BIOMARKERS, 2001, VOL. 6, NO. 4, 262–273

Mutational spectrum of *N*-hydroxy-*N*-acetyl-4-aminobiphenyl at exon 3 of the *HPRT* gene

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Received 14 August 2000, revised form accepted 26 September 2000

4-Aminobiphenyl is a human bladder carcinogen present in many environmental sources, including cigarette smoke. It can be metabolized in two steps to the mutagen *N*-hydroxy-*N*-acetyl-4-aminobiphenyl (*N*-OH-AABP). In this study the mutational spectrum of *N*-OH-AABP-exposed human lymphoblastoid cells (TK6) was determined using *HPRT* as the target gene. Three large, HAT (hypoxanthine–aminopterin–thymidine)-cleaned TK6 cultures were independently treated with 20 μM *N*-OH-AABP for 24 h, allowed to recover for 4 days, then continuously exposed to 40 μM 6-thioguanine to select for induced mutants. Contemporary control cultures received vehicle in place of *N*-OH-AABP. *N*-OH-AABP treatment gave an 11-fold increase in mutation frequency. Mutations were delineated in exon 3 of the *HPRT* gene directly from genomic DNA extracted from both treated and untreated cells using the polymerase chain reaction, denaturing gradient gel electrophoresis (DGGE) and dideoxy sequencing. DGGE analysis showed *N*-OH-AABP increased both the number and type of mutations as compared with controls. The major background mutation was a G(197) → A transition. The major *N*-OH-AABP-induced changes were G(209-211) → T transversions (30%), a seven-base repeat at position 185 (17%) and an A(215) → T transversion (2%). The shift in the control spectrum of a transition to that of transversions and insertions suggest that the electrophile *N*-OH-AABP forms bulky adducts at the same sites on exon 3 of *HPRT* as do many other bulky electrophiles, causes replication errors by similar mechanisms, but induces at least one potentially signature mutation.

Keywords: HPRT, aminobiphenyl, denaturant gradient gel electrophoresis, TK6, mutational spectrum.

Abbreviations: ABP, 4-aminobiphenyl; bp, base-pair; DGGE, denaturing gradient gel electrophoresis; DMSO, dimethylsulphoxide; HAT, hypoxanthine–aminopterin–thymidine; HPRT, hypoxanthine–guanine phosphoribosyl transferase: *HPRT*, gene encoding HPRT; MF, mutation frequency; *N*-OH-AABP, *N*-hydroxy-*N*-acetyl-4-aminobiphenyl; PCR, polymerase chain reactions; Pfu, *Pyrococcus furiosus* DNA polymerase; TE buffer, 10 mM Tris–HCl, pH 7.4, containing 2 mM EDTA; 6-TG, 6-thioguanine; wt, wild type.

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Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online © 2001 Taylor & Francis Ltd
http://www.tandf.co.uk/journals
DOI: 10.1080/13547500010017367

Introduction

Interaction of environmental chemicals and their metabolites with biological macromolecules can result in cytotoxic and genotoxic effects. Exposure to chemicals such as 4-aminobiphenyl (ABP) can result from many types of occupational and/or environmental sources. The most widespread and significant source of exposure to ABP is, however, tobacco smoke (Patrianakos and Hofmann 1979, Vineis 1994). ABP is a known pro-mutagen and bladder pro-carcinogen (Vineis 1994). Other potential target organs of ABP include the mammary gland and colon (El-Bayoumy 1992). Humans can metabolize ABP in two steps to the mutagen *N*-hydroxy-*N*-acetyl-4-aminobiphenyl (*N*-OH-AABP). Interaction of *N*-OH-AABP with DNA can occur, inducing both mutagenicity and cytotoxicity (Vineis 1994).

When analysing genotoxic effects from electrophilic metabolites of environmental xenobiotics, such as aromatic amines (e.g. ABP) and polycyclic aromatic hydrocarbons (e.g. benzo[*a*]pyrene), similarities in the preferred nucleophilic base are often observed. The offending mutagen can sometimes be distinguished by analysing both the mutations it induces and the DNA sequence context in which it induces these alterations. Elucidation of these base/sequence context alterations induced by physical effects or xenobiotics has been termed 'mutational spectrometry' (derived from Benzer and Freese 1958).

In this study, we elucidated the mutational spectrum caused by *N*-OH-AABP at exon 3 of the *HPRT* gene in human lymphoblastoid TK6 cells. Hypoxanthine–guanine phosphoribosyl transferase (HPRT) is a soluble cytoplasmic protein that can be detected in all human tissues at low levels (Stout and Caskey 1988, Melton *et al*. 1981) and HPRT mutants can be selected in cell culture with a guanine analogue, 6-thioguanine (6-TG), that is toxic to cells containing functioning HPRT. Furthermore, the TK6 cell line provides an important link between *in vitro* studies and the most easily obtained and culturable human cells, lymphocytes. The denaturing gradient gel electrophoresis (DGGE) technique has been used to analyse mutational spectra in 6-TG-resistant mixed mutant cells generated *in vitro* by exposure to numerous agents such as ultraviolet light and benzo[*a*]pyrene diol epoxide (Keohavong *et al*. 1991, Keohavong and Thilly 1992a). In this study, DGGE was used to evaluate the spectrum of mutations induced by *N*-OH-AABP. Clear differences between control and *N*-OH-AABP-induced mutants in migration patterns during DGGE and in sequencing analyses were observed. Specificity for a 'hot spot' region in exon 3 of *HPRT* in the *N*-OH-AABP-exposed cultures was identified, as was a unique site of mutation outside of the hot spot that led to a novel repeat. The results indicate the high mutational specificity of this ABP metabolite and add to the database used in elucidating mechanistically similar mutagens in molecular epidemiology studies.

Methods

Cell culture

TK6 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 (Gibco-BRL Life Technologies, Inc., Gaithersburg, MD) medium containing 10% foetal bovine serum (Hyclone Laboratories, Inc., Logan UT) and 1% penicillin–streptomycin (Gibco-BRL) at 37 ¡C in a 5% CO_2, humidified atmosphere. Cells were maintained at a density of $2–5 \times 10^5$ cells ml^{-1}. Cultures were split twice per week. Frozen cell aliquots were thawed and kept in culture for at least 2 weeks before treatment with test chemicals. Cell viability and numbers were routinely determined microscopically on a haemacytometer using Trypan blue stain.

Chemicals

N-OH-AABP (generously provided in > 99% purity by Dr Fred Kadlubar of the National Center for Technological Research, Jefferson, AR), was stored neat at –80°C. Stock solutions (200×) were prepared fresh on treatment days by dissolving in analytical grade dimethylsulphoxide (DMSO; Sigma Chemical Co., St Louis, MO) and kept on ice until cell exposure. 6-TG (Sigma) was prepared for use as a 20 mM stock solution by dissolving in 1 M NaOH then diluted into aliquots and frozen at –80°C until use.

Chemical treatment and generation of mixed mutant cultures

Prior to treatment with test chemicals, TK6 cells were treated with 1% HAT (hypoxanthine–aminopterin–thymidine) selective medium (Gibco-BRL) in order to reduce the *HPRT* mutant background level to a minimum. Surviving cells were considered pheno- and genotypically normal and were used for bulk generation of mixed mutants.

Cultures of $1–3\times10^8$ TK6 cells were treated with 20 μM *N*-OH-AABP for 24 h and washed with medium plus additives. Surviving cells were then incubated in fresh medium for 3–4 days in order to allow for recovery and expression of the mutant phenotype. The selection of *HPRT* mutant cells *en masse* was then achieved by exposure to medium containing 40 μM 6-TG. Cultures were incubated for 2–3 weeks and passaged in selective medium during that period as necessary. Contemporary control cultures of $1–3\times10^8$ TK6 cells treated with vehicle (DMSO) only were also incubated with 40 μM 6-TG. The resulting cultures of 6-TG-resistant mixed mutants (both treated and control) were split into aliquots and frozen at –80°C in complete medium containing 10% DMSO for subsequent molecular analyses. Three replicates of experiments, each with one control culture and one *N*-OH-AABP-exposed culture, were performed.

Extraction of DNA

Frozen aliquots of mixed mutants generated in bulk were thawed, rinsed, lysed, and cleared of RNA and protein with RNase A and proteinase K (Gibco-BRL). DNA was extracted by the phenol/chloroform/iso-amyl alcohol method and precipitated with ethanol. The total isolated DNA was then dissolved in 1 ml of TE buffer (10 mM Tris–HCl, pH 7.4, 2 mM EDTA) and stored at 4°C until used.

Polymerase chain reaction (PCR) amplification of HPRT exon 3

Amplification of exon 3 was performed directly from extracted genomic DNA of control and exposed cultures. The oligonucleotides used as primers were: P1, 5′-CAT ATA TTA AAT ATA CTC AC-3′; P2, 5′-TCC TGA TTT TAT TTC TGT AG-3′; P3, 5′-GAC TGA ACG TCT TGC TCG AG-3′; P4 5′-TCC AGC AGG TCA GCA AA-3E and P2-GC clamp 5′-GCC GCC TGC AGC CCG CGC CCC CCG TGC CCC CGC CCC GCC GCC GGC CCG GGC GCC TCC TGA TTT TAT TTC TGT AG-3′ (Midland Certified Reagent Co., Midland, TX). From genomic DNA, a 224 base-pair (bp) fragment was generated and amplified using P1 and P2, which are complementary to the flanking regions of the exon 3 coding region. The reaction mixture consisted of DNA polymerase buffer [100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris–HCl (pH 8.8), 20 mM $MgSO_4$, 1% Triton X-100 and 1 mg ml^{-1} bovine serum albumin], 300 μM of each dNTP, 1 μm each of P1 and P2, 100–250 ng of DNA, and 2 units of *Pyrococcus furiosus* (Pfu) DNA polymerase (Stratagene, La Jolla, CA) in a total volume of 25 μL.

The conditions for PCR amplification were a hot start at 95°C for 3 min followed by Pfu addition and 36 cycles of 94°C for 1 min, 45°C for 1 min and 72°C for 2 min. Bis-acrylamide electrophoresis (8% gel) and visualization by ethidium bromide staining with UV transillumination confirmed the amplification of the 224 bp fragment. The remainder of the amplified product was then purified by electrophoresis on an 8% bis-acrylamide gel, the bands excised and the product eluted into 20 μl of TE buffer for the following nested PCR.

Two nested PCR reactions using the 224 bp fragment as template were required in order to generate a fragment of 204 bp containing a low melting temperature domain of 104 bp and a fragment of 180 bp containing an 80 bp high melting temperature domain.

The radiolabelled reaction mixture for the 204 bp fragment amplification contained the same reactants as the 224 bp fragment amplification with the following changes: primer P3 was used instead of P2 and the concentration of unlabelled dATP was decreased to 100 μM dATP. The conditions for PCR amplification included a hot start at 95°C for 3 min followed by addition of Pfu and [α-^{32}P]ATP (DuPont, Boston, MA) and 36 cycles at 94°C for 1 min, 45°C for 1 min, 53°C for 45 s and 72°C for 2 min. The amplified 204 bp product was then gel purified by electrophoresis on an 8% bis-acrylamide gel. The bands were excised and the product eluted into 20 μl of TE buffer.

Primers used to generate the 180 bp fragment were P4 and P2-GC clamp. The radiolabelled reaction mixture contained the same reactants and concentrations as mentioned above. The PCR amplification included a hot start at 95°C for 3 min followed by addition of Pfu and [α-^{32}P]ATP and 36 cycles at 94°C

for 1 min, 53 °C for 45 s, and 72 °C for 2 min. The amplified 180 bp PCR product was confirmed and purified by gel electrophoresis.

DGGE analysis

HPRT exon 3 mutant and wild type (wt) sequences were separated by DGGE taking advantage of the GC-rich regions and high and low melting domains (Stout and Caskey 1988, Keohavong and Thilly 1989, 1992a). A denaturing gradient of 15–30 % urea/formamide was used for DGGE analysis of the 204 bp fragments containing a low melting domain (104 bp). The 180 bp fragment containing the high melting domain (higher GC content) required a 36–45 % denaturing gradient. Prior to loading of the gel-purified 180 bp and 204 bp ^{32}P-labelled PCR products, samples were boiled for 5 min and left at 65 °C for 1–2 h for duplex reannealment in Tris–HCl (10 mM), pH 7.5, containing 2 mM EDTA and 250 mM NaCl. Samples from control and *N*-OH-AABP-exposed cultures from the three independent experiments were loaded on a 12.5 % polyacrylamide gel (bis-acryl 1:37.5) containing 15–30 % (for the 204 bp fragment) and 36–45 % (for the 180 bp fragment) denaturants. The gels were electrophoresed for 12 h at 150 V submerged in a 60 °C Tris–acetate (40 mM) buffer containing 1 mM EDTA. The gels were dried (Hoefer Scientific Instruments model SE 1160 Slab Gel Dryer) and radioautographs were generated.

Visible and distinguishable bands from exposed and control samples were isolated by excision from the gel and elution into TE buffer. They were further amplified using the PCR conditions described above and sequenced as double stranded DNA (Keohavong and Thilly 1989).

Results

In a set of preliminary clonogenicity experiments with TK6 cells, the average *HPRT* mutation frequency (MF) observed in cultures of about 4×10^6 cells exposed to 20 μM *N*-OH-AABP for 24 h was 17×10^6 versus an MF of 1.5×10^{-6} in controls (data not shown). Thus, *N*-OH-AABP gave a relative mutation induction of 11-fold and an absolute mutation induction of 15.5×10^{-6}. Extrapolating the same rate of mutation induction in the bulk cultures with $\sim 3 \times 10^8$ cells suggests 5100 independent mutants formed in each *N*-OH-AABP-treated culture, 450 (9 %) of which were 'background' (i.e. the number formed in control cultures) and 4650 (91 %) of which were mutagen-induced. Mutants were selected from bulk cultures by continuous growth in 6-TG for 2–3 weeks, then surviving cells were seeded into fresh flasks and allowed to expand for 2–3 weeks. Frozen aliquots of mixed mutants generated in bulk from exposure to *N*-OH-AABP and contemporary controls were thawed and cultured in complete medium 2 weeks prior to analysis in order to assure viability of the mutants and to expand the mutant clones. DNA was then extracted from 1×10^8 cells per culture.

The *HPRT* gene consists of nine exons containing 657 bp of coding region. Exon 3 (184 bp) represents 28 % of the entire coding region. Wild type exon 3 is composed of an 80 bp high melting domain (positions 220–299) and a low melting domain containing 104 bp (positions 300–403) (Keohavong *et al.* 1991). DGGE is a method that allows separation of homo- and heteroduplexes of DNA based on the relative strengths of their double strand complementarity. Point mutation occurring within the 104 bp region can be detected by DGGE, as the mixed mutant and wt duplexes focus at different relative migratory positions (determined by urea/formamide concentration) of the denaturing gel, with the high melting domain serving as a tightly hydrogen-bonding 'clamp', keeping the two strands together (Fisher and Lerman 1983, Keohavong and Thilly 1992a). To detect mutations occurring in the remaining 80 bp high melting domain, an artificial, higher temperature-melting domain rich in GC content was included during the 180 bp fragment PCR amplification and used as a clamp (Keohavong and Thilly 1992b). After extraction of DNA, amplification of *HPRT* exon 3 using primers P1

and P2 resulted in a PCR product of 224 bp. The excised and gel-purified DNA fragment was then used as a template for PCR amplification of 180 bp high melting and 204 bp low melting fragments.

DGGE analysis of the 204 bp fragments containing the low melting domain showed little contrast between controls and exposed cultures from the three experiments (data not shown). This indicated that the type(s) of mutations induced at that region of exon 3 of the *HPRT* were primarily background mutations and not due to exposure to *N*-OH-AABP. Since little contrast was observed between control and exposed cultures, no further analysis was performed on this fragment.

DGGE analysis of the 180 bp fragment containing the high melting point domain, however, showed stark differences between control and *N*-OH-AABP-exposed cultures from the three independent experiments (figure 1). Each pair of lanes indicates the matched set of control and exposed cultures from each experiment. The band at the bottom of each lane is the wt/wt homoduplex. Bands shown 'melting' at lower denaturant concentrations represent mutant/wt heteroduplexes, mutant/mutant homoduplexes, or mutant/mutant heteroduplexes.

There were similarities in the bands observed from the three control cultures (Expts 1–3 '**C**' in figure 1). The presence of fewer bands in the control lanes compared with those of *N*-OH-AABP exposed cultures indicated the lower frequency and types of mutations in the control populations. These mutations

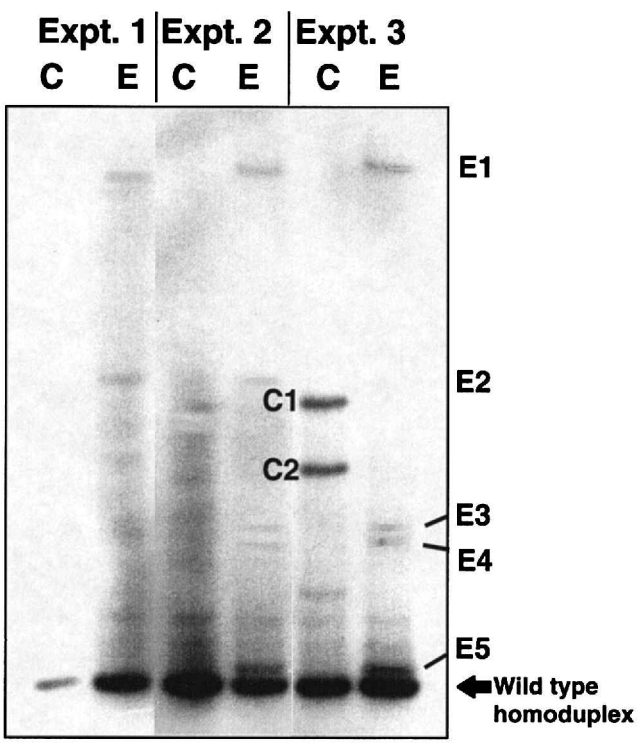

Figure 1. First DGGE analysis of the high-melting domain. Lanes from each of the three independent bulk exposure experiments are shown [**C**, control (vehicle-exposed) cultures; **E**, *N*-OH-AABP-exposed cultures]. Bands **C1–C2** and **E1–E5** were excised for further analysis.

could have occurred spontaneously or be due to the vehicle used in control cultures (DMSO). Some bands were not present in all three of the controls, suggesting variability in the level and type of background mutations arising in the untreated populations, or in survival factors affecting the expression of those mutants.

Analysis of the 180 bp fragments containing the high melting point domain from the *N*-OH-AABP-exposed cultures showed similarities within replicates (Expts 1–3 '**E**' in figure 1). The intensities of these bands, however differed slightly. A few mutant bands were observed in lanes containing fragments from *N*-OH-AABP-exposed cultures that were also present in one or two of the control lanes, indicating the possibility that they represented background or spontaneous mutations and were not a result of *N*-OH-AABP treatment or that *N*-OH-AABP could induce mutations indistinguishable from background.

Several distinctive and reproduced bands in the replicates of controls and exposed were excised from the gel, eluted into buffer and further amplified using the same conditions described above. Two of the equally abundant bands in the control lanes, (**C1** and **C2** in figure 1) and five of the equally abundant bands in the exposed lanes (**E1–E5** in figure 1) were selected for further analysis. Each amplified product was then gel-purified and analysed by a second DGGE.

The second DGGE analysis of the seven excised bands is shown in figure 2. Each lane represents one original band from the first DGGE. Lanes 1 and 2 in figure 2 correspond to the two excised bands from the control lanes. Lanes 3 to 7 show the five excised bands from the *N*-OH-AABP-exposed cultures. The migration of samples in lanes 1 and 2 (**C1** and **C2**) resulted in similar mutant bands. From the control lanes loaded with amplified material of control cultures, the four bands were excised for further sequence analysis, as were the most prominent and distinctive bands from the lanes containing material from *N*-OH-AABP-exposed cultures.

Fourteen 180 bp fragments were selected from the second DGGE experiment for sequencing analysis. Results are shown in table 1. Four bands corresponded to background mutations and were present in all control groups. Sequencing showed all background mutations occurred at position 197, resulting in a G to A transition. Band **a** was a wt/wt homoduplex with no mutation detected. Bands **b** and **c** were wt/mutant heteroduplexes with one strand bearing wt 197G and the other bearing mutant 197A. Band **d** was a mutant/mutant homoduplex with transition of 197G to A.

The remaining ten fragments sequenced came from the *N*-OH-AABP-exposed cultures. The ten bands sequenced from the exposed groups were not detected in the control sequences, indicating that these were indeed *N*-OH-AABP-induced mutations. Band **e** (figure 2) came from band **E1** (figure 1), the latter of which was clearly replicated in the three independent experiments. Sequence analysis (figure 3) showed band **e** was a mutant/mutant homoduplex containing an insertion of a repeated, seven-base sequence (CATCACA) at position 185. A potential explanation for this insertion relies on the slippage-misalignment-model (Hiroshi *et al.* 1994), which suggests that even a one-base repeat or insertion can induce misalignment, followed by deletion of a loop-out intermediate. These data suggest that the formed loop was read and transcribed, after which the strand straightened and was read again, resulting in the repeat of seven bases.

Three of the 14 sequenced bands, **f**, **k**, and **m**, were wt/wt homoduplexes. These data indicate that bands **E2–E5** in figure 1 were wt/mutant heteroduplexes.

Table 1. Summary of denaturant gradient gel electrophoretic and dideoxy sequencing analysis for background and *N*-OH-AABP-induced mutants.

DGGE 1	% of detected bands	DGGE 2		Type of mutation	Coding change
Control					
C1	64.7	**a–d**	**a**	wt/wt homoduplex	None
			b	197 G > A transition, mutant homoduplex	Cys to Tyr
			c	197 G > A transition, wt/mutant heteroduplex	Cys to Tyr
			d	197 G > A transition, wt/mutant heteroduplex	Cys to Tyr
C2	13.7			Same as **C1, a–d**	
Exposed					
E1	16.7	**e**	**e**	Insertion of 7-base repeat (ATCACAC) at 185, mutant homoduplex	Insertion of two His followed by one frameshift
E2	29.4	**f, g, h, i, j**	**f**	wt/wt homoduplex	None
			g	mutant/mutant heteroduplex	
				209 G > T transversion	Gly to Val,
				211 G > T transversion	Gly to Cys
			h	211 G > T transversion, mutant/mutant homoduplex	Gly to Cys
			i	mutant/mutant heteroduplex	
				209 G > T transversion	Gly to Val,
				211 G > T transversion	Gly to Cys
E3	1.3	**k, l**	**k**	wt/wt homoduplex	None
			l	wt/mutant heteroduplexes	
				207 G > C transversion	Lys to Arg
				208 G > C transversion	Gly to Val
				209 G > C transversion	Gly to Ala
				210 G > C transversion	Gly to Gly (silent)
				211 G > C transversion	Gly to Arg
				212 G > C transversion	Gly to Ala
E4	3.5	**m, n**	**m**	wt/wt homoduplex	None
			n	215 A > T transition, wt/mutant heteroduplex	Tyr to Phe
E5	1.2			Same as **E4, m/n**	

Band **g** in figure 2 was sequenced (figure 4) to reveal that it was a mutant heteroduplex with each strand having a G to T transversion; these occurred at positions 209 and 211. Band **h** was a mutant homoduplex with a 211G to T transversion. Sequencing of band **i** and **j** showed them to be mutant/wt heteroduplexes, each with G to T transversions at the same hot spots within exon 3, positions 209 and 211, respectively.

Sequence analysis of band **l** showed it to be a collection of a mixture of wt/mutant heteroduplexes bearing mutations in positions 207 to 212. This region is composed of six consecutive guanines. In each case transition from G to A was observed. The last analysed heteroduplex sequence (band **n**), exhibited only one mutation, a 215A to T transversion (data not shown).

Potential changes in the amino acid sequence as result of the induced mutations are also shown in table 1. In the DGGE analysis of the four control fragments, the

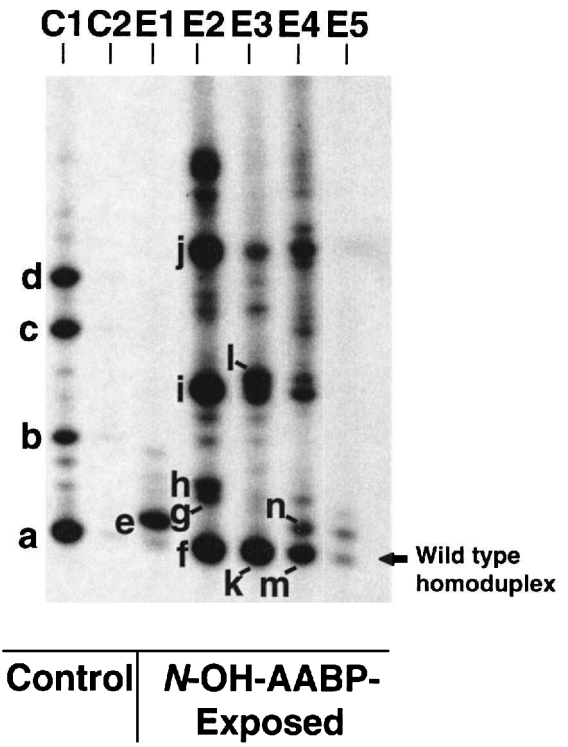

Figure 2. Second DGGE analysis of the high-melting domain of bands **C1–C2** and **E1–E5**, which were excised from the first DGGE shown in figure 1, reamplified and reannealed. Lanes **C1–C2** are mutant bands from the control cultures; lanes **E1–E5** are the mutant bands from *N*-OH-AABP-exposed cultures. Letters **a–n** show the bands excised from this second DGGE for sequencing analysis.

only background mutation detected was a transition from G to A that would result in the change of Cys(66) to Tyr.

Mutations due to exposure to *N*-OH-AABP generated a more complex spectrum of amino acid changes. The slippage due to insertion/repeat of seven bases at position 185 (band **e**) would cause incorporation of two histidines and the seventh inserted base would cause a complete alteration of the remaining sequence of amino acids encoded by exon 3. In the *N*-OH-AABP-induced mutants, bands **g** to **j** showed the same G to T transversions at 209 and 211, therefore potentially yielding the same amino acid changes Gly(70) → Val and Gly(71) → Cys. The 209G to T transversion could potentially cause a splice variant resulting in exclusion of 110 bases from the message coded by exon 3 (O'Neill *et al*. 1998), which would also presumably yield an inactive enzyme. *N*-OH-AABP-induced mutant band **1**, where the run of six guanines (207–212) were mutated, showed transversions from G to C and would yield the following changes in amino acids: Lys(69) → Asn; Gly(70) → Arg; Gly(70) → Ala; Gly(70) → Gly (i.e. silent); Gly(71) → Arg; and Gly(71) → Ala. The silent mutation at 210 must have been accompanied by at least one other mutation (in exon 3 or outside of it) in order for HPRT to be inactivated. The mutation in band **n**, an A to T transversion, would result in Tyr(72) → Phe.

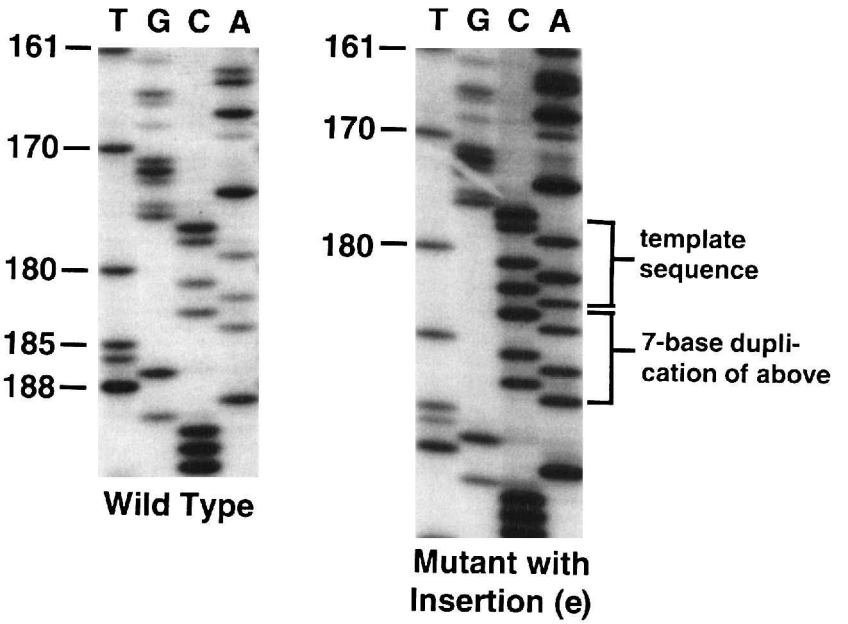

Figure 3. Sequencing analysis of band **e** (see figure 2) from *N*-OH-AABP-exposed mutants. This band showed an insertion of seven bases (see also table 1). Left sequence from wild type *HPRT* exon 3. Right: sequence from mutant band **e**. Numbers at left of gels show the base number within the coding region of the exon.

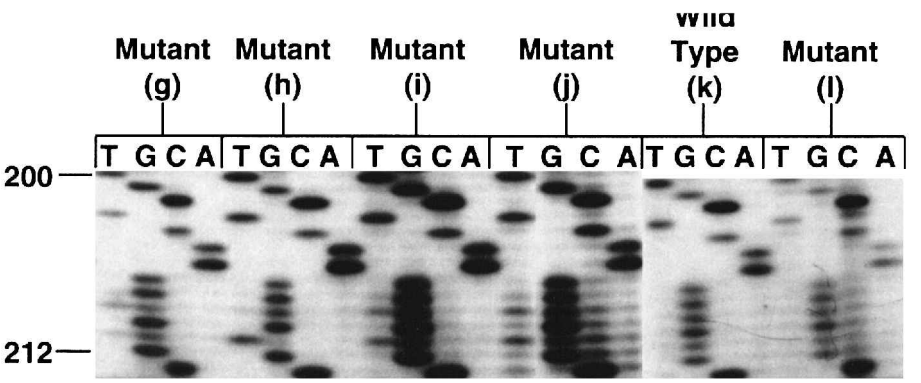

Figure 4. Sequencing analysis of bands **g–l** from the second DGGE analysis of *N*-OH-AABP-exposed mutant bands (see figure 2) showing changes in the 'hot spot' region of six sequential guanines (see also table 1). Numbers at left of gels show the base number within the coding region of *HPRT* exon 3.

None of the observed mutations introduced a termination signal (nonsense mutation) by changing an amino acid codon to a stop codon, such as TAG, TAA or TGA. In contrast, such induced stop codons are formed in exon 3 by benzo[*a*]pyrene diol epoxide (Keohavong and Thilly 1992b). Of course, any stop codons introduced by *N*-OH-AABP outside of the exon 3 coding region would not have been detected in this work.

The HPRT enzyme is a homodimer that catalyses the reversible formation of IMP and GMP from their respective bases, hypoxanthine and guanine, and the phosphoribosyl donor 5-phosphoribosyl-1-pyrophosphate (Xu and Grubmeyer 1998). Exon 3 codes for amino acids 46–106 of HPRT. The X-ray crystallographic structure (Eads *et al*. 1994) indicates that Lys(69) is one of the residues in contact with the ligands and is capable of interacting with multiple ligand atoms by hydrogen bonding or hydrophilic contacts. Induction of mutation by *N*-OH-AABP at exon 3 was very specific, with changes at Gly(70) most often observed. Changes from Gly(70) to any other amino acid could easily affect Lys(69)'s interactions with ligand atoms. Inspection of molecular models from the crystal structure shows that the mutations would cause the ε-NH_2 group of Lys(69) to lose contacts with the 2′O of GMP and to disrupt normal hydrogen bonds with exocyclic N2 and purine N3 of GMP. Additionally, Gly(70) sits very near the dimerization face in each half of the homodimer of HPRT. Altered side chains in this residue could well perturb dimerization and hence function of HPRT.

The mutation found in the control (background) cultures would change Cys(66) to Tyr. Again, there are two possible outcomes of this change that would result in an inactive enzyme. The phenol side chain could affect contact of Lys(69) with GMP due to Tyr's ability to form hydrogen bonds with the ε-NH_2 group of Lys(69). In addition, the bulkiness of Tyr could perturb the position of the ε-NH_2 group in three dimensional space. Finally, Cys(66) also lies near the homodimerization face, and a change to Tyr could cause improper contacts and/or alignment with the other half of the homodimer.

Discussion

Cultures of mixed mutants were generated with *N*-OH-AABP and surviving cells were grown up in 6-TG selective medium. Use of such an approach brings up several related questions: Does this method confer a growth advantage for some types of mutation over others? If an advantage does indeed occur, is the final percentage frequency of each mutation detected by this method representative of the initial frequency of each mutation? and, Is an overrepresented mutation truly due to multiple independent mutations at the same site, or is it due to overgrowth of a single mutant clone? One way to address these questions is to use labour-intensive experiments where each mutation represented in the spectrum is generated from an independent culture, or by sophisticated molecular analyses of each clone. Our experiments included three independent treated and three independent control publications. Each yielded essentially the same pattern, suggesting that similar mutagenic processes contributed to the same extent in each culture. The fact that the pattern of mutations found in *N*-OH-AABP-treated cultures were substantially different from that found in control cultures indicates mutagen-induced species were faithfully detected; i.e. there was no evidence of endogenous mutations with a growth advantage compromising the analysis. The types of mutations detected can be rationalized with the known properties of *N*-OH-AABP (purine adduction), also lending credence to the spectrum. The questions raised above have previously been tested (Keohavong and Thilly 1992b) with a similarly bulky mutagen, benzo[*a*]pyrene diol epoxide (BPDE), wherein the mutational spectrum of BPDE at exon 3 of TK6 *HPRT* was determined using essentially the same conditions used here. To test for growth advantage, these authors grew the same mixed mutant

cultures for additional tens of generations and re-analysed the mutation spectrum. They detected no changes. If growth advantage/selection had occurred under the mass culture/mixed mutant conditions, continued differential growth should have exaggerated the differences between the intensities of mutant bands. In the final analysis, however, we cannot be sure if mutants that could survive *N*-OH-AABP selection, but were inherently less viable, were not lost between induction and analysis. This is an inherent problem with any mutational assay that involves clonal expansion. Ultimately, it is the mutations that provide viable cells, rather than the entire spectrum of mutations induced (by *N*-OH-AABP in this case), that are of potential significance for human health effects and, most importantly, that can be used as biomarkers.

The main hot spot for point mutations induced by *N*-OH-AABP in exon 3 of *HPRT* in TK6 cells is the GC-rich region ^{205}AAG GGG GGC^{213}. Within this region, all of the guanines were mutated to differing degrees by *N*-OH-AABP. The main types of mutation observed were point mutation at guanine, with G to T transversion dominating, followed by G to C transversion. Additionally, a distinctive type of mutation was generated by *N*-OH-AABP, an insertion of a repeat sequence at position 185. The only detected background mutation was a G to C transition at position 197. None of the *N*-OH-AABP-induced point mutations were detected in control cultures. Inspection of molecular models generated from the crystal coordinates of GMP-bound HPRT showed that all of these mutations could conceivably be causes of enzyme inactivation.

Analysis of the published *HPRT* mutation database (Cariello *et al*. 1998) shows that induction of point mutations at the stretch of Gs in constitutive positions 207–212 appear common, especially amongst bulky electrophiles. The *N*-OH-AABP-induced insertion of a seven base repeat, however, appears new and novel, and could be a signature for *N*-OH-AABP in DNA samples used for molecular epidemiological studies.

Acknowledgements

We thank Dr Fred Kadlubar for the generous gift of *N*-OH-AABP, B. Z.-R. was supported in part by NIEHS-NRSA F31-ES05691.

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