells were incubated for up to 24 h. The media were removed, the cells were washed with HBSS, and the monolayer was removed with 0.25% trypsin-EDTA. The cells and media were then stored at −20°C. Precursor and metabolites were extracted from the media or cells following addition of an equal volume of chloroform:methanol (2:1), followed by a second extraction with an equal volume of chloroform. The aqueous phases were purged of methanol with nitrogen, and then incubated with either 15 units/ml aryl sulfatase (pH 5.1 with 0.1 M sodium acetate), or 1500 units/ml β-glucuronidase (pH 7.4 with 0.1 M potassium phosphate) to hydrolyze conjugated metabolites to the aglycones or aglycones, which were then extracted with chloroform.

[³H]-1-Nitropyrene metabolites were analyzed by HPLC techniques (Fig. 1a) analogous to those previously described (34), except that the solvents were water and acetonitrile:methanol (1:1). Cochromatography with synthetic nonradiolabeled compounds was used to establish the identity of the radiolabeled metabolites. Similarly, [³H]BaP metabolism was determined by cochromatography of the radiolabeled metabolites with authentic metabolites (Fig. 1a) as previously described (31).

Instrumentation. Scintillation counting was performed by using a Packard Tri-Carb 460 liquid scintillation spectrometer. HPLC analyses were performed by using a Varian 5000 instrument equipped with a Rheodyne injector, and either a Varian UV100 detector or an Isco (Lincoln, NE) UA5 absorbance monitor equipped with a 10-μl HPLC flow cell. Fractions of 0.5 or 1 ml were collected, and the presence of radiolabeled compounds was evaluated following the addition of either Scintisol or Scintiverse-LC (Fisher). For analysis of [³H]-1-nitropyrene metabolites, the solvent gradient was a linear increase of 45% solvent B in 3 min. Solvent A was water, and solvent B was acetonitrile:methanol (1:1). The column was a 0.39 × 30-cm C₁₈-μBondapak reversed phase column (Waters Associates) at a flow of 2 ml/min. The spectra of the 1-aminopyrene was obtained with a Hewlett-Packard 1040 diode array spectrometer as the compound eluted from the HPLC column.

RESULTS

A mutagenicity assay was developed in the HepG2 cells (Table 1). MNNG and 4-NQO were used as positive controls in these tests, and exposure of the HepG2 to 6.8 μM and 5.3 μM, respectively, resulted in the net induction of 86 and 48 mutants/10⁶ clonable cells. The addition of 1-nitropyrene to HepG2 resulted in a marked increase in the frequency of mutations at the HGPRT gene locus. The mutation frequency of 1-nitropyrene plateaus at concentrations in excess of approximately 10 μM. The cytotoxicity of 1-nitropyrene, MNNG, and 4-NQO ranged from 0 to 30% at the concentrations tested.

Additionally the same chemicals induced unscheduled DNA synthesis (Table 2) in HepG2 cells. The administration of 4

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**Table 1** Mutagenicity at the HGPRT gene locus in HepG2 cells

<table>
<thead>
<tr>
<th>Chemical</th>
<th>μM</th>
<th>% of survival after chemical treatment</th>
<th>Mutants/10⁵ clonable cells²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Nitropyrene</td>
<td>2.0</td>
<td>109</td>
<td>18.3 ± 3.6</td>
</tr>
<tr>
<td>4.0</td>
<td>112</td>
<td>75.7 ± 15.1</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>95</td>
<td>487 ± 115</td>
<td></td>
</tr>
<tr>
<td>20.3</td>
<td>65</td>
<td>525 ± 93</td>
<td></td>
</tr>
<tr>
<td>MNNG</td>
<td>6.8</td>
<td>76</td>
<td>86.3 ± 12.1</td>
</tr>
<tr>
<td>4-NQO</td>
<td>5.3</td>
<td>70</td>
<td>47.9 ± 19.1</td>
</tr>
</tbody>
</table>

² The percentage of clonable cells in each group before selection with 6TG was approximately 25-30%.

³ Mutation frequency: mean (± SD) of 2 replicates in 3-4 experiments minus the spontaneous frequency of the DMSO (<0.5%) control (22.8 ± 3.5 mutants/10⁵ clonable cells).

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**Fig. 1.** High pressure liquid chromatographic analysis of (a) benzo(a)pyrene metabolites and (b) 1-nitropyrene. Conditions are described in "Materials and Methods." In each case nonradiolabeled metabolites (−−−) were co-injected with radiolabeled metabolites (−−−−−) isolated from incubation of HepG2 with radiolabeled precursors. Retention times for BaP metabolites are as follows: BaP-9,10-dihydrodiol, 7.9 min; BaP-4,5-dihydrodiol, 18.8 min; BaP-7,8-dihydrodiol, 23.9 min; BaP-1,6-dione, 36.8 min; BaP-3,6-dione, 40 min; BaP-6,12-dione, 42.8 min; BaP-4,5-oxide, 44.2 min; BaP-9-phenol, 48 min; BaP-3-phenol, 51.8 min; BaP, 64.2 min. Retention times for 1-NP metabolites are as follows: NP-4,5-dihydrodiol (not shown), 7.1 min; N-acetyl-1-aminopyrene, 10.1 min; 1-aminopyrene, 15.1 min; NP-4,5-oxide and NP-9,10-oxide, 17.8 min; 1-pyreneol, 18.2 min; NP-6-phenol and NP-8-phenol, 20.6 min; NP-3-phenol, 24.4 min; NP, 28.2 min; 1-nitrosopyrene, 30.1 min.
with the accumulation of the [3H]-1-nitropyrene into the cells, followed by metabolism to water-soluble conjugates which are transported into the medium. Support for this hypothesis was derived from HPLC examination of the radiolabeled compounds in the medium and cells (Fig. 1b; Table 3). After 4 h of incubation, the precursor concentration in the medium had dropped to 82.2%, with 2.0 and 1.1% of the total compounds as [3H]-1-aminopyrene in the medium and cells, respectively. This demonstrated that nitroreduction of [3H]-1-nitropyrene to [3H]-1-aminopyrene was occurring in the cells as the absence of nitroreduction was noted when [3H]-1-nitropyrene was incubated in medium plus serum but devoid of cells. In addition, the metabolite that had identical elution times as 1-aminopyrene had the same UV-visible spectra as authentic 1-aminopyrene (not presented). The only time significant amounts of nonconjugated metabolites other than [3H]-1-aminopyrene were detected in the media was at $t = 0.1$ h.

Since the metabolism of [3H]-1-nitropyrene indicated the formation of low levels of phenols and dihydrodiols, to ascertain the presence of microsomal oxidative pathways, we incubated [14C]benzo(a)pyrene with the HepG2 cells (Table 3). The metabolism of [14C]benzo(a)pyrene was similar to that reported earlier by Diamond et al. (29). As with the [3H]-1-nitropyrene, there was a redistribution of the radiolabel from organic soluble material in the medium (73.1%) to organic nonextractable material in the medium (91.9%) over 24 h (Fig. 3). Associated with this was the detection of radiolabeled compounds in the organic soluble fraction of the cells at 4, 9, and 24 h. HPLC analysis of the organic extracts from the cells and medium at 4 h (Fig. 1a; Table 3) demonstrated the presence of both phenols and diols.

Since glutathione, sulfate, and carbohydrate conjugates of xenobiotics occur in vivo, the aqueous fractions of the cells and media were incubated with aryl sulfatase and β-glucuronidase to hydrolyze the conjugates to the corresponding asulfones and aglycones (Table 3). Sulfate conjugates of [3H]-1-nitropyrene-trans-4,5-dihydrodiol, [3H]-1-nitropyrene-6-ol, and/or [3H]-1-nitropyrene-8-ol, and [3H]-1-nitropyrene-3-ol were detected, accounting for 11.7% of the radiolabeled material in the aqueous phase of the medium at 24 h. Incubation of the 24-h medium aqueous phase with β-glucuronidase resulted in less than 5% of the radiolabel partitioning into chloroform as aglycones. Of the metabolite detected, 23.8% comigrated with [3H]-1-aminopyrene. The remaining 83.9% of the conjugates in the medium at 24 h have not been characterized, but exhibit a chromatographic behavior consistent with glutathione conjugates. Incubation of the aqueous fraction from [14C]benzo(a)pyrene-treated cells with aryl sulfatase demonstrated that at 4 h 36.8% and 10.7% of the radiolabeled compounds comigrated in HPLC with 3-hydroxybenzo(a)pyrene and benzo(a)pyrene-9,10-diol. As with [3H]-1-nitropyrene, less than 5% glucuronide conjugates were detected.

**DISCUSSION**

The present study indicates that the human liver-derived cell line HepG2 can be useful in demonstrating mutagenicity and genotoxicity. One of the most interesting aspects of this cell line is that it retains some biosynthetic and metabolic capabilities of normal human liver parenchymal cells, thereby retaining liver cell functions. Diamond et al. (29) have previously demonstrated that cocultivation of HepG2 with Chinese hamster V79 cells mediated the induction in V79 cells of mutations at the HGPRT gene locus, as measured by the induction by
benzo(a)pyrene of 6TG resistance. Dearfield et al. (30) later demonstrated that HepG2 cells metabolized cyclophosphamide to genotoxic intermediates that induced increases in sister chromatid exchanges. Our data show that 1-nitropyrene was found to be directly mutagenic and genotoxic (Tables 1 and 2) in the HepG2 cell line. This suggests that the cell line possesses the enzymes necessary for the metabolic activation of 1-nitropyrene to genotoxic metabolites (at passages higher than 95).

Diamond et al. (29) also demonstrated that HepG2 cells at passages 45 to 62 metabolized benzo(a)pyrene to specific phenolic and dihydrodiol derivatives. This metabolism resulted in the formation of two DNA adducts, i.e., as a result of reaction of the anti and syn isomers of the benzo(a)pyrene-7,8-diol-9,10-epoxide with deoxyguanosine. In our studies, we find that cells at passages greater than 95 are still capable of metabolizing benzo(a)pyrene in a manner similar to that reported by Diamond et al. (29). Very few unconjugated benzo(a)pyrene metabolites were found in the cells and media (Table 3). Hydrolysis of the media with aryl sulfatase resulted in the release of 3- and 9-hydroxybenzo(a)pyrene, benzo(a)pyrene-7,8 and 9,10-diol, and benzo(a)pyrene quinones. As reported by Diamond et al. (29), and as shown with 1-nitropyrene (Table 3), the uridine diphosphoglucuronic acid transferase pathway is not active in these cells, as evidenced by the absence of glucuronide conjugates of benzo(a)pyrene metabolites (Table 3).

The nitrated polycyclic aromatic hydrocarbons are a class of chemicals which are environmental contaminants (35, 36). 1-Nitropyrene is one of the most abundant of the chemicals in this class. These compounds are produced in most combustion processes, and are found in diesel emissions, coal-powered plant emissions, cigarette smoke, cooked meat products, and gas burner emissions (4, 37–39). The occurrence, mutagenicity, and carcinogenicity of these compounds have been reviewed (36).

1-Nitropyrene is activated in vitro and in vivo through the enzymatic reduction of the nitro group to the corresponding nitroso-, hydroxylamino-, and aminopyrenes (3, 40). The formation of the N-aryldihydroxyamine is responsible for genotoxicity of many of the nitroarenes, and is apparently responsible for DNA adduction in S. typhimurium (41), human diploid fibroblasts (35, 41), and in target tissues in vitro (42). For some nitroarenes, esterification of the hydroxylamine through an O-esterificase is necessary for activation. In the HepG2 cells, we find 1-nitropyrene to be mutagenic (Table 1). This then indicates that nitroreduction is occurring under the aerobic conditions in which these cells were cultured.

In addition to metabolism through nitroreduction, 1-nitropyrene is metabolized by liver microsomes in vitro and in vivo to aryl epoxides, yielding a mixture of stable K-region nitropyrene epoxides (43), or nitropyrene epoxides which rearrange to form nitropyrenols (3, 44). The K-region epoxides have been shown to react directly with DNA in S. typhimurium (41), human diploid fibroblasts (35, 41), and in target tissues in vitro (42). For some nitroarenes, esterification of the hydroxylamine through an O-esterificase is necessary for activation. In the HepG2 cells, we find 1-nitropyrene to be genotoxic (Table 1). This then indicates that nitroreduction is occurring under the aerobic conditions in which these cells were cultured.

Since we are unable to determine the nature of the "unidentified" radiolabeled products in the aqueous fraction following extraction with chloroform, we cannot quantitate the rates of nitroreduction and arene oxidation in these cells. However, we can conclude that the ratio of nitroreduction to arene oxidation is quite high in these cells as compared to microsomes in vitro, where low oxygen tension is evidently needed to allow nitroreduction (45). In addition, while nitroreduction seems to be the dominant pathway for metabolism in S. typhimurium, selected cultures of obligate and facultative anaerobes, and mixed intestinal microflora from rats and man in vitro and in vivo (41, 46), it has not been shown to be the predominant pathway in eukaryotic cells in vitro and in vivo, explaining perhaps the lack of mutagenicity in many of these cells.

Evidence for the presence of nitropyrene arene oxidation comes from the HPLC analysis of the cultures (Table 3).
Although few nonconjugated phenol or dihydrodiol derivatives were detected, sulfate esters of 1-nitropyrene-4,5-dihydrodiol and 1-nitropyren-(6 or 8)-ol were detected. Incubation of 1-NP with liver microsomes from several species has revealed that in most cases formation of the 6- and 8-phenols is preferred over the 3-phenol (3, 40). This is consistent in the HepG2. In addition, in many species the 4,5-dihydrodiol is formed in differing amounts, sometimes ranging from one-half to twice as much as the 6- and 8-phenols (34, 45). From our results we can conclude that the diols are formed about one- to twice as much as the 6- and 8-phenols (34, 45). From our results we can conclude that the diols are formed about one- to twice as much as the 6- and 8-phenols. We have demonstrated that microsomal mixed function oxidases specific for the epoxidation of arenes are present in addition. The formation of diols with these compounds (Table 3) would be consistent with the presence of epoxide hydrolase. Dearfield et al. (30) have measured the levels of cytochrome b$_5$, benzphetamine-y-demethylase, and ethoxyresorufin-O-deethylase were below detection limits.

It was shown earlier (47) that 1,6-dinitropyrene induces DNA repair and damage in rat and human hepatocytes. More recently (48, 49) 1,6- and 1,8-DNP have been shown to be carcinogenic in rats and mice while 1-NP was not carcinogenic. These researchers (49) concluded that the carcinogenicity of 1-NP shown earlier (50) was possibly due to contamination of the preparation with DNPs.

The 1-NP used in our study was greater than 99% pure which was checked on gas chromatography to be free from DNP contamination. Work done in our laboratory on the mutagenicity of the DNPs in HepG2 cells, under similar experimental conditions used for 1-NP, have indicated that 1,6- and 1,8-DNP are devoid of mutagenic activity (51, 52). Therefore, in this study the possibility of 1,6-DNP contamination could not alter the mutagenicity of 1-NP in HepG2 cells. While several investigators have used HepG2 cells to activate carcinogens and subsequently detect the mutations in cocultured cells, our results demonstrate that one can detect mutagenic events in the HepG2. We also have determined that the enzymes responsible for the repair of this genetic damage are intact in these cells as evidenced by the induction of unscheduled DNA synthesis (Table 2). Clearly, this ability to measure mutagenicity and genotoxicity directly in a single cell line of human origin and epithelial cell type, combined with the capacity for metabolic activation and proliferation makes the HepG2 cell line relevant as a model system for short-term testing of human mutagens and carcinogens.

ACKNOWLEDGMENTS

The authors wish to thank L. Diamond for HepG2 cells, R. Mer melstein for 1-nitropyrene, M. C. Biaglow and G. J. DeMarco for technical assistance, and M. Birkel for preparing the manuscript.

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