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Comparison of the mutant frequencies and mutation spectra of three non-genotoxic carcinogens, oxazepam, phenobarbital, and Wyeth 14,643, at the λc*II* locus in Big Blue® transgenic mice

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Abstract

Oxazepam (OX), a widely used benzodiazepine anxiolytic, phenobarbital (PHE), a drug used for convulsive disorders, and Wyeth 14,643 (WY; [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid), a hypolipidemic agent, are all hepatocarcinogenic in B6C3F1 mice. They have been classified as "non-genotoxic" carcinogens since they are non-DNA reactive in *in vitro* assays and are either negative or weakly positive in *Salmonella typhimurium* (Ames assay). Male B6C3F1 Big Blue® transgenic mice were fed 2500 ppm of OX or PHE or 500 ppm of WY in their diet, while a control group of mice received diet alone for 180 days. The mutant frequency (MF) of *cII* in the control mice, after correction for clonality, was $6.2 \pm 2.8 \times 10^{-5}$. The MF values for mice fed OX, PHE, and WY were $10.0 \pm 3.6 \times 10^{-5}$ ($P < 0.05$), $7.9 \pm 1.3 \times 10^{-5}$ ($P = 0.1$) and $17.4 \pm 4.2 \times 10^{-5}$ ($P < 0.01$), respectively. The mutation spectrum (MS) at *cII* from the PHE-fed mice was significantly different ($P < 0.05$) from that of the control mice even though the MF was not, whereas the MS spectra of mice fed OX ($P = 0.4$) and WY ($P = 0.7$) were not significantly different. The PHE-derived spectrum differed from the spontaneous spectrum in the lower occurrence of G:C>C:G transversions (17 vs 1.6%) and the higher incidence of A:T>T:A transversions (3.4 vs 9.5%). Prior to correction for clonal expansion, each treated group exhibited a high incidence of frameshift mutations at the homopolymeric run of guanines at bp 179–184 (OX 21%, PHE 21%, WY 16% of the total mutations); this was not the case with the control group (6%). Even after clonal correction, more than 10% of the mutations were frameshifts in the treated mice, while 5% were frameshifts in the control mice. Despite this hypersensitive region of the gene, our findings suggest that the *cII* locus is less sensitive than the *lacI* locus to mutation induction by non-DNA reactive carcinogens.

Keywords: Oxazepam; Phenobarbital; Wyeth 14; 643; *cII* assay; Mutation spectra

1. Introduction

Concern has been expressed regarding the possible side-effects of chronic exposure of humans to drugs, especially if they have been found to elicit tumors in rodents. Two compounds to which humans are being extensively and chronically exposed are OX and PHE. OX (Serax®) is an active metabolite of the central nervous system depressants Valium and Librium. PHE has been used for about five decades to treat seizures [1]. A third compound, WY (Wyeth 14,643; [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid), is a precursor of the clofibrates and a model hypolipidemic agent that induces the proliferation of liver peroxisomes. These three compounds have been found to be hepatocarcinogenic in mice after feeding for 2 years [2–4]. Despite their tumorigenic properties, the mechanisms by which they cause tumors in rodents are not understood because they are not genotoxic in short-term *in vitro* assays. OX does not induce gene mutations *in vitro* in the Ames assay or sister chromatid exchanges in Chinese hamster ovary cells [2], nor does it enhance the production of micronuclei in mouse peripheral blood erythrocytes *in vivo* [5]. WY is a member of a novel class of hepatocarcinogenic

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Abbreviations: OX, oxazepam; PHE, phenobarbital; WY, Wyeth 14,643; PCR, polymerase chain reaction; MF, mutant frequency; MS, mutation spectrum; TMX, tamoxifen; and DMN, dimethylnitrosamine.

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agents, known as peroxisome proliferators (PPs) that are not mutagenic *in vitro* [6], do not cause unscheduled DNA synthesis in hepatocytes [7], and do not bind to DNA *in vitro* or *in vivo* [8,9]. However, PPs cause a significant increase in the level of the H_2O_2-generating peroxisomal fatty acid oxidation enzyme system in the liver [10–12]. PHE, on the other hand, has been classified as a weak mutagen in the Ames assay, since it induces a 2-fold increase in the mutagenicity of *Salmonella typhimurium* TA1535 in the absence of S9 [13,14]. However, using ^{32}P-postlabeling techniques, no bulky adducts were formed by PHE in Sprague–Dawley rats [15] or B6C3F1 mice [16]. The weak mutagenicity of PHE, coupled with its lack of DNA adduct formation, suggests that it, too, is tumorigenic through a mechanism other than the conventional genotoxic route.

Several possible mechanisms have been proposed to explain the tumorigenicity of carcinogens such as OX, PHE, and WY. These include oxidative stress [4], stimulation of cell division by mitogenesis [17], compensatory cell division due to cytotoxicity of the agent [18], and disruption of cell-to-cell communication [19]. Since the three compounds are mitogens, the fidelity of DNA repair could be compromised during the shortened G1, G2, and S phases [20]. Also rapid cell division could increase the amount of unprotected DNA exposed to endogenous reactive oxygen species, which are known to be mutagenic [21].

Although OX and WY are non-genotoxic *in vitro* and PHE is a weak genotoxin, we hypothesized that chronic exposure of rodents to these compounds might elicit a genotoxic response *in vivo*. The availability of transgenic rodents containing stably integrated prokaryotic genes that can be readily recovered for mutational analysis facilitates the study of mutagenesis in mammals *in vivo* [22]. One such rodent model is the Big Blue® transgenic mouse harboring the λLIZ shuttle vector containing the *lacI* gene that can be used as a surrogate gene for analysis of mutations in the genome. Previously, we found that chronic feeding of OX and WY for 6 months to Big Blue® mice resulted in an increase in the MF of the *lacI* gene in the liver [23,24]. This was not the case in PHE-fed mice [25]. Sequencing of the mutants from the mice fed OX, PHE, and WY resulted in a significant change in the mutation spectra reminiscent of oxidative damage similar to that reported for other organisms. As a result of this finding and the chronic exposure of humans to these drugs, we deemed it important to determine whether a second transgene, the *cII* gene [26], on the λLIZ shuttle vector would respond similarly to these compounds. This study would confirm our original findings at the *lacI* locus and would be important for validating the *cII* assay. The *cII* assay has a number of advantages over the *lacI* assay in that it is a simpler direct selection assay, is less expensive, and involves the sequencing of a smaller target gene. We and others have shown that the spontaneous MF [27] and the induced MF elicited by DMN [28] and TMX [29], as well as the induced MS, were similar at the *lacI* and *cII* loci. It was, therefore, of interest to determine if this was the case with OX, PHE, and WY. For the present study, DNA samples were isolated from the same liver tissue from the same mice from which the *lacI* data [23–25] had been generated.

2. Materials and methods

2.1. Animals and treatment

Age-matched 10-week-old male B6C3F1 Big Blue® transgenic mice bearing multiple copies of the λLIZ shuttle vector harboring the *cII* gene stably integrated into chromosome 4 were obtained from Taconic Farms. The mice were housed randomly, 3–4 per cage, and were quarantined for 7 days before treatment was initiated. For untreated control and exposed animals, powdered NIH-07 feed containing antioxidant was supplemented with either 0 (N = 10), 2500 ppm OX or PHE (N = 4), or 500 ppm Wyeth 14,643 (N = 6) and provided *ad lib.* to the mice for 180 days. Mice in all the treatment groups were weighed and killed by CO_2 asphyxiation the day after the feeding of the drugs was halted. The livers were harvested, weighed, and flash-frozen in liquid N_2 and stored at −80° until the MF and MS were determined.

2.2. Isolation of DNA

DNA was isolated using the RecoverEase® protocol (Stratagene Cloning Systems®). Briefly, 50 mg of liver was homogenized in ice-cold disruption buffer to lyse the cells and release the nuclei. The homogenate was filtered through a nylon mesh filter into a 50-mL tube and centrifuged at 1000 *g* for 10 min at 4°. The supernatant was decanted, and the tube was dried to remove the disruption buffer. A 70-μL aliquot of digestion buffer containing RNAce-It® (20 μL/mL of buffer, Stratagene Cloning Systems) and 70 μL of warmed proteinase K solution (50°) was added to the nuclear pellet, and the tube was incubated for 45 min at 50°. The contents of the tube were dialyzed for 48 hr against 10 mM Tris/1 mM EDTA buffer, pH 7.4. The viscous DNA was harvested and stored at 4° until packaging.

2.3. cII assay

λDNA was excised from hepatic DNA using RecoverEase® (Stratagene) and packaged into phage particles using Transpack® packaging extract. The *cII* assay selects for mutants by exploiting the critical role of the *cII* gene product in the commitment of λ to lysogeny following infection of a permissive *Escherichia coli* host. The CII protein activates the transcription of the λ *cI* repressor and λ integrase genes essential for lysogeny. The level of the CII protein present in *E. coli* is negatively regulated by FtsH protease encoded by the *hflB* gene, while the *E. coli hflA*

gene encodes the HflKC membrane complex, which modulates the protease function of FtsH [30]. The *E. coli* strain used for the *cII* assay is *hfl*$^{-}$*;* thus, high levels of CII will direct lysogeny by the infecting λ phage. If low levels of CII are present due to a mutation in *cII* or the altered expression of *cI,* lysis occurs and a visible plaque can be seen. The *cII*$^{-}$ phage grow at 24° but not at 37°, while *cII*$^{+}$ phage grow at 37°. Therefore, mutated phage can be selected and easily quantified by growth at 24°, and non-mutated phage can be quantified at 37° [26].

Phage were screened for mutations by mixing 200 μL of *E. coli* G1250 cells with 100 μL of packaged phage and plating on TBI plates (1.2% Bacto agar, 1% Bacto-tryptone, 0.5% NaCl, 0.0001% thiamine hydrochloride). The plates were incubated at 24° for 46 hr, and the mutant plaques were counted, picked, and stored in 500 μL of SM buffer [5.8 g NaCl, 2.0 g $MgSO_4 \cdot 7H_2O$, 50 mL 1 M Tris–HCl (pH 7.5), 5 mL 2% (w/v) gelatin]. For titration, a 20-μL aliquot of a 1:100 dilution of the packaged phage was mixed with *E. coli* G1250 cells, plated on TBI plates, and incubated at 37° for 16 hr. The mutant plaques were confirmed by replating on TBI plates at 24°. A well-isolated, clear plaque was picked for sequencing.

The MF of λ*cII* was determined by dividing the number of mutant plaques by the total number of plaque forming units (p.f.u.) evaluated from each animal. Student's *t*-test was performed to assess significant differences in the MF between the treatment and the control groups. Based on the sequencing data, the MF of the OX-, PHE-, and WY-treated mice were corrected for clonal expansion, and the data were re-evaluated statistically for differences in the MF from the control mice.

2.4. Sequencing of the mutants

Although it would be ideal if all the mutants from each mouse could be sequenced, this is frequently unrealistic due to the time, effort, and cost involved. Experience in both our laboratory and that of others[1] has suggested that between 80 and 100 mutants should be sequenced from each treatment group to obtain a representative spectrum. To do this, we choose approximately the same number of mutants from each animal so that the total sequenced from each treatment group was close to 100 mutants. To obtain a representative sample from each animal and to avoid bias, mutants were chosen from different plates from each packaging reaction of phage for sequencing.

The *cII* gene was amplified from the bacteriophage in a PCR with an upstream primer, 5′-CCGCTCTTACACATTCCAGC-3′, located at position -112 to -92 and a downstream primer, 5′-TATGAGTTGAAGCCGTCTCC-3′, located at position 345 to 365 on a Perkin Elmer® Gene Amp System. Each 100-μL reaction mixture comprised 82.5 μL of a 1:20 dilution of the phage in water, 5 μL of PCR 10x buffer, 20 pmol of each upstream and downstream primer, 2.5 mM dNTPs, and 5 U of *Taq* DNA polymerase. Agarose gel electrophoresis confirmed the success of the PCR. The 400 bp fragment containing the *cII* region was purified on a QiaQuick PCR purification column (Qiagen, Inc.) and concentrated to 10 μL using a vacuum centrifuge. Cycle sequencing of 100 ng of purified DNA was performed in the presence of 5 pmol of the upstream sequencing primer and 2–3 μL of Big Dye® ready reaction mix (Perkin Elmer Applied Biosystems). The products were precipitated with ethanol and dried.

The samples were reconstituted in 3 μL of deionized formamide, and 1 μL was run on an ABI 377 Sequencer®. The sequence was analyzed using the Sequencher Program (Gene Codes Corp.) to identify mutations. The spectrum from each treated group was compared with the spectrum from the control group using the hypergeometric program of Adams and Skopek [31].

3. Results

3.1. MF

To assess whether OX, PHE, and WY were mutagenic at *cII,* the MF was determined in the livers of two groups of four mice fed 2500 ppm of OX or PHE in the diet for 180 days. The doses for OX and PHE were chosen to mimic the dose used in the NTP bioassay and the IARC study [2,3] in which tumors were found in B6C3F1 mice. Six mice were fed 500 ppm of WY for 6 months, which corresponded with the dose at which the chemical was hepatocarcinogenic in a 2-year bioassay [4]. Data for the control group was gathered from four control mice from the WY experiment and six control mice from the OX/PHE experiment. The MF of both control groups was assessed separately, but because there was no significant difference between the MF of the two groups, they were pooled for further analysis. The MF of the control group was $7.1 \pm 2.9 \times 10^{-5}$, while those from the OX, PHE, and WY groups were $14.5 \pm 5.6 \times 10^{-5}$, $12.2 \pm 1.7 \times 10^{-5}$ and $18.2 \pm 4.1 \times 10^{-5}$, respectively (Table 1). The differences in MF between the control and treated groups were significant for the three compounds before correction for clonal expansion.

To accurately assess the MF at the *cII* locus, the frequency was corrected for clonal expansion and for mutants that did not show a mutation after sequencing both the sense and antisense strands of the coding region. Clonal expansion refers to the recovery of multiple mutations at the same site in the same tissue from the same animal and is assumed to arise as daughter cells of a single, mutated cell. Although these identical mutations could be unique and arise independently, as a conservative estimate, they are counted as a single event. Clonal expansion is of particular concern in rapidly dividing tissues, a situation that occurs after mitogen

[1] de Boer J, personal communication. Cited with permission.

Table 1
Mutant frequency (MF) of *cII* in the livers of control, oxazepam, phenobarbital, and Wyeth 14,643-treated Big Blue® mice

Mouse number	No. mutants	No. plaques ($\times 10^5$)	Mutant frequency ($\times 10^{-5}$)
Control			
1	78 (67)[a]	8.13	9.6 (8.2)
2	35 (32)	4.89	7.2 (6.5)
3	27 (23)	3.69	7.3 (6.2)
4	83 (80)	6.84	12.1 (11.7)
5	21 (18)	2.81	7.5 (6.4)
6	11 (11)	4.00	2.8 (2.8)
7	23 (20)	4.07	5.7 (4.9)
8	20 (14)	3.02	6.6 (4.6)
9	13 (11)	4.31	3.0 (2.6)
10	27 (25)	3.00	9.0 (8.3)
			7.1 ± 2.9[b] (6.2 ± 2.8)
Oxazepam			
1	34 (23)	2.07	16.4 (11.1)
2	32 (22)	3.35	9.6 (6.6)
3	56 (38)	2.60	21.5 (14.6)
4	47 (35)	4.46	10.5 (7.8)
			14.5 ± 5.6[b]* (10.0 ± 3.6)**
Phenobarbital			
1	19 (12)	1.48	12.8 (8.1)
2	52 (33)	3.65	14.2 (9.0)
3	24 (14)	2.33	10.3 (6.0)
4	23 (17)	2.04	11.3 (8.3)
			12.2 ± 1.7[b]* (7.9 ± 1.3)
Wyeth 14,643			
1	115 (106)	5.66	20.3 (18.7)
2	194 (191)	9.53	20.4 (20.2)
3	80 (72)	6.66	12.0 (10.8)
4	57 (54)	3.80	15.0 (14.2)
5	93 (92)	5.17	17.9 (17.8)
6	117 (114)	5.01	23.4 (22.8)
			18.2 ± 4.1[b]* (17.4 ± 4.2)***

[a] The number in parentheses refers to the number of mutants after correction for clonal expansion and for mutants that lacked a mutation in the *cII* coding sequence. The corresponding corrected MF are also in parentheses.

[b] Mean ± SD of the MF. The MF was calculated by dividing the number of mutants recovered from each mouse by the total number of plaque forming units evaluated from each mouse.

* Significantly different ($P < 0.05$) before correction for clonal expansion.

** Significantly different ($P < 0.05$) after clonal expansion, compared with control MF.

*** Signficantly different ($P < 0.01$) after clonal expansion, compared with control MF.

stimulation, which has been reported for OX, PHE, and WY treatments.

In all the treated groups, but to a lesser extent in the control group, clonal expansion contributed significantly to the MF in many of the mice. Thus, within each group, clonal correction reduced the MF considerably (Table 1). However, after correction, the MF at the *cII* locus was still significantly different in the mice that received OX and WY. In the OX- and WY-exposed mice, the MF at *cII* was $10.0 \pm 3.6 \times 10^{-5}$ ($P < 0.05$) and $17.4 \pm 4.2 \times 10^{-5}$ ($P < 0.01$), respectively, compared with the MF of the control group, $6.2 \pm 2.8 \times 10^{-5}$. In the PHE-exposed mice, the MF of *cII* was $7.9 \pm 1.3 \times 10^{-5}$, which was not significantly different from the control value ($P > 0.1$). It should be noted that in all the animals from the PHE-treated group, the number of mutants decreased by more than 28% after clonal correction (mouse #1: 5 out of 19 mutants, mouse #2: 17 out of 52 mutants, mouse #3: 5 out of 24 mutants, and mouse #4: 5 out of 23 mutants). Nine mutants (17.3%) were recovered from mouse #2 of this group with an A>T substitution at bp 124 in the *cII* gene and 7 mutants (13.5%) with a +1 frameshift of G in the homopolymeric run of guanines at bp 179–184. Compared with the other treatment groups, the WY-exposed mice exhibited the highest MF. The overall occurrence of clonal expansions in the WY-treated group was much lower in comparison to the occurrence of clonal expansions in the PHE-treated mice, with a total of only 16 mutants eliminated in the WY-treated group. The majority (8 out of 16) of the clonal expansions in the WY-treated group were from mouse #1, while 2 were from mice #2, #3, and #6, and 1 was from mice #4 and #5.

3.2. Classes of mutations in the control mice

In each of the treatment and control groups, a minimum of 15 mutants from each mouse were sequenced. The two control spectra, one generated from the experiment with PHE and OX and the second from the experiment with WY, were pooled since there were no significant differences between the two spectra when analyzed using the program of Adams and Skopek [31]. Of the 311 mutants collected from the control animals, 180 were chosen randomly and sequenced. A mutation could not be identified in 16 of these mutants. A total of 164 mutations were identified in the *cII* gene with 17 mutants removed from the spectrum due to clonal expansion. The sequencing data shown in Table 2 reflect the number of mutants in each class after correction for clonality. The majority of the mutations were base pair substitutions with G:C>A:T transitions comprising 39.5% of the spectrum, while G:C>T:A and G:C>C:G comprised 21.8 and 17.0%, respectively. Compared with the treated groups, the percentage of frameshifts was relatively low (6.1%).

3.3. Classes of mutations in the treated mice

Of the 169 mutants collected from the OX group, 131 were randomly chosen and sequenced. Mutations were identified in 108 of the randomly chosen mutants, while 23 (17.6%) lacked a mutation in the coding sequence. Of the 108 mutations, 29 (26.8%) were identified as clonal, yielding a final number of 79 individual mutations (Table 2). Sixty-five of these (82.3%) were base pair substitutions, 7 of which (10.8%) generated stop codons. There were 11 frameshift mutations, 2 double mutations, and 1 complex mutation. No significant difference ($P = 0.5$) was noted between the corrected OX spectrum and the pooled cor-

Table 2
Classification of *cII* mutations, after clonal correction, recovered from the liver of oxazepam; phenobarbital- and Wyeth 14,643-treated Big Blue® mice

	Control		Oxazepam		Phenobarbital		Wyeth 14,643	
	No.*	%	No.	%	No.	%	No.	%
Base pair								
Substitutions	134	91.2	65	82.3	53	84.1	80	86.0
Transitions								
G:C > A:T	58	39.5	25	31.7	26	41.3	35	37.6
At CpG sites	38	65.5	21	84.0	21	80.7	23	65.6
A:T > G:C	8	5.4	6	7.6	6	9.5	7	7.5
Transversions								
G:C > T:A	32	21.8	14	17.7	12	19.0	22	23.7
At CpG sites	9	28.0	5	35.7	1	8.3	9	39.1
G:C > C:G	25	17.0	12	15.2	1	1.6	11	11.8
At CpG sites	7	28.0	3	25.0	0	0	2	18.0
A:T > T:A	5	3.4	4	5.1	6	9.5	3	3.2
A:T > C:G	6	4.1	4	5.1	2	3.2	2	2.2
Other mutations	13	8.8	14	17.7	10	15.9	13	10.3
−1 Frameshifts	6	4.1	6	7.6	5	7.9	8	8.6
+1 Frameshifts	3	2.0	5	6.3	4	6.4	4	4.3
Deletions	1	0.7	0	0	0	0	0	0
Insertions	0	0	0	0	0	0	0	0
Complex changes	0	0	1	1.3	0	0	0	0
Double mutants	3	2.0	2	2.5	1	1.6	1	1.1
Total no. of mutants	**147**	**100**	**79**	**100**	**63**	**100**	**93**	**100**

* Control spectrum was derived from pooling of the spontaneous spectra from the oxazepam, and phenobarbital, experiment and the Wyeth 14,643 experiment

rected control spectrum. However, the percentage of G:C>A:T transitions at 5′-CpG-3′ sites increased notably, from 65.5% in the control mice to 84.0% in the treated mice. The proportion of frameshifts (13.9%) in the treated group was 2-fold greater than in the control group (6.1%).

Of the 118 mutants from the PHE-treated animals, 105 were randomly chosen and sequenced. Mutations were identified in 95 of the randomly chosen mutants, while 10 of them (9.5%) lacked a mutation at *cII*. However, 32 of the mutations (33.7%) were clonal, leaving a final number of 63 mutations. Fifty-three of these (84.1%) were identified as base pair substitutions, 9 of them (16.7%) generating stop codons. There were also 9 frameshift mutations and 1 double mutation. Unlike the OX-treated group, the mutation spectrum of the PHE-treated mice was found to be significantly different ($P < 0.05$) from the control spectrum (Table 2). The most significant change in the spectrum was a 10-fold decrease in the percentage of G:C>C:G transversions in the exposed group (1.6%) compared with the control group (17.0%). In addition, base pair substitutions increased at adenines in the PHE-treated group. Nearly a 2-fold increase in the percentage of A:T>G:C transitions (9.5 vs. 5.4%) and a 2.8-fold increase in A:T>T:A transversions (9.5 vs. 3.4%) was noted. Similar to the findings in the OX-treated mice, a higher percentage of frameshift mutations (14.3%) were noted in the PHE-treated mice compared with the control mice (6.1%). Also, the percentage of G:C>A:T transitions at 5′-CpG-3′ sites increased from 65.5 to 80.7% in the PHE-treated group.

From the collection of 656 mutants isolated from the WY-treated mice, 119 were sequenced. Of these, 11 lacked a mutation in the *cII* gene (9.2%) and 15 (12.6%) were clonal, resulting in a final number of 93 mutants. Eighty of these (86.0%) were base pair substitutions (Table 2) with 10 of them (12.5%) creating stop codons. The spectrum also included 12 frameshift mutations and 1 double substitution. The spectrum from the WY-treated mice was not significantly different from that of the control ($P = 0.7$). However, analogous to the spectrum from the PHE-treated mice, there was a decrease in the frequency of G:C>C:G transversions, from 17.0 to 11.8%. As with the other treatment groups, there was also an increase in the occurrence of frameshift mutations, from 6.1 to 12.9%.

It should be noted that before correction for clonality, the mutants from the OX-, PHE-, and WY-treated groups exhibited a high incidence of frameshift mutations at the homopolymeric run of guanines at bp 179–184, while this was not the case in the control group. The original incidence of frameshifts in the control group at this hot spot was 5.8% (3.5% of +1, 2.3% of –1), which decreased to 4.8% (2.1% of +1, 2.7% of –1) after correction for clonality. In the OX-treated group, the incidence of frameshifts in this region was significantly higher at 21.3% (15.7% of +1, 5.6% of –1) that decreased to 11.4% (6.3% of +1 and 5.1% of –1) after correction for clonal expansion. Similarly, in the PHE-treated group, the frequency of frameshifts decreased from 21.0% (18.9% of +1, 2.1% of –1) to 9.5% (6.3% of +1, 3.2% of –1) after correction for clonality. However, in the WY-exposed animals, the percentage of frameshifts at this region before correction was 15.7% (8.3% of +1 and 7.4%

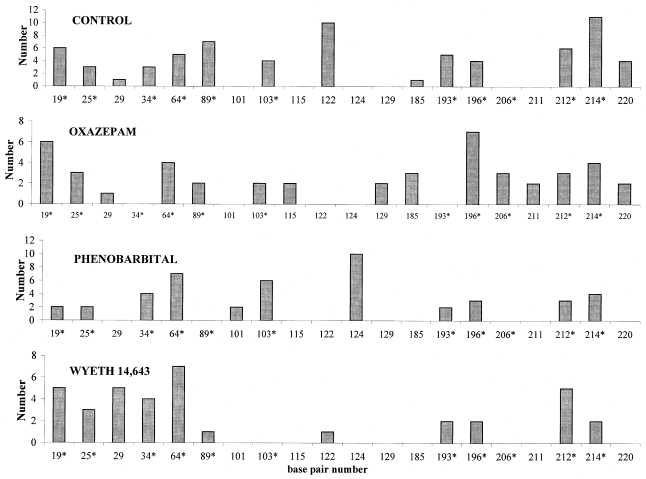

Fig. 1. Incidence of base pair substitutions recovered at the same site from control and treated Big Blue® mice. This figure shows the sites in the *cII* gene at which multiple mutations were found in the liver from control, OX-, PHE-, and WY-treated mice. The number of mutants with identical mutations from the same animal has not been corrected for clonal expansion. Key: (*) indicates a 5′-CpG-3′ site at the nucleotide base.

of –1), which decreased to 10.9% (4.3% of +1 and 6.6% of –1) after correction.

3.4. Potential "hot spots"

Sites at which multiple mutations were found in the four spectra are shown in Fig. 1. In the spontaneous spectrum, base pair substitutions were most commonly found at bp 19, 89, 122, and 214, suggesting that these sites may be "hot spots," i.e. areas of the gene more prone to spontaneous mutation induction. It should especially be noted that three of these four sites are 5′-CpG-3′ sites (19, 89, and 214), with the predominant mutations at these sites being C>T transitions. A significant difference in the control spectrum compared with each of the three treated groups was the substantial decrease (WY), or nonexistence of mutations at bp 122 in the spectra from the OX- and PHE-treated mice. Unique substitutions at bp 115, 129, 206, and 211 were recorded in the OX-derived spectrum, which were not induced by any of the other compounds. The major difference in the sites of mutation in the PHE spectrum was the high frequency of A:T>T:A transversions at bp 124. The multiplicity of mutations throughout the gene was less frequent in the WY-derived spectrum.

4. Discussion

To determine the sensitivity of the *cII* gene to mutation induction by the three non-DNA reactive hepatocarcinogens (OX, PHE, and WY), the MF and MS were determined. Despite the increases in MF at *cII* in the OX- and WY-treated groups, the mutation spectra from these two groups were not significantly different from the control spectrum. This may be due, in part, to the relatively high frequency of G:C>C:G mutations (Table 2) in the two spectra collected from the control mice. Before pooling, the percentage of G:C>C:G transversions was 17% in the control spectrum from the OX and PHE experiment and 11.8% from the WY spectrum. Since these two control spectra were not significantly different after using the hypogeometric analysis of Adams and Skopek [31], they were combined. A similarly elevated proportion of G:C>C:G transversions was observed at *cII* by Harbach *et al.* [27], who found that 10% of the total mutations in the spontaneous mutation spectrum in the liver, lung, and spleen of transgenic mice were G:C>C:G transversions. Both our observations and those of Harbach *et al.* [27] suggested that this relatively high incidence of G:C>C:G transversions was neither an isolated phenomenon, nor unusual.

A major concern regarding the use of this Big Blue® mouse mutagenicity assay is the cost of labor and materials in determining the potential mutagenicity of xenobiotic compounds. This is exacerbated when the experimental design dictates that the animals are fed a chronic regimen for extended periods of time such as 6 months. Another aspect of these initial long-term studies is the decision not to assess dose- and time-related effects on MF and MS until it has been shown that the compound in question is mutagenic *in vivo.* One important issue in this regard is the observation that mutations at the non-transcribed *lacI* and *lacZ* transgenes accumulate over time in animals exposed chronically to *N*-ethyl-*N*-nitrosourea (ENU) and benzo[*a*]pyrene (B[a]P) [32]. While ENU and B[a]P are potent genotoxic mutagens, OX, PHE, and WY are not; thus, it is unlikely that an increase in MF would be evident in mice treated for shorter periods of time with OX, PHE, and WY. Likewise, a change in spectra would not be evident at earlier time points. This issue of dose- and time-related responses in *in vivo* mutagenicity assays is an important area for future research. These parameters are poorly understood at present, as many metabolic pathways including certain DNA repair pathways may be different at transgenes than at endogenous genes.

4.1. Lack of mutations at the cII locus

A varying proportion of the mutant phage DNA samples sequenced in the sense and antisense direction did not yield a mutation in the *cII* coding region. Specifically 18, 10, and 9% of all DNA isolated from mutant plaques sequenced in the OX, PHE, and WY groups, respectively, did not show a mutation at *cII*, compared with a control rate of 8%. The lack of a mutation at the *cII* locus in some mutants with purported mutations in the *cII* assay has been documented before. Following exposure of Big Blue® mice to TMX, 10 and 16% of the mutants from the control and TMX-treated animals, respectively, lacked a mutation at the *cII* gene [30]. Similarly Harbach *et al.* [27] found that 19% of 182 spontaneous *cII* mutants from the liver, lung, and spleen of Big

Blue® mice did not elicit a mutation at the *cII* locus. However, 75% of those mutants that lacked a mutation at *cII* had a mutation at *cI*. Since clear mutant plaques could develop, not only in the absence of an active *CII* protein but also as the result of a defective gene product, it is possible that a small proportion of mutants that are recovered in the assay are, in fact, due to mutations at *cI*. In the present study, it was deemed unnecessary to sequence these putative *cI* mutants since they made up a relatively small proportion of the mutants.

4.2. Incidence of frameshifts

In the three exposed groups, the incidence of +1 and –1 frameshift mutations at the homopolymeric run of guanines between bp 179 and 184, after clonal correction, was higher than that of the control group; thus, these frameshift mutants contributed to the overall mutagenesis at the *cII* locus. Although the mechanism of formation of frameshift mutations is believed to be due to slippage by DNA polymerases at nucleotide repeats during DNA replication [33] while bypassing bulky adducts, this mechanism may not apply for these three drugs. This is because these compounds do not form bulky adducts. If slippage by the polymerase is the mechanism for these frameshifts, then it might be expected that a high incidence of spontaneous frameshift mutations at the homopolymeric run of adenines at bp 241–246 would also occur. Since this was not the case, polymerase errors due to bulky adducts in homopolymeric runs is an unlikely mechanism. Previously, we proposed that the mutations at *lacI* in the OX- and PHE-treated mice were the result of oxidative stress following chronic administration of the two drugs [23,25]. A common DNA lesion formed by oxidative stress is an apurinic/apyrimidinic (AP) site, in which DNA replication opposite this lesion generally results in the incorporation of adenine residues [34]. However, in the presence of an AP site in a homopolymeric run of guanines, it might be envisaged that slippage of the polymerase could easily occur, resulting in a frameshift. Another possible mechanism for frameshifts was suggested by Jackson *et al.* [35] who reported that frameshift mutations could be produced in a plasmid containing a homopolymeric run of guanines or microsatellite repeats following treatment with hydrogen peroxide to generate oxidative stress. They suggested that strand breaks could increase the probability of strand misalignment leading to the formation of insertion or deletion loops. Since oxidative damage rarely occurs at adenines, this would also explain the low or nonexistent frequency of +1 and –1 frameshifts at the homopolymeric run of adenines between bp 241 and 246. Thus, the induction of frameshift mutations in the homopolymeric run of guanines at bp 179–184 in the *cII* gene may be the result of oxidative damage to guanine residues resulting in apurinic sites or strand breaks, followed by polymerase errors.

4.3. cII vs lacI MF and MS

The MF and MS at the *cII* locus in this experiment were compared with the results obtained at the *lacI* locus of the λ transgene from the same mice. The increase in MF at *cII* from the OX- and WY-treated mice was similar to that reported for *lacI* [23,24], while the absence of a significant change in the MF in the PHE-treated mice at *cII* mirrored the data reported at the *lacI* locus [25]. OX, PHE, and WY treatments led to a significant increase in G>T and G>C transversions at *lacI*, but not at *cII*. As a result, the OX- and WY-induced spectra at *cII* were not significantly different from the control spectrum. In contrast, PHE exposure resulted in a significant decrease in the frequency of G>C mutations at *cII*, with a concomitant increase in mutations at adenines resulting in AT>GC and AT>TA mutations. Thus, a significant change in the mutation spectrum at *cII* without a significant increase in the mutant frequency was noted in the PHE-treated group compared with the control group.

Other studies, with varying results, have compared the responses at the *lacI* and the *cII* loci in Big Blue® mice. The MF of the *hprt*, *lacI*, and *cII* loci in the lymphocytes of Big Blue® mice exposed to *N*-methyl-*N*-nitrosourea (MNU) and B[a]P were compared [36]. The *hprt* gene was the most sensitive locus to both agents followed by *lacI*, while the response at *cII* was not elevated significantly compared with the control MF. Subsequently, Harbach *et al.* [27] found that the spontaneous MF and MS at the *lacI* and *cII* loci from the liver, spleen, and lung of Big Blue® mice were similar, except for a decrease in the proportion of G:C>A:T transitions and an increase in G:C>C:G transversions at *cII* compared with *lacI*. More recently, we found that the increases in MF at the *lacI* and *cII* loci were comparable when mice were treated with DMN [28]. However, it was reported that the *lacI* locus in Big Blue® rats was more sensitive than the *cII* locus to mutation induction by TMX [37]. An increase in the MF at *lacI* was found after feeding 10 or 20 mg/kg TMX for 6 weeks, whereas the MF at *cII* was statistically significant only at the higher dose [29,37]. These studies suggested that the *cII* locus might be less sensitive than the *lacI* locus to mutagenesis. Our present study with OX, PHE, and WY support the contention that the *cII* locus may be less sensitive than the *lacI* locus to *in vivo* mutagenesis. In conclusion, although we have found that chronic feeding of OX and WY to mice increases the MF at two transgenic loci *in vivo*, the relevance of this finding to mutation induction in humans taking these drugs chronically is unknown.

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