

Reactive Oxygen Species Generated by PAH *o*-Quinones Cause Change-In-Function Mutations in *p53*

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Polycyclic aromatic hydrocarbons (PAHs) in tobacco smoke may cause human lung cancer via metabolic activation to ultimate carcinogens. *p53* is one of the most commonly mutated tumor suppressor genes in this disease. An analysis of the *p53* mutational database shows that G to T transversions are a signature mutation of lung cancer. Aldo-keto reductases (AKRs) activate PAH *trans*-dihydrodiol proximate carcinogens to yield their corresponding reactive and redox-active *o*-quinones, e.g., benzo[*a*]pyrene-7,8-dione (BP-7,8-dione). We employed a yeast reporter system to determine whether PAH *o*-quinones or the ROS they generate cause change-in-function mutations in *p53*. *N*-Methyl-*N*-nitroso-*N*-nitro-guanidine, a standard alkylating mutagen was used as a positive control. MNNG caused a dose-dependent increase in mutant yeast colonies and at the highest concentrations 8–14% of the yeast colonies were mutated and were characterized by G:C to A:T transitions in the *p53* DNA binding domain. Treatment of *p53* cDNA with micromolar concentrations of (±)-*anti*-7,8-dihydroxy-9 α ,10 α -epoxy-7,8,9,10-tetrahydro-benzo[*a*]pyrene, (*anti*-BPDE, an ultimate carcinogen) or sub-micromolar concentrations of BP-7,8-dione in the presence of redox-cycling conditions (NADPH and CuCl₂) also caused *p53* mutations in a dose-dependent manner. We found that no mutants were observed with PAH *o*-quinones or NADPH alone. *p53* mutagenesis by BP-7,8-dione was attenuated by ROS scavengers and completely abrogated by a combination of superoxide dismutase and catalase, indicating that both superoxide anion and hydroxyl radicals were the responsible mutagens. The bulk of the mutations detected were single-point mutations and were not random in occurrence. Over 46% of BP-7,8-dione-induced mutations were G:C to T:A transversions, consistent with the formation of 8-oxo-dGuo or its secondary oxidation products. In addition, 25% of these mutations were at hotspots in *p53* which are known to be mutated in lung cancer. Together these data suggest that PAH *o*-quinones generate an endogenous mutagen (ROS) which leads to *p53* inactivation. These observations provide an alternative route to G to T transversions that dominate in *p53* in lung cancer.

Introduction

Lung cancer is a leading cause of death of both males and females in the U.S. population (1). One of the genes most commonly mutated in this disease is the *p53* tumor suppressor gene, which normally exerts a brake on the cell-cycle and prevents cells with DNA-damage from entering into S-phase (2). When *p53* is mutated, cells that would otherwise be arrested and enter into apoptosis now have a growth advantage so that they will survive. Lung cancer appears to be unique in the type of mutations observed in *p53*, there is a preponderance of G to T transversions, and this is in fact a signature mutation for the disease (3).

Exposure to tobacco smoke has long been known as the major risk factor for the development of lung cancer (4, 5). Of the mutagens present in cigarettes, polycyclic aromatic hydrocarbons (PAHs)¹ are candidates for causing the mutations in *p53*. Since PAHs require metabolic activation to exert their mutagenic effects, the question arises as to whether activated PAH-metabolites can cause

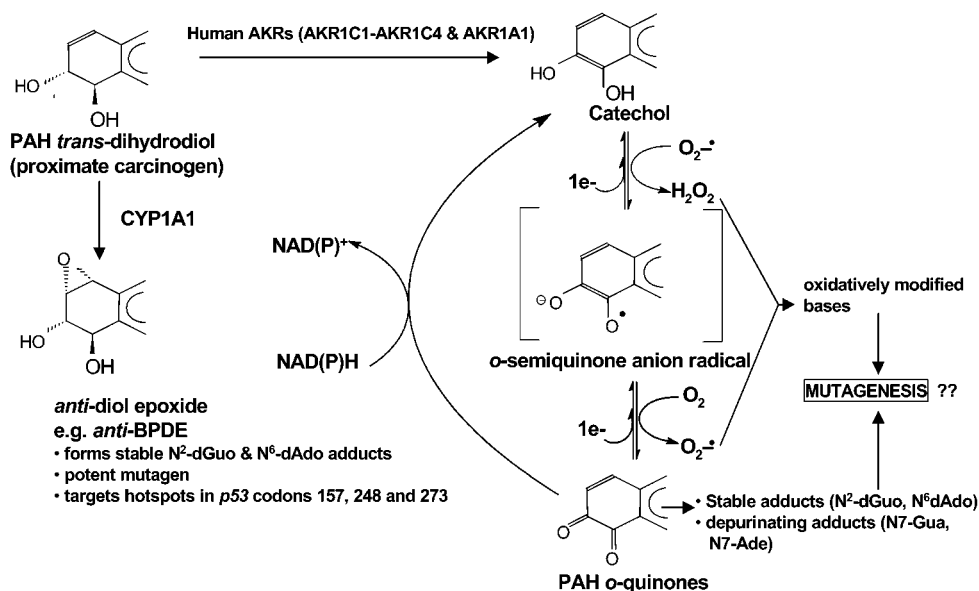
the G to T transversions observed. Three principal pathways of PAH activation have been proposed. These include the formation of *anti*-diol-epoxides by the combined action of CYP1A1 and epoxide hydrolase (6, 7), the formation of radical cations by CYP peroxidase (8), and the formation of reactive and redox-active *o*-quinones by dihydrodiol dehydrogenase members of the aldo-keto reductase (AKR) superfamily (9). Of these the most widely accepted pathway of activation is the formation of *anti*-diol-epoxides. In this metabolic sequence benzo[*a*]pyrene (BP), is converted to the (–) *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene (BP-7,8-diol) which is subsequently converted to (+)-*anti*-7,8-dihydroxy-9 α ,10 α -epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, (+)-*anti*-BPDE, Scheme 1. Up until now only the diol-epoxides have been examined for their ability to cause DNA adducts and mutations in *p53*.

¹ Abbreviations: AKR, Aldo-keto reductase; *anti*-BPDE, (±)-*anti*-7,8-dihydroxy-9 α ,10 α -epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; BP-diol, (±)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene; BP-7,8-dione, benzo[*a*]pyrene-7,8-dione; CYP, cytochrome P450; DD, dihydrodiol dehydrogenase; MNNG, *N*-methyl-*N*-nitroso-*N*-nitroguanidine; NP-1,2-dione, naphthalene-1,2-dione; 8-oxo-dGuo, 7,8-dihydro-8-oxo-2'-deoxyguanosine; PAH, polycyclic aromatic hydrocarbon; ROS, reactive oxygen species; SOD, superoxide dismutase.

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Scheme 1. Activation of PAH to Mutagens by Aldo-Keto Reductases



(\pm)-*anti*-BPDE forms stable N^2 -deoxyguanosine adducts in p53 (10, 11). If these adducts are unrepaired, translesional DNA synthesis by error-prone bypass polymerases can introduce an A opposite the bulky adduct, which upon replication, leads to a G to T transversion (12). Discrete codons on p53 were shown to be more prone to dG adduct formation with (\pm)-*anti*-BPDE (codons 157, 158, 245, and 273) and these corresponded to "hot spots" mutated on p53 in patients with lung cancer, suggesting a causal relationship (11).

Studies of the p53 mutational database have compared the mutational pattern in lung-cancer patients versus nonlung cancer patients. In this analysis, the frequency of each of the possible 12-different base substitutions was examined and identified a preponderance of G to T transversions. This mutational pattern appears to be unique to lung cancer. This analysis also examined the mutational spectrum in which the percentage of all G to T transversions were plotted against codon number in the p53 gene and identified hot spots or codons most prone to undergo this mutation (13). There are also now sufficient data in the database to compare the p53 mutational spectra between smokers and nonsmokers with lung cancer. These data show that there is coincidence between the "hot spots" mutated and sites of adduction seen with (\pm)-*anti*-BPDE in vitro and in cell culture (13, 14). In recent experiments human bronchial epithelial cells were treated with (\pm)-*anti*-BPDE and the incidence of G to T transversions in p53 was scored in codon 157 using a restriction fragment length polymorphism-PCR protocol (14). These experiments showed a dose-dependent frequency of G to T transversions in this codon and a similar approach was used to score high G to T transversions in codons 248, 249, and 250. Thus, a compelling inferential case has been developed to relate (\pm)-*anti*-BPDE adducts to the occurrence of p53 mutations that are observed in smokers with lung cancer.

Other studies have implicated a role of reactive oxygen species in lung carcinogenesis (15, 16). This may be supported by the finding that one allele of *hOGG1*, a mammalian base excision repair enzyme that excises 8-oxo-dGuo by acting as an AP-glycosylase/lyase, has been shown to be deleted in some human lung cancers

(17, and references cited therein). *hOGG1* is located on the distal end of the short arm of chromosome (3p25/26) and nearly 100% of cells derived from small cell lung cancers (which represent one-third of all lung cancers) are characterized by a loss of heterozygosity at chromosome 3. This suggests that during the development of lung cancer individuals lose the ability to excise the most common DNA lesion associated with ROS exposure and this could increase the mutational load. Because of the well-established association of smoking with lung cancer, these observations raise the issue of whether there is a known pathway of activation of tobacco carcinogens that increases the production of ROS.

Dihydrodiol dehydrogenase members of the AKR superfamily oxidize PAH *trans*-dihydrodiols (precursors of the diol-epoxides) to catechols that are air-sensitive (9). These catechols undergo two 1 electron oxidations leading to the concurrent generation of ROS (18). As a result of this autoxidation event a PAH *o*-quinone [e.g., benzo[*a*]pyrene-7,8-dione (BP-7,8-dione)] is produced. Because the PAH *o*-quinones are Michael acceptors they have the ability to form stable adducts (e.g., BP-7,8-dione- N^2 -dGuo and BP-7,8-dione- N^6 -dAdo) and depurinating adducts (e.g., BP-7,8-dione- N^7 -Gua and BP-7,8-dione- N^7 -Ade) which may have mutagenic potential (19, 20). In addition, in the presence of reducing equivalents PAH *o*-quinones can be converted back to the catechol, Scheme 1. Alternatively, the *o*-quinone can undergo two 1e enzymatic reductions via CYP-NADPH reductases back to the catechol (21). In each of these instances trace amounts of PAH *o*-quinones establish futile redox-cycles that will amplify ROS multiple times.

In humans at least five members of the aldo-keto reductase superfamily have dihydrodiol dehydrogenase activity (AKR1C1-AKR1C4, and AKR1A1). Of these AKR1C1 is inducible by planar aromatics and ROS (22), and differential display shows that it is highly expressed in nonsmall cell lung carcinoma (23). This high expression has been correlated to a poor prognostic outcome. One interpretation of these data is that AKRs are overexpressed as a result of PAH exposure and are involved in their metabolic activation in situ.

In this study, we used a yeast reporter system to score change-in-function mutations in p53 DNA caused by products of the AKR pathway, e.g., PAH *o*-quinones or the ROS they generate during redox-cycling. We find that sub-micromolar concentrations of PAH *o*-quinones are highly mutagenic on *p53* provided they are used under redox-cycling conditions. Furthermore, the mutations seen on *p53* are predominantly G to T transversions which are consistent with the formation of 8-oxo-dGuo and/or its secondary oxidation products. These transversions were not random and occurred on many of the 22 most commonly mutated codons in lung cancer. These findings provide an alternative route by which PAH-metabolites can mutate a tumor suppressor gene implicated in this disease.

Materials and Methods

Chemicals and Reagents. Adenine, L-leucine, L-tryptophan, NADPH, Superoxide dismutase (type I from bovine erythrocytes), and catalase (from bovine liver) were purchased from Sigma (St. Louis, MO). YEASTMAKER Yeast Transformation and Plasmid Isolation Kit and all yeast culture media were obtained from CLONTECH (Palo Alto, CA). NP-1,2-dione and BP-7,8-dione were synthesized according to the published routes (24, 25). *N*-Methyl-*N'*-nitro-*N*-nitroso-guanidine (MNNG) and (\pm)-*anti*-BPDE were obtained from the National Cancer Institute, Chemical Carcinogen Repository (Kansas City, Missouri). Other reagents were of the highest grade available.

Caution: All PAHs are potentially hazardous and should be handled in accordance with "NIH Guidelines for the Laboratory Use of Chemical Carcinogens".

Yeast Strains, Media, and Plasmids. The ade reporter yeast strain, yIG397, and gap-repair expression vector pss16 were kindly provided by Dr. Richard Iggo (Swiss Institute for Experimental Cancer Research, 1066 Epalinges, Basel, Switzerland) (26). Basic methods for yeast manipulations were carried out as described (27). Liquid media contained 0.67% yeast nitrogen base, 2% dextrose, 1% casamino acids, and 20 μ g/mL of adenine. Solid media for prototrophic selection of appropriate plasmids contained 0.67% yeast nitrogen base, 2% dextrose, 2% agar with complete additions minus the relevant amino acids and nucleosides to select for auxotrophic markers.

Preparation of p53 cDNA Fragment and Gapped Vector. Full-length wild-type human *p53* cDNA was RT-PCR-amplified from HepG2 cell RNA. The amplimers used were 5'-(forward primer) 5'dCTGCCATGGAGGAGCCGCAGTCA-3' (where the underline is the initiation codon) and 5' (reverse primer) 5'-dGATCAGTCTCAGTCAGGCCCTTC-3' (where the underline is the termination codon). The cDNA was subcloned into the PCR cloning vector pCRII-DNA (Invitrogen, Carlsbad, CA). Sequencing revealed that PCR had not introduced any mutations into *p53*. The insert was released with *Eco*RI and resolved by agarose gel electrophoresis. The gapped repair vector was prepared for homologous recombination using *Hind*III/*Stu*I, and the vector fragment purified by gel electrophoresis.

p53 Mutagenesis Assay. The purified p53 DNA fragment (200 ng) was incubated with MNNG (10% methanol, v/v), (\pm)-*anti*-BPDE or PAH *o*-quinone (8% DMSO, v/v) in 10 mM sodium phosphate buffer (pH 7.4) in the absence or presence of redox-cycling conditions, e.g., 1 mM NADPH and 100 μ M CuCl₂ for 1 h at 37 °C. After mutagenesis, the yIG397 yeast strain (grown to a OD₆₀₀ 0.6–0.9) was co-transformed with a mixture of 50–100 ng of the pSS16 gapped-vector, 50–100 ng of the mutagen treated p53, and 100 μ g of carrier DNA (herring testis) using the lithium acetate procedure according to the YEASTMAKER Yeast transformation System Kit (CLONTECH). The cells were collected, resuspended in 150 μ L of TE buffer and plated on synthetic minimal medium minus leucine plus minimal adenine (5 μ g/mL) and incubated for 3 days at 35 °C. The yIG397 strain has an adenine reporter gene under the control of the p21

promoter stably integrated into its genome. Wild-type p53 stimulates the reporter gene expression whereas change-in-function mutations of p53 do not. Yeast colonies expressing wild-type p53 are white, and yeast colonies expressing mutant p53 are red. Red colonies were clearly identifiable after 3 days at 30 °C but the color is more intense after an additional 2 days at 4 °C. The mutation incidence was expressed as (number of red colonies – number of spontaneous red colonies)/total number of colonies \times 100.

Recovery of p53 Plasmids from Yeast and DNA Sequencing. p53 expression plasmids were rescued from transformed yeast following lysis with glass beads (Sigma, St. Louis, MO) as described (27). Briefly, yeast colonies were cultured in 5 mL of SC-*leu* for 2 days. An aliquot of the culture medium (1.5 mL) was transferred to an Eppendorf tube, the yeast were collected by centrifugation at 10 000 rpm for 10 s and resuspended in 0.1 mL of 50 mM TrisHCl (pH 8.0), 62.5 mM Na₂-EDTA, 2.5 M LiCl, and 4% Triton X-100. After addition of 0.1 mL of phenol:chloroform (1:1, v/v) and 0.2 g of acid-washed glass beads (Sigma, St. Louis, MO) the cells were disrupted by vigorous vortexing for 2 min. The upper aqueous phase was removed following centrifugation at 12 000 rpm for 2 min. The DNA was precipitated by adding 0.2 mL of ethanol. After washing with 70% ethanol and drying, the DNA was dissolved in 20 μ L of Tris-EDTA, pH 8.0. Plasmid DNA (1 μ L) was used for transformation into XL-1 blue electro-competent *E. coli* cells (Stratagene, Cedar Creek, TX). The transformation was carried out using a Bio-Rad (Hercules, CA) Gene Pulser Electroporator according to the instruction manual. The DNA binding domain of p53 (codons 126–339) was sequenced on both strands with S6 (5'-dCTGGGACAGCCAAGTCTGT3') and R6 (5'-dCCTCATTCAGCTCTCGGAA3') primers on an Applied Biosystems 373A automated sequencer in the DNA sequencing facility at the Cell Center at the University of Pennsylvania School of Medicine.

Statistical Analysis. (1) Frequency of Base Substitutions. There are 12 possible base substitutions that can occur. Each of the four bases could be mutated to any one of the remaining three. Since the mutagenicity assay does not distinguish whether the initial hit occurred on the coding or noncoding strand the number of possible base substitutions is effectively reduced to six: C \rightarrow T (transition), C \rightarrow G (transversion), G \rightarrow T (transversion), A \rightarrow G (transition), A \rightarrow C (transversion), and A \rightarrow T (transversion). The statistical test used was based on the probability that any one of these six mutations was preferred over a random distribution. To test the hypothesis that BP-7,8-dione caused G \rightarrow T mutations (G:C to T:A transversions) more frequently than other mutations, we used a χ^2 goodness-of-fit test. Under the null hypothesis that mutations occur randomly, all six particular mutations should occur with equal frequency. For our primary comparison, we retained all six possible mutations and tested the general fit of the data to the randomness assumption. This produces a χ^2 statistic with 5 degrees of freedom. As a secondary analysis, we tested whether G \rightarrow T transversions occurred more frequently than all other mutations combined. This leads to a test with 1 degree of freedom. We also estimated the proportion of mutations that were G \rightarrow T mutations, along with an exact 95% binomial confidence interval. We performed an a priori sample size calculation to plan the number of mutations we needed to sequence. We estimated that we would need to sequence 83 mutations to have 80% power to detect an increase in frequency of G \rightarrow T mutations so that they represented 30% of all mutations. In this calculation, we allowed for an increased frequency of G \rightarrow A mutations (to >20%), as well. We did an interim analysis at $N = 63$ mutations, and because we found a much higher frequency of G \rightarrow T mutations than anticipated, we stopped sequencing earlier than planned.

Similar logic was applied to the analysis of the MNNG mutations. Here an interim calculation at $N = 17$ mutations was performed and because 88% of the mutations were G:C \rightarrow A:T transitions there was no further need to sequence additional mutations.

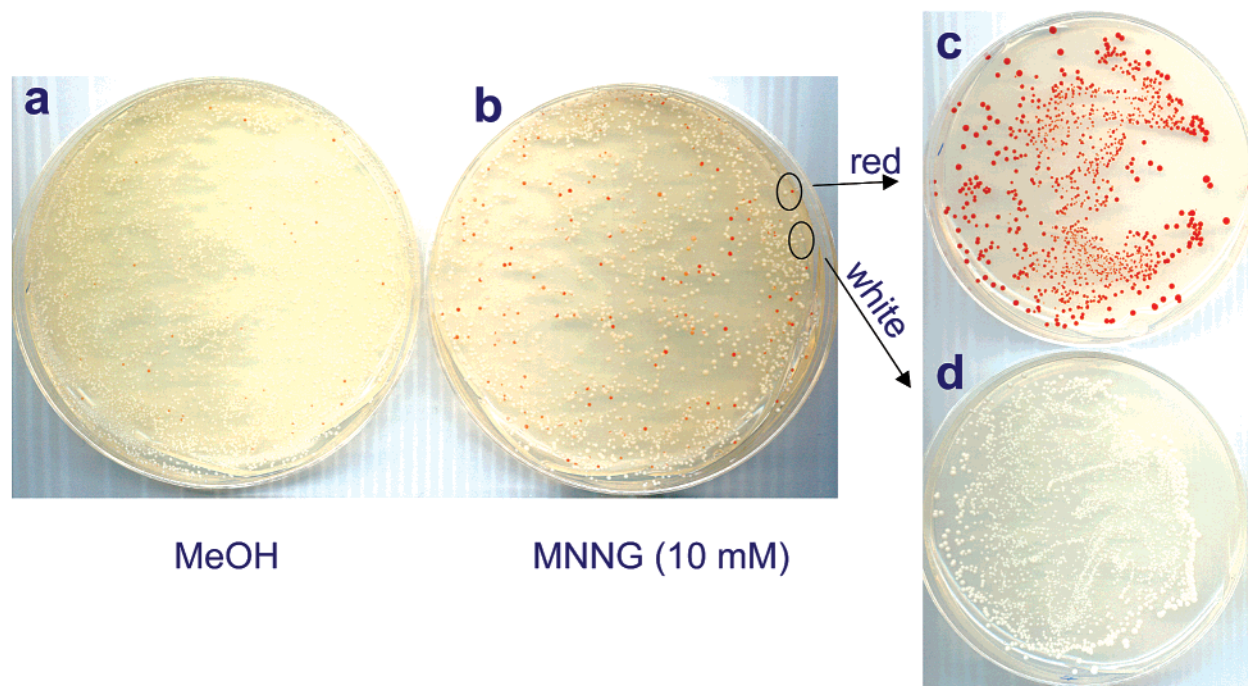


Figure 1. Validation of the *p53* mutagenesis assay using MNNG. Mutagenesis by vehicle (MeOH) (a); mutagenesis observed with MNNG (10 mM) (b); retransformation of yeast with *p53* expression plasmid from red colonies turns yeast reporter strain red (c); and retransformation of yeast with *p53* expression plasmid from white colonies turns yeast reporter strain white (d).

(2) Frequency of Mutations at Hotspots. It is known from the *p53* mutational database that mutations in 22 codons (from amino acids 154–275) account for more than 50% of the mutations in lung cancer. If the mutations in our experiment were also associated with lung cancer, we would expect them to occur preferentially in one or more of these 22 defined “hot spots.” We sequenced from amino acids 126–339 or 213 codons. If the 63 mutations we identified were occurring randomly across those locations, we would expect the number of mutations occurring in “hot spots” to be proportional to the relative number of hot spots within the 213 codons we sequenced. This would mean that 22/213 mutations, or 10%, would occur in hot spots, if the mutations were occurring randomly. We tested this hypothesis of randomness by testing whether the observed frequency of mutations occurring in hot spots was statistically consistent with a true underlying binomial probability of 10%.

Results

p53 Mutagenesis Assay. To perform the mutagenesis assay we treated naked ds-*p53* cDNA with a test mutagen, and then mixed the *p53* cDNA with the gap-repair plasmid *pss16* and transformed a *yIG397* yeast strain. This yeast strain contains an adenine reporter gene under the control of the *p21* promoter stably integrated into its genome. In vivo, the *p53* fragment and gap-repair plasmid undergo homologous recombination. Wild-type *p53* will bind to the *p21* promoter to activate the adenine reporter and as a consequence yeast colonies turn white. By contrast, plasmids containing *p53* mutated in the DNA-binding domain will not bind to the *p21* promoter so the adenine reporter gene is not activated, and in the presence of limiting adenine, colonies turn red, Scheme 2.

MNNG-Induced Mutations in the DNA Binding Domain of *p53*. The mutagenesis assay was validated by incubating the *p53* cDNA fragment with a common alkylating agent, MNNG, and then using the *p53* to co-transform the *ade* yeast reporter strain *yIG397* in the presence of the gapped vector. The number of total

Scheme 2. Adenine Reporter System for Detection of *p53* Mutagenesis

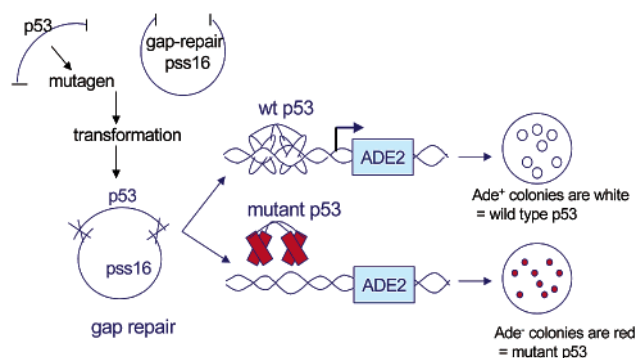


Table 1. Detection of *p53* Mutation by MNNG in the Yeast Gap Repair Assay^a

MNNG (mM)	total colony no.	red colony no.	% red colony
0	1060	4	0.4
2.5	1012	26	2.6
5.0	400	32	8.0
10	157	23	14.6

^a *p53* cDNA fragment was incubated with 0, 2.5, 5.0, and 10 mM *N*-methyl-*N*-nitroso-*N*-nitro-guanidine (MNNG) for 1 h at 37 °C in 10 mM phosphate buffer (pH 7.4). Then the *p53* fragment was co-transformed with gapped vector into the *ade2* reporter yeast strain using lithium acetate. The transformants were grown on leu-synthetic media containing 5 μg/mL adenine. After 3–4 days of incubation at 35 °C the number of total and red colonies were counted and the % red colony numbers scored.

colonies observed (both white and red) was constant until concentrations of MNNG became cytotoxic. The number of red colonies formed increased in a dose-dependent fashion, (Table 1). At 10 mM MNNG 14.6% of the yeast colonies turned red while the vehicle treatment turned only 0.4% of the yeast colonies red (Figure 1, panels a and b). Plasmids isolated from the red colonies following treatment of *p53* with vehicle alone contained no *p53*

Table 2. DNA Sequencing of MNNG-Treated p53^a

yeast colony	codon no.	p53 mutation	AA change
white	1	none	
	2	none	
red	1	CTG → TTG	L→L
		CGT → CAT	R→H
	2	CAG → TAG	Q→Stop
	3	CCC → CTC	P→L
	5	TGC → TTC	C→F
	6	GGA → GAA	G→E
	9	GTG → ATG	V→M
	10	GGC → GAC	G→D
		GGA → GAC	G→D
		GAA → AAA	E→K
	11	GGG → GAG	G→E
	17	TCA → TTA	S→L
		ATC → ATT	I→I
		GCC → GTC	A→V
	26	CCT → TTT	P→F
27	CCC → CTC	P→L	
30	AAG → TAG	K→Stop	

^a After restriction digestion analysis, the plasmids from MNNG-treated p53 transformants (two white colonies and 12 red colonies) were analyzed by DNA sequencing using primers for both strands. The mutations in the p53 DNA binding domain are shown.

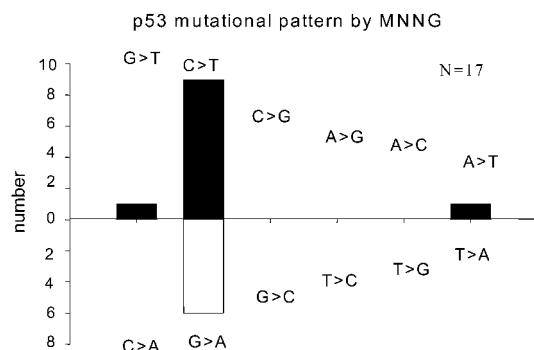


Figure 2. p53 Mutational pattern induced by the alkylating agent MNNG. The incidence of mutations observed in red colonies following treatment of wild-type p53 with MNNG is shown.

insert suggesting that vector religation had occurred rather than gap-repair.

To further validate the assay, we characterized the p53 gene from MNNG-treated cells. The expression plasmids were rescued from randomly selected colonies, two white and five red colonies. Restriction digestion analysis with *SacI/KpnI* revealed that all the colonies, both white and red, contained the p53 expression plasmids, suggesting that the p53 gene correctly recombined into the gapped vector. To confirm that these plasmids were responsible for the difference in the color of the yeast colonies, several plasmids from each of the white and red colonies were transformed back into the ade reporter yeast strain yIG397 (Figure 1, panels c and d). They produced 100% white and red colonies, respectively, indicating that the mutation in the p53 DNA binding domain accurately predicted the change in color of the yeast colonies (Figure 1, panels c and d).

To identify the mutations in p53 induced by MNNG, the rescued plasmids were sequenced through the p53 DNA binding domain (codons 126–339). No mutations were detected in p53 rescued from the white colonies (Table 2). The mutation pattern (Figure 2) shows that among the 12 red colonies examined, three had more than one-mutation. Of the total of 17 mutations, 15 were G:C → A:T transitions or 88% versus other possible muta-

Table 3. Detection of p53 Mutation by (±) anti-BPDE in the Yeast Gap Repair Assay^a

(±) anti-BPDE (μM)	total colony no.	red colony no.	% red colonies
0	1524	5	0.3
2.5	1112	10	0.9
5.0	944	11	1.2
10	608	9	1.5
20	169	12	7.1

^a p53 mutagenesis was determined in the presence of 0, 2.5, 5.0, 10, and 20 μM anti-BPDE as described in Table 1. The purity of (±) anti-BPDE was confirmed prior to the assay by RP-HPLC to detect either (±) tetraols or (±) trans-ring opened thioether conjugate [(±)-10ε-[(2-hydroxyethyl)thio]-7β,8α,9ε-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene]. The quantitative conversion of (±) anti-BPDE to a thioether conjugate in the presence of 2-mercaptoethanol ensured that (±) anti-BPDE was not hydrolyzed (29).

Table 4. DNA Sequencing of (±) anti-BPDE-Treated p53^a

yeast colony	codon no.	p53 mutation	AA change
3	164	AAG → AAT	K→N
5	182	TGC → TGA	C→Stop
15	243	ATG → ATC	M→I
2	247	AAC → ACC	N→T
22	248 ^b	CGG → CCG	R→P
18	262	GGT → CGT	G→R
21	283	CGC → AGC	R→S

^a (±) anti-BPDE-induced mutations in p53 DNA binding domain were determined in plasmids from seven red colonies as described in Table 2. ^b Hot-spot mutated in lung cancer.

tions. Statistical analysis showed that G:C → A:T transitions occurred with a $p < 0.0001$ over the other 5 possible mutations and with a $p < 0.0001$ over all the other mutations combined. These data are consistent with other mutagenesis studies using this alkylating agent (28).

Gap-Repair Assay and p53 Mutagenesis by (±) anti-BPDE. To address whether this gap-repair assay detects p53 mutations mediated by PAH metabolites, (±)-anti-BPDE was incubated with p53 cDNA and its mutagenicity was analyzed using this assay. The purity of the (±) anti-BPDE was assured by trapping the diol-epoxide as its thioether conjugate in aqueous solution (29). The basis of this analytical method is that unhydrolyzed (±)-anti-BPDE will yield one dominant thioether conjugate [10ε-[(2-hydroxyethyl)thio]-7β,8α,9ε-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene] whereas hydrolyzed (±)-anti-BPDE will yield a mixture of the (±)-trans and (±)-cis tetraols as well as a thioether conjugate (29). The (±)-anti-BPDE used in these experiments formed the thioether conjugate only.

In the mutagenicity assays we found that the number of red colonies increased in a dose dependent fashion, suggesting that this assay can also detect (±)-anti-BPDE-induced p53 mutations (Table 3). To identify the mutations observed, the p53 expression plasmids were rescued from randomly selected red colonies and sequenced through the DNA binding domain (Table 4). A large proportion of the red colonies contained plasmid with no p53 insert, indicating that with (±)-anti-BPDE a large number of gap-repair vectors self-ligated. Thus, most of the red colonies were false-positives, reducing the number of mutant p53 available for sequencing. In addition, at the higher concentrations of (±)-anti-BPDE used, the number of total yeast colonies fell precipitously indicating that this mutagen is quite cytotoxic in this assay. Of the

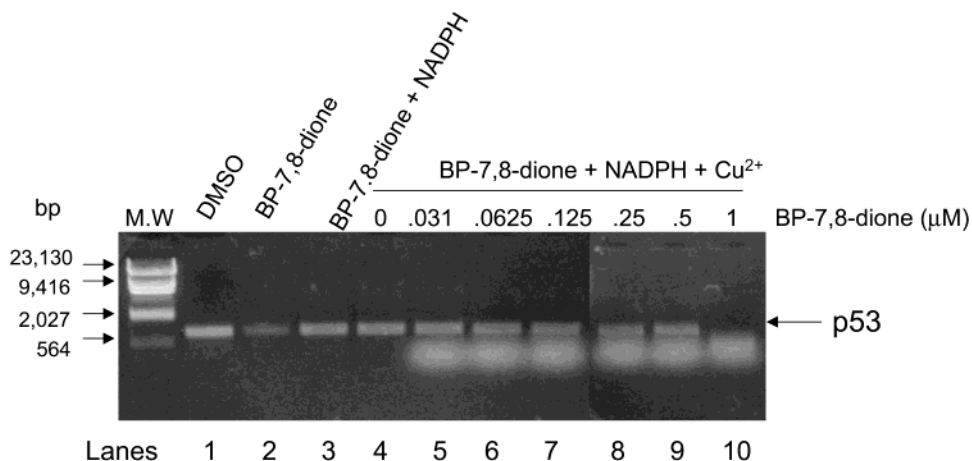


Figure 3. p53 cDNA Strand Scission by BP-7,8-dione. p53 fragment was incubated with vehicle (DMSO) alone, (lane 1); with 1 μ M BP-7,8-dione alone, (lane 2); 1 μ M BP-7,8-dione in the presence of 10 mM NADPH (lane 3), or 0, 0.031, 0.0625, 0.125, 0.25, 0.5, 1.0 μ M BP-7,8-dione in the presence of 1 mM NADPH plus 100 μ M CuCl_2 for 1 h at 37 $^\circ\text{C}$ (lanes 4–10). The treated p53 was analyzed by 0.7% agarose gel electrophoresis and the position of mol. wt. markers is shown, *Hind*III ladder of λ DNA.

Table 5. NP-1,2-Dione and BP-7,8-Dione Mutate p53 under Redox-Cycling Conditions^a

treatment colonies	NPQ			BPQ		
	total	red	%red	total	red	%red
quinone alone	922	11	1.2	3380	32	0.9
quinone + NADPH quinone (μ M) + NADPH + CuCl_2	896	13	1.5	3160	31	1.0
0	798	16	2.0	3290	34	1.0
0.031	812	31	3.8	3284	41	1.2
0.0625	1232	54	4.4	3080	51	1.7
0.125	756	46	6.1	2536	69	2.7
0.25	484	45	9.3	2200	113	5.1

^a p53 mutagenesis was determined in the presence of a 20 μ M PAH *o*-quinone (NP-1,2-dione or BP-7,8-dione) alone, 20 μ M quinone in the presence of 1 mM NADPH, or 0, 0.031, 0.0625, 0.125, 0.25 μ M quinone in the presence of 1 mM NADPH plus 100 μ M CuCl_2 as described in Table 1.

eight mutants sequenced it was found that they were all transversions and that one occurred in a known hot spot mutated in lung cancer. Attempts to obtain larger numbers of (\pm)-*anti*-BPDE mutants for sequencing were thwarted by the high incidence of false positives (self-ligated plasmid). Limitations of the in vitro yeast gap-repair assay are discussed later.

PAH *o*-Quinones Induce p53 Mutations Under Redox-Cycling Conditions. To examine the mutagenicity of PAH *o*-quinones on p53, representative compounds NP-1,2-dione and BP-7,8-dione were tested in the gap-repair system. We tested the quinones in the absence or presence of redox-cycling conditions using either NADPH or NADPH plus CuCl_2 (Table 5). No mutant colonies were seen with either the *o*-quinone alone or the *o*-quinone plus NADPH. In contrast, the number of red colonies increased in a concentration-dependent manner when sub-micromolar concentrations of *o*-quinones were incubated with p53 cDNA in the presence of NADPH and CuCl_2 , indicating that NADPH and CuCl_2 were essential for the mutagenicity of the *o*-quinone. At 0.25 μ M NP-1,2-dione and BP-7,8-dione the number of red colonies were 9.3 and 5.1%, of the total, respectively.

We previously reported that PAH *o*-quinones cause strand scission of Φ X174RF DNA in the presence of NADPH and CuCl_2 (30). To determine whether this was an issue in the current assay, p53 cDNA was incubated

Table 6. Effects of ROS Scavenging Agents on BP-7,8-dione-Induced p53 Mutation^a

treatment	% red colonies	% inhibition
CS [BP-7,8-dione]	3.2	
CS + SOD (210 μ M)	1.0	69
CS + catalase (880 μ M)	0.9	72
CS + SOD + catalase	0.2	94
CS + tiron (10 mM)	0.4	88
CS + mannitol (5 mM)	1.9	41
CS + Na-benzoate (5 mM)	1.5	53

^a p53 mutagenesis was determined in the presence of 0.25 μ M BP-7,8-dione plus 1 mM NADPH and 100 μ M CuCl_2 (CS = complete system) or CS supplemented with various ROS scavengers indicated as described in Table 1. The background red colony number (1%) was subtracted from the % red colonies. The effects of the ROS scavengers were evaluated as % inhibition of the red colony number.

with BP-7,8-dione alone, or in the presence of either NADPH, or NADPH and CuCl_2 and analyzed using agarose gel electrophoresis. As shown in Figure 3, the p53 cDNA was completely degraded at 1 μ M BP-7,8-dione while fragmentation was negligible at 0.25 μ M BP-7,8-dione (the maximum concentration used in the mutagenicity assay) in the presence of NADPH and CuCl_2 . This suggested that the red colonies detected in the assay were due to mutation of p53 and not DNA fragmentation.

Superoxide Anion Radical and Hydroxyl Radical Are Responsible for the p53 Mutagenesis by PAH *o*-Quinones. We have reported that PAH *o*-quinones can generate reactive oxygen species (ROS) in the presence of NADPH and CuCl_2 (29). To determine whether ROS are involved in the PAH *o*-quinone-induced p53 mutagenesis, various free-radical scavengers were incubated with p53 cDNA and BP-7,8-dione in the presence of NADPH and CuCl_2 . SOD alone or catalase alone reduced the mutagenicity of the *o*-quinones whereas their combination almost completely abrogated the mutagenicity indicating that the ROS responsible for the mutations are both $\text{O}_2^{\cdot-}$ and hydroxyl radical (Table 6). These findings were further supported by the ability of Tiron (a chemical scavenger for $\text{O}_2^{\cdot-}$), mannitol and sodium-benzoate (hydroxyl radical scavengers) to attenuate mutagenesis.

p53 Mutations Induced by BP-7,8-dione Under Redox-Cycling Conditions (NADPH and CuCl_2). To identify the mutations observed with PAH *o*-quinones

Table 7. DNA Sequencing of BPQ-Mutated p53

yeast	codon no.	p53 mutation	AA change
A22	132	AAG → AGG	K→R
A4	135	TGC → CGC	C→R
A57	135	TGC → TGA	C→Stop
B90	135	TGC → TTC	C→F
	330	CTT → ATT	L→I
I7	138	GCC → GAC	A→D
A10	139	AAG → GAG	K→E
BQ27	149	TCC → TAC	S→Y
	198	GAA → TAA	E→Stop
	281	GAC → TAC	D→Y
I20	151	CCC → TCC	P→S
BQ10	152	CCG → CTG	P→L
A36	154 ^a	GGC → GTC	G→V
BQ11	154 ^a	GGC → TGC	G→C
	146	TGG → TTG	W→L
BQ9	155	ACC → ATC	T→I
A11	157 ^a	GTC → ATC	V→I
	130	CTC → GTC	L→V
A40	163 ^a	TAC → CAC	Y→H
A5	164	AAG → TAG	K→Stop
A3	164	AAG → TAG	K→Stop
BPQ2	167	CAG → TAG	Q→Stop
	235	AAC → GAC	N→D
A17	170	ACG → CCG	T→P
BQ2	176 ^a	TGC → TGA	C→Stop
A48	178	CAC → AAC	H→N
A52	179 ^a	CAT → TAT	H→Y
BQ24	180	GAG → AAG	E→L
BQ25	183	TCA → TAA	S→Stop
A39	193 ^a	CAT → CCT	H→P
A32	196	CGA → GGA	R→G
A9	198	GAA → TAA	E→Stop
BQ6	198	GAA → TAA	E→Stop
BPQ5	199	GGA → TGA	G→Stop
A15	204	GAG → CAG	E→Q
	300	CCC → GCC	P→A
	306	CGA → TGA	R→Stop
B96	224	GAG → TAG	E→Stop
A37	236	TAC → TAA	Y→Stop
I22	241	TCC → TTC	S→F
A23	242 ^a	TGC → TTC	C→F
BQ14	243	ATG → ATC	M→I
I1	244 ^a	GGC → GAC	G→D
A6	244 ^a	GGC → GAC	G→D
A58	244 ^a	GGC → GAC	G→D
I27	245 ^a	GGC → TGC	G→C
I12	246	ATG → ATA	M→I
BQ8	249 ^a	AGG → ATG	R→M
BQ17	249 ^a	AGG → AGC	R→S
A34	259	GAC → TAC	D→Y
BQ23	262	GGT → GAT	G→D
A18	264	CTG → CGG	L→R
A45	267	CGG → CTG	R→L
A19	273 ^a	CGT → CTT	R→L
A43	278 ^a	CCT → CAT	P→H
	279	GGG → GAG	G→E
	162	ATC → ATA	I→I
B100	281	GAC → TAC	D→Y
A53	283	CGC → AGC	R→S
A44	306	CGA → TGA	R→Stop
A55	314	TCC → TTC	S→F
A12	317	CAG → TAG	Q→Stop
B89	339	GAG → TAG	E→Stop

^a Reported hot spots in human lung cancer.

under redox-cycling conditions, plasmids were rescued from the red colonies and sequenced. The presence of the *p53* gene in the plasmids was confirmed by restriction digestion (*SacI/KpnI*) (data not shown). The sequencing data obtained are summarized in Table 7.

Among the 53 colonies examined, seven had more than one point mutation. Of the total of 63 mutations sequenced, 29 were G:C to T:A transversions or 46%.

Statistical analysis showed that G:C to T:A transversions occurred with a $p < 0.0001$ over the other five mutations possible and with a $p < 0.0001$ over all the other mutations combined. The mutation pattern is shown in Figure 4 and is remarkably similar to that seen for lung cancer and dissimilar to that seen for all other cancers.

We next tested the hypothesis that the 63 mutations we identified occurred randomly across the region. In this test, we compared the observed proportion of mutations occurring in hot spots against a binomial probability of 10%, which is the relative proportion of hot spots within the region. We defined a hot spot as any of the 22 codons most commonly mutated in lung cancer which are found in this region. We found that 16 of the 63 mutations, or 25.4%, occurred in hot spots. This was statistically significantly different (exact p value = 0.0007) from the expected value of 10%.

Discussion

We have adapted a yeast reporter system to detect mutations in p53 induced by PAH-metabolites. In one step this system detects change-in-function mutations in p53 induced by these metabolites. In addition, DNA can be easily isolated and sequenced to determine the mutational pattern (base substitution or frame-shift) and mutational spectrum (incidence of a specific base substitution through the coding region). This assay is highly efficient with some mutagens in that more than 90% of the red colonies contain point mutations in the DNA binding domain of p53, often corresponding to hotspots observed in cancer. The use of microbial systems to study mutagenesis has been well established, but most systems rely on bacterial reporters. Since yeast are eukaryotes, they use many of the same repair systems found in mammals including OGG1, an AP glycosylase/lyase that excises 8-oxo-dGuo (31), and several bypass DNA polymerases capable of error-free and error prone translesional synthesis (32). Yeast systems such as this one provide functional assays for the inactivation of tumor suppressor genes and can select biologically relevant mutations. While we used yeast to test mutagenesis paradigms in vitro, the gap-repair system was originally designed to retrieve RT-PCR amplified p53 from tumor samples. For in vivo studies this amplification procedure could be used to isolate mutated p53 from cells and tissues treated with PAH-metabolites. Thus, the yeast-reporter system has several advantages over traditional microbial-based mutagenesis assays.

By adapting this yeast reporter system, we were able to detect change-in-function mutations in p53 caused by the alkylating agent MNNG, a representative *anti*-diol-epoxide, (\pm)-*anti*-BPDE, and representative reactive and redox-active PAH *o*-quinones (NP-1,2-dione and BP-7,8-dione). The inclusion of the latter allowed the assessment of the mutagenic properties of activated metabolites that arise from the AKR pathway of PAH-activation on the *p53* gene.

We found that the alkylating agent MNNG caused single base-pair changes in the p53 DNA binding domain with a dominant preference for the formation of G:C to A:T transitions. This result is consistent with the mutations observed with this agent in the Ames Test and other mutagenesis assays where over 82% of the mutations observed were G:C to A:T transitions (28, 33, 34). The

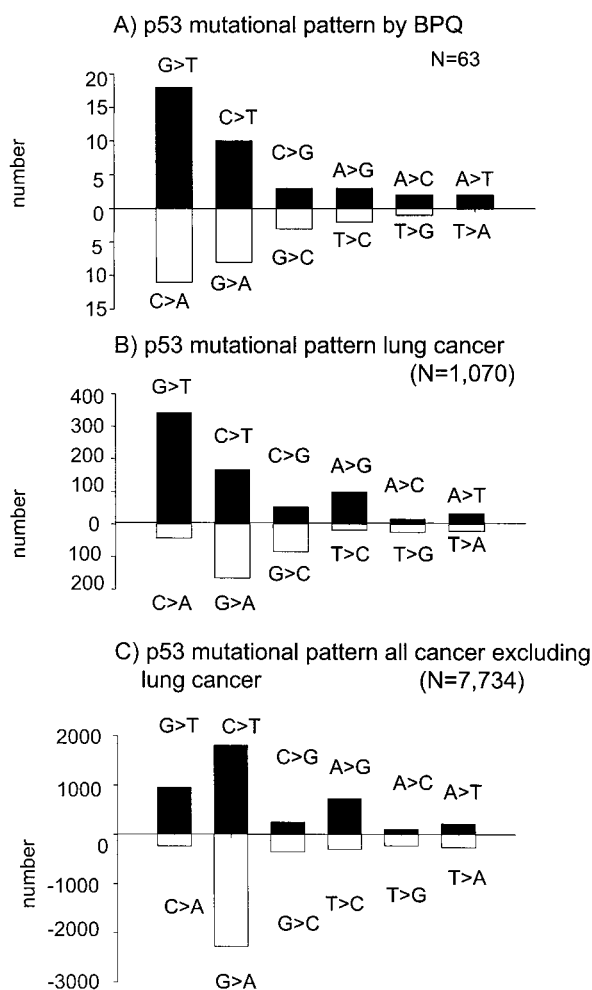
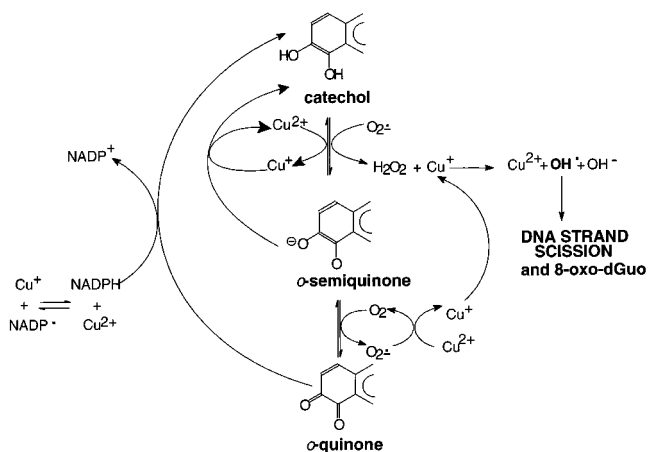


Figure 4. Comparison of p53 mutational pattern induced by the reactive and redox-active BP-7,8-dione with that seen in lung cancer. The mutational pattern observed in red colonies following treatment of wild-type p53 with BP-7,8-dione under redox-cycling conditions (a); the mutational pattern observed in patients with lung cancer (b); and (c) the mutational pattern observed in all cancers excluding lung cancer.

high incidence of these mutations can be explained by the preferential alkylation of guanine bases to yield *O*⁶-methylguanine. This result validates the assay for detecting the incidence of mutations observed with other test mutagens.

When (\pm)-*anti*-BPDE and PAH *o*-quinones were compared as direct acting mutagens, the former caused single base-pair mutations. Although 12-base substitutions are possible (four bases being mutated to one of the remaining three), our assay does not distinguish between whether the initial hit was on the coding or noncoding strand. Therefore, six possible mutations can arise, and of these, three were observed with (\pm)-*anti*-BPDE, in each instance transversions rather than transitions were observed. By comparison the PAH *o*-quinones, NP-1,2-dione, and BP-7,8-dione were nonmutagenic by themselves. Our experiments showed that it was difficult to obtain large numbers of (\pm)-*anti*-BPDE mutants for sequencing because of the high incidence of false-positives (self-ligated plasmid) and may reflect some limitations of the gap-repair assay. The higher frequency of the false positives could be explained if p53 treated with (\pm)-*anti*-BPDE is less prone to undergo homologous recombination into the gap-repair plasmid. This may be due to

Scheme 3. Role of Cu²⁺/Cu⁺ in the PAH *o*-Quinone Redox Cycling and ROS Generation



significant distortion in the p53 treated cDNA due to either bulky adduct formation or intercalation of the hydrocarbon leading to a bias against agents that cause bulky adducts. If this bias exists, this would also explain why BP-7,8-dione is not mutagenic by itself, assuming that stable adducts instead of depurinating adducts form. In vitro, BP-7,8-dione forms both stable and depurinating adducts (19, 20).

Sub-micromolar concentrations of NP-1,2-dione and BP-7,8-dione caused significant mutations when redox cycling was stimulated with NADPH and CuCl₂, re-emphasizing that only trace amounts of *o*-quinones need be formed to observe significant mutation. The concentrations of *o*-quinone that were mutagenic were insufficient to cause strand scission. At higher concentrations of *o*-quinone, complete DNA cleavage was not observed due to the absence of a larger smear on the gel. There are two explanations as to why complete scission was not observed. First, multiple double-strand breaks are required to cause complete destruction of the DNA. Double-strand breaks will only be produced when sufficient reactive oxygen species (ROS) are produced to yield single strand breaks that align. This event is infrequent and will occur only if sufficient ROS is produced by the *o*-quinone. Second, DNA-damage may not be random. BP-7,8-dione is a planar aromatic hydrocarbon and its intercalation into DNA may be governed by sequence context. In fact our data support site-specific mutation by BP-7,8-dione even under redox-cycling conditions.

BP-7,8-dione mutation of p53 was attenuated by the addition of SOD and catalase, and completely abolished with both ROS scavenging enzymes. These data strongly indicate that the mutagens responsible were superoxide anion and hydroxyl radical.

Mutations observed with PAH *o*-quinones required the presence of both NADPH and CuCl₂; the absence of the transition metal led to a complete attenuation of mutagenicity. We have previously documented the requirement for CuCl₂ to observe PAH *o*-quinone mediated DNA strand scission (30) (also see Scheme 3). Although, this transition metal is not present at high "free" concentrations in the cell it is present at high amounts on nucleoprotein complexes (35) and is closely associated with DNA bases (36), particularly guanine. Further, we have shown that exposure of rat hepatocytes to BP-7,8-dione causes significant strand scission of DNA, suggesting that the metal-ions and cofactors necessary for

o-quinone-mediated DNA damage do exist in sufficient quantities in whole cells to cause DNA strand scission (37). Therefore, the inclusion of Cu²⁺ is considered physiologic in these studies.

There are several roles for CuCl₂ in the sequence that takes place. First, Cu²⁺ catalyzes the futile redox-cycle between the catechol and *o*-semiquinone radical, Scheme 3. Second, the production of Cu⁺ in this futile cycle can initiate Cu⁺-mediated Fenton chemistry to produce OH[•] radical. Third, when *o*-semiquinone radical subsequently autoxidizes to the *o*-quinone, Cu²⁺ can oxidize the superoxide that is produced to increase the formation of Cu⁺. Fourth, it is unclear whether it is Cu⁺ mediated production of OH[•] radical or a Cu(I)(OOH[•]) species that ultimately causes the oxidative damage of DNA (38–40).

PAH *o*-quinones were previously examined as direct-acting mutagens in *Salmonella typhimurium* tester strains (41). They were found to have a greater mutagenic efficiency than the test mutagens for each strain and were found to be predominantly frame-shift mutagens. However, BP-7,8-dione was 10–5500 times less mutagenic than (±)-*anti*-BPDE depending on the *Salmonella* strain used. The presence of an activating system (Aroclor-induced rat liver S9 with a NADPH-generating system) failed to increase the mutagenicity of *o*-quinones even though this would promote redox-cycling. In these earlier assays, the ROS were produced extracellularly and were unlikely to reach the *his* operon to cause mutations. The present assay circumvents this problem since the target sequence is incubated directly with the mutagen in vitro.

The mutations caused by PAH *o*-quinones in the p53 gene were all point mutations. Of the six possible base substitutions that can be detected in the assay over 46% were G to T transversions. This mutational pattern is similar to that seen in patients with lung cancer and dissimilar to that seen in other human cancers. The high preponderance of these mutations may be explained if the primary DNA-lesion is the formation of 8-oxo-dGuo, which could occur either by attack of a OH[•] radical or by attack of a Cu(I)(OOH[•]) species (38–40). Once 8-oxo-dGuo is formed there are several routes to G to T transversions. First, if 8-oxo-dGuo is repaired in yeast by a monofunctional glycosylase (Mut Y homolog) an apurinic site will form (42). If an apurinic site is left unrepaired upon replication an A will be introduced opposite that site, leading to a G to T transversion (43). Second, if 8-oxo-dGuo is left unrepaired, the presence of the additional C8-keto group will ensure base mis-pairing with A (44). Again, upon replication of the daughter strand a G to T transversion will occur. Third, 8-oxo-dGuo can undergo secondary oxidation to produce oxaluric acid, oxazalone or cyanuric acid, and when these lesions are by-passed by DNA polymerase they also yield G to T transversions (45). In related work, we have demonstrated that incubation of salmon testis DNA in vitro with PAH *o*-quinones under redox-cycling conditions does increase the formation of 8-oxo-dGuo using EC-HPLC detection methods (46). 8-Oxo-dGuo and its secondary oxidation products are not the only deoxyguanosine lesions that arise from ROS attack. Evidence also exists for the formation of 5′8-purine deoxyribonucleosides which are removed by the nucleotide-excision repair pathway (47). However, their mutagenic potential is unknown. In summary, the formation of 8-oxo-dGuo and its secondary oxidation products provides the most

straightforward routes to the G to T transversions observed.

If PAH *o*-quinones produced by AKRs cause DNA lesions via ROS these lesions should be observed in vivo. We have previously shown that when isolated rat hepatocytes were treated with BP-7,8-diol there was significant strand scission which was attenuated with AKR inhibitors demonstrating that the ROS lesions are AKR dependent (37).

The occurrence of single-point mutations in the p53 gene by ROS generated by the redox-cycling of PAH *o*-quinones was unexpected. A priori, we anticipated that ROS lesions would be random throughout the p53 gene and that multiple mutations would be seen upon sequencing DNA from a single mutant yeast colony. Although higher concentrations of *o*-quinone caused extensive strand scission and substantially lowered the efficiency of yeast transformation, multiple mutations in p53 were relatively rare (7/53 = 13% of plasmids). There are several possible explanations for this observation that warrant further study. First, the formation of discrete point mutations may result if the effects of ROS are directed by the intercalation of the PAH *o*-quinone with the DNA. Second, multiple mutations that might arise due to ROS amplification may also overwhelm the gap-reporter system so that only colonies containing single hits are viable. We favor the latter explanation as evident by the reduced transformation efficiency observed at higher *o*-quinone concentrations that correlated with increases in DNA fragmentation.

Mutations in approximately 22 codons of p53 account for almost 50% of the mutations observed in human lung cancer. Of these codons 273, 249, and 245 are among the three most frequently mutated in lung cancer (3). In our study, 16/63 (25.4%) of the mutations were in the 22 “hot spots” and (7/16) were G to T transversions as well. In lung cancer, the frequency of G to T transversions in hot spots may reflect both the incidence of mutation at that position and biological selection for cells in which mutated p53 provides a growth advantage. In both instances the G to T transversions must originate in these positions by exposure to the responsible mutagen. In the case of (+)-*anti*-BPDE there is a high correlation between sites adducted and sites mutated suggesting that with this ultimate carcinogen the sequence context of adduct formation may be more important than subsequent biological selection. However, (+)-*anti*-BPDE is not the only ultimate carcinogenic species that can be derived from tobacco smoke. Tobacco smoke is a mixture of PAH, nicotine, and free radicals (*o*-semiquinones) (48, 16). Mixtures of PAH may give rise to different proportions of activated metabolites (diol-epoxides, *o*-quinones, radical cations), while nicotine will form nicotine-derived nitrosamines (49), and *o*-semiquinones will amplify ROS production (16). It is important to recognize that many of the same codons in p53 are mutated in smoke inaccessible tissues the difference being that G to T transversions are found with lower incidence. Such findings suggest that these mutations may be selected based on their growth advantage to the cell. It is also unlikely that ROS and ROS derived from PAH *o*-quinones will yield the same mutational spectrum as *anti*-BPDE, because the lesions are fundamentally different and will be repaired and selected differently.

The importance of this study is that we show that *o*-quinones derived from PAH give rise to ROS that lead

to a predominant pattern of G to T transversions in p53. Many but not all are located on codons designated as "hot spots" associated with lung cancer. The frequency of all G to T transversions at these hot spots is not identical to the mutational spectra observed in smokers with lung cancer and likely reflects differences in biological selection between yeast and tumor cells. We did observe G to T transversions in codons 245, 249, and 273 and by providing a growth advantage to mammalian cells these could contribute to the dominant mutations detected in lung cancer. Our observations become more powerful when they are coupled with the finding that AKR1C isoforms are highly overexpressed in nonsmall cell lung carcinoma (23). This suggests that PAH o-quinones and the ROS they generate are produced in targets of PAH carcinogenesis. This has been validated recently in this laboratory where we have shown that AKR1C isoforms are highly expressed in A549 human lung carcinoma cells and that these cells can convert DMBA-3,4-diol to one of the most highly reactive and redox active o-quinones, DMBA-3,4-dione (50).

In conclusion, we speculate that PAH o-quinones are formed in human lung by the inducible AKR1C isoforms following exposure to the parent hydrocarbon. This results in ROS formation which in turn causes G to T transversions in p53. While there is not an exact congruence between G to T transversions seen in our *in vitro* system with that observed in p53 in lung cancer, the difference could be explained by the use of cDNA as opposed to chromatin and/or biological selection. The next step will be to use this assay to determine the mutagenic potential of PAH o-quinones on p53 in mammalian cells.

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Footnote Added in Revision. After this paper was reviewed, Ohnishi and Kawanishi (*Biochem. Biophys. Res. Commun.* (2002) 290, 778–782) showed that BP-7,8-dione induces double-base lesions at 5'-TG-3' sites, CGC, and poly(C) sequences in the p53 tumor suppressor gene. The sequence complementary to codon 273, ACG was preferentially targeted. These lesions had a strict requirement for CuCl₂ and NADPH indicating that redox-cycling and the production of ROS was essential. These *in vitro* fragmentation experiments support mutation of p53 by PAH o-quinone derived ROS reported in this paper.

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