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The role of polycyclic aromatic hydrocarbon-DNA adducts in inducing mutations in mouse skin

Dhrubajyoti Chakravarti[1,*], **Divya Venugopal**[1], **Paula C. Mailander**[1], **Jane L. Meza**[2], **Sheila Higginbotham**[1], **Ercole L. Cavalieri**[1], and **Eleanor G. Rogan**[1]

1 *Eppley Institute for Research in Cancer and Allied Diseases, 986805 Nebraska Medical Center, Omaha, NE 68198-6805*

2 *Department of Preventive and Societal Medicine, 984350 Nebraska Medical Center, Omaha, NE 68198-4350*

Abstract

Polycyclic aromatic hydrocarbons (PAH) form stable and depurinating DNA adducts in mouse skin to induce preneoplastic mutations. Some mutations transform cells, which then clonally expand to establish tumors. Strong clues about the mutagenic mechanism can be obtained if the PAH-DNA adducts can be correlated with both preneoplastic and tumor mutations. To this end, we studied mutagenesis in PAH-treated early preneoplastic skin (1 day after exposure) and in the induced papillomas in SENCAR mice. Papillomas were studied by PCR amplification of the H-*ras* gene and sequencing. For benzo[*a*]pyrene (BP), BP-7,8-dihydrodiol (BPDHD), 7,12-dimethylbenz[*a*] anthracene (DMBA) and dibenzo[*a,l*]pyrene (DB[*a,l*]P), the codon 13 (GGC to GTC) and codon 61 (CAA to CTA) mutations in papillomas corresponded to the relative levels of Gua and Ade-depurinating adducts, despite BP and BPDHD forming significant amounts of stable DNA adducts. Such a relationship was expected for DMBA and DB[*a,l*]P, as they formed primarily depurinating adducts. These results suggest that depurinating adducts play a major role in forming the tumorigenic mutations. To validate this correlation, preneoplastic skin mutations were studied by cloning H-*ras* PCR products and sequencing individual clones. DMBA- and DB[*a,l*]P-treated skin showed primarily A.T to G.C mutations, which correlated with the high ratio of the Ade/Gua-depurinating adducts. Incubation of skin DNA with T.G-DNA glycosylase eliminated most of these A.T to G.C mutations, indicating that they existed as G.T heteroduplexes, as would be expected if they were formed by errors in the repair of abasic sites generated by the depurinating adducts. BP and its metabolites induced mainly G.C to T.A mutations in preneoplastic skin. However, PCR over unrepaired *anti*-BPDE-N^2dG adducts can generate similar mutations as artifacts of the study protocol, making it difficult to establish an adduct-mutation correlation for determining which BP-DNA adducts induce the early preneoplastic mutations. In conclusion, this study suggests that depurinating adducts play a major role in PAH mutagenesis.

Keywords

Polycyclic aromatic hydrocarbons; SENCAR mouse skin; H-*ras*; Mutations

Correspondence to: D. Chakravarti, Eppley Institute for Research in Cancer and Allied Diseases, 986805 Nebraska Medical Center, Omaha, NE 68198-6805, Tel: (402) 559-2951, Fax: (402) 559-8068, E-mail: dchakrav@unmc.edu.

1. Introduction

Polycyclic aromatic hydrocarbons (PAH) are environmental carcinogens [1–3] associated with skin, lung, pharynx, oral and other cancers [4–6]. Initiation of these diseases is often associated with mutations in H-, K- and N-*ras* genes [7–13]. These *ras* mutations are thought to be induced by the PAH-DNA adducts generated in exposed cells [14–18].

In the cell, PAH are oxidized by cytochrome P450s to form electrophilic derivatives (*e.g.*, diolepoxides and radical cations) that react with DNA to form adducts [19]. PAH react primarily with the purine bases in DNA, forming bulky stable and depurinating adducts [20–24]. The adduct-forming reaction is moderately sequence-selective [25–32]. In the cell, PAH-DNA adduct formation blocks replication and induces base and nucleotide excision repair (BER and NER) activities during a 24 h-long period [33–39]. The stable adducts undergo NER, whereas the abasic sites generated from the spontaneous loss of the depurinating adducts undergo BER [38,40–42]. During this period, some PAH-induced abasic sites are converted into heteroduplex mutations by BER errors [39,43,44], and a subpopulation of the stable adducts are removed by error-free NER [45,46]. As cells re-enter S-phase, the heteroduplex mutations are "fixed" in daughter DNA molecules by one round of replication [44]. In addition, some cells carrying the unrepaired stable adducts acquire mutations as DNA polymerases go over the adducted bases, incorporate incorrect bases and fail to remove these mistakes by proofreading [47–57]. Two rounds of replication are needed to fix this latter type of mutations into daughter DNA molecules [58,59].

Studies in the SENCAR mouse skin cancer model [14–16] have led to suggestions that stable and depurinating PAH-DNA adducts can both contribute to the formation of the tumorigenic preneoplastic mutations [16,42,60,61]. These ideas were mainly inspired by correlating PAH-DNA adducts (which form within hours of exposure) with oncogenic H-*ras* mutations found in mouse skin tumors (which form 7–9 weeks later) [14–16]. However, such correlations can provide limited information about the nature of mutagenesis. PAH induce both oncogenic and non-oncogenic mutations in the preneoplastic stage [43,58,59]. Cells that acquire the oncogenic mutations become clonally-expanded (based on their transforming potentials and tissue specificities) to establish the tumors [62–65]. Therefore, oncogenic mutations found in the tumors cannot describe the mutational specificity of PAH-DNA adducts. Knowing the mutational specificity in the preneoplastic tissue is important, because it can reveal the critical lesions for cancer. *In vitro*, the mutational specificity is obtained from studies that examine polymerase errors during the filling synthesis of nicks or gaps similar to those generated by the excision steps of NER and BER [66,67], or those during bypass over PAH-DNA stable adducts (translesion synthesis), similar to those left unrepaired by NER [68,69]. The knowledge obtained from such studies is limited to the specific DNA polymerases, and sometimes cannot explain mutagenesis *in vivo*. For example, mouse skin papillomas induced by benzo[*a*]pyrene (BP) show both codon 13 (GGC to GTC) and 61 (CAA to CTA) mutations in the H-*ras* gene [16]. Translesional synthesis errors over BP diolepoxide (BPDE)-N^2dG adducts at the central G of GGC form the GTC mutation [49], but the BPDE-N^6dA adducts do not form the CTA mutation at the central A of CAA [69,70].

A number of studies in PAH-treated cells in culture or PAH-exposed tissues of animals have been conducted to understand the *in vivo* mutational specificity (discussed in Section 4). Results of these studies need to be interpreted with caution, because cell death from DNA damage-induced apoptosis and necrosis, and from fatal mutations [71–73], as well as selective proliferation and regression of oncogenically-mutated cells [74–76], can induce dramatic changes in preneoplastic mutation spectra. For example, some of the initial mutations (6 h- 1 d) observed in dibenzo[*a,l*]pyrene (DB[*a,l*]P)-treated mouse skin are enriched or lost in the later preneoplastic period (days 2–7) [43,44]. Therefore, early preneoplastic spectra can

provide important information about PAH mutational specificity in the target cells. In both DB[*a,l*]P- and DB[*a,l*]P-diolepoxide-treated mouse skin, the early mutation period appears to be between 6 h - 1 d [43,44]. In the present study, we have used the 1 d spectra as a time point for learning about PAH mutational specificity *in vivo*.

Errors made by repair and by translesion synthesis may produce quite different spectra of mutations. For example, if error-prone BER induces mutations from PAH-induced abasic sites, the spectra will be determined primarily by the specificities of gap-filling errors of BER polymerases. If DNA polymerase β or ι is involved, the majority of base substitutions should be A to G transitions [66,77,78]. On the other hand, translesional synthesis errors of DNA polymerases over PAH-DNA stable adducts can produce a greater variety of base substitution mutations, dictated by factors such as the structure and conformation of the adducts, their DNA sequence contexts, and the DNA polymerase [79,80]. The classical replicative polymerases are inefficient in bypassing bulky stable adducts or extending DNA synthesis beyond the adducted sites. Therefore, bypass polymerases are now thought to be more important for generating mutations by this mechanism. For example, DNA polymerases η/ζ/ι may incorporate an A or a G opposite BPDE-N^2dG adducts in the template strand (leading to G to T or G to C mutations) [57,81,82]. In comparison, BPDE-N^6dA adducts induce fewer mistakes and show little misincorporation preference [82].

The extent to which mutagenesis by errors in repair or translesion synthesis contributes to cancer can be understood from studies that examined the roles of quiescent or proliferating cells in tumor formation. Since ~95% of the epidermal cells in telogen skin are quiescent, this question can be easily addressed *in vivo* [74]. One study reported that 7,12-dimethylbenz[*a*]anthracene (DMBA) induces 3-fold (males) and 13-fold (females) more skin tumors when the skin of the mice is treated (initiation only) in the resting (telogen) phase rather than the proliferative (anagen) phase [83]. In another study, mouse skin was treated with 5-fluorouracil either 1 d before or 1 d after treatment with DMBA [84]. In that study, 5-fluorouracil (stops DNA replication and selectively kills cycling cells) only marginally reduced the yield of benign tumors (papillomas) and did not reduce the yield of carcinomas. This result suggests that following DMBA treatment, either the non-proliferating cells acquire the oncogenic mutations, or DNA replication/cell proliferation in the first couple of days is not critical for tumor formation. If the first idea is correct, DMBA-treated quiescent skin cells would be expected to have mutations induced by erroneous repair. In a third study, DMBA did not show a relationship between stable DNA adducts and tumor latency [85]. Our studies with DB[*a,l*]P, which forms predominantly depurinating DNA adducts [23,24], suggest that quiescent skin cells form preneoplastic mutations by error-prone BER [39,43,44]. DMBA also forms predominantly depurinating DNA adducts [21]; therefore, it would be expected to form preneoplastic mutations by error-prone repair.

Biological evidence for the role of proliferating cells in tumorigenesis was obtained from a study with BPDE in the regenerating rat liver model [48]. BPDE forms predominantly stable DNA adducts [22,86] and it formed the most number of tumors when early S-phase liver cells were exposed [48]. This result is consistent with studies suggesting that NER of the BPDE-induced stable adducts is error-free [45,54], and that mutations are induced in the proliferating cells by errors in translesion synthesis. Taken together, it appears that the PAH or their metabolites that form predominantly depurinating adducts may form preneoplastic mutations by error-prone repair in quiescent cells, whereas the PAH that form mainly stable adducts would induce mutations by errors in translesion synthesis in proliferating cells. PAH such as BP, which forms significant amounts of both types of adducts [22,86], appear to induce mutations from both stable and depurinating adducts. For example, the relative proportions of BP-induced depurinating adducts show a strong correlation with the relative abundance of Ade- or Gua-specific oncogenic H-*ras* mutations in skin tumors [16]. In addition, BP-induced stable

DNA adducts do not show a linear relationship with tumor yield [75,87]. On the other hand, deficiencies in some NER genes increase BP-induced mutagenesis and tumor formation [88–90].

In view of the complexities in mutagenesis by PAH, we have conducted a comprehensive study to examine PAH-induced mutations in preneoplastic mouse skin and in papillomas, both to examine the mutations and their relationship with DNA adducts, and to obtain insights into the mutagenic mechanisms.

2. Materials and Methods

2.1. Chemicals

The PAH and their derivatives (BP, BPDHD, *anti*-BPDE, DMBA, DB[*a,l*]P and *anti*-DB[*a,l*]PDE) were either purchased from the Chemical Carcinogen Repository of the National Cancer Institute or synthesized in our laboratory as described previously [91,92]. *These PAH are hazardous and handled according to NIH guidelines* [93].

2.2. Treatment of SENCAR mice and harvesting preneoplastic skin

Eight wk-old female SENCAR mice (National Cancer Institute-Frederick Cancer Center, Frederick, MD) were treated once on an area of shaved dorsal skin with 100 μL acetone (solvent) or a PAH in 100 μL acetone (initiation) at doses indicated in Table 1. Animals (3 mice/treatment group) were sacrificed 24 h later by asphyxiation under CO_2, and the treated area of dorsal skin harvested for the analysis of preneoplastic mutations. In addition, samples of unexposed ventral skin were also harvested from these mice to verify the absence of pre-existing H-*ras* mutations. Harvested skin samples were flash-frozen in liquid N_2, wrapped in aluminum foil and stored at −80 °C until use.

2.3. Papillomas

Tumors induced by the initiation-promotion protocol in previously described experiments and stored frozen at −80 °C [16,91,94] were analyzed for mutations. To obtain these tumors the PAH-treated mice were promoted by twice weekly doses of 3 nmol 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) in 100 μL acetone, until papilloma induction was complete (7–9 wks).

2.4 Analysis of H-ras mutations

Chromosomal DNA from the papillomas and preneoplastic skin samples was isolated as previously described [16,43,44]. For analysis of the clonal H-*ras* mutations in the papillomas, a 550 bp-long segment spanning the exon 1-2 region was PCR amplified with primers MRF and MRR and the product directly sequenced as previously described [16]. Direct sequencing of PCR products from tumors detects mutations that exist in at least 10% of the DNA molecules [95]. This approach provides adequate sensitivity, since clonally expanded oncogenic H-*ras* mutations are typically present in 14–47 % of cells in PAH-induced papillomas [43]. Linear amplification reactions for sequencing were conducted with dye-deoxy nucleotides and previously described primers [16] and the reaction products were resolved in a Beckman/Coulter CEQ2000XL 8-capillary DNA sequencer (Genomics Core Research Facility, University of Nebraska, Lincoln). When a clonally-enriched H-*ras* mutation was present in papilloma DNA, the sequencing electropherogram showed an overlapped peak corresponding to the mutant and wild-type bases.

To examine the induction of preneoplastic mutations in PAH-treated mouse skin, the same H-*ras* gene exon 1-2 segment was PCR amplified with primers MFPS and MRER from skin DNA as previously described [44,96]. MRER (contains an *Eco*RI site) is an altered MRR, and MFPS (contains a *Pst*I site) is an altered MRF, and they were useful for restriction digesting the PCR

products and direct cloning into the *EcoR*I/*Pst*I sites of pUC 18 to form recombinant plasmids, which were then transformed into *E.coli* Top10 cells (Invitrogen Life Technologies, Carlsbad, CA). Single colonies carrying the recombinant plasmids were identified on the basis of the IPTG/X-gal blue-white colony color assay, grown in small cultures, re-verified for recombinant plasmid content by dot-blot experiments conducted with a non-radioactively-labeled H-*ras* PCR product as probe (ECL direct labeling and detecting system, Amersham Pharmacia, Piscataway, NJ), and a ZetaProbe membrane (Biorad Laboratories, Hercules, CA). Cultures grown from ligation experiments that resulted in 80% or greater cloning efficiency were used to isolate recombinant plasmids and sequence the H-*ras* inserts. Sequencing reactions were conducted with M13 forward and reverse primers.

In addition to preneoplastic skin, two DB[*a*,*l*]P-induced papillomas (A43 and B32) were examined by the PCR-cloning-sequencing procedure. This procedure can detect mutations present in lower frequency than direct sequencing. For example, the detection limit is 2% when one mutation is scored in 50 recombinant plasmids. Mutation frequency was calculated by dividing the number of mutations by the product of the number of plasmids and the length of the sequenced region, as described previously [44,96,97].

2.5 T.G-DNA glycosylase assay for the detection of heteroduplex mutations induced by repair errors

Errors in excision repair would form heteroduplex mutations. For example, A.T to G.C mutations would be formed either as G.T heteroduplexes (substitution of A with G across T) or as A.C heteroduplexes (substitution of T with C across A). Since PAH react primarily with purines, repair will excise the purine lesions and resynthesize the gap. Thus, A.T to G.C mutations would really be A to G mutations, *i.e*., G.T heteroduplexes. To detect G.T heteroduplexes in skin, we employed the T.G-DNA glycosylase (TDG) assay [44,96,97]. TDG removes T residues from G.T heteroduplexes to form G.AP sites [44,96]. We used the *Methanobacterium thermoautotrophicum* TDG (Trevigen, Gaithersburg, MD), which removes T residues from G.T heteroduplexes, and G residues from G.G duplexes (G.C to C.G mutations, formed by incorporation of G in a C-gap), but has no activity on A.C heteroduplexes.

After TDG treatment, skin DNA is re-purified by phenol:chloroform extraction and ethanol precipitation to remove any remaining enzyme and subjected to mutation analysis by the PCR-cloning-sequencing procedure. Abasic site-containing DNA is refractory to PCR [44,98]. By generating abasic sites, TDG renders G.T and G.G heteroduplex-containing DNA refractory to PCR, which would result in a drastic reduction in the proportion of A.T to G.C (and G.C to C.G) mutations, if present. The reduction of A.T to G.C mutations is taken as evidence for the presence of G.T heteroduplexes. In DB[*a*,*l*]P-treated mouse skin, the effect of TDG on A.T to G.C mutations is only observed until 1 d (the repair period) [39]. In the post-repair period, DNA replication converts the G.T heteroduplexes into A.T and G.C pairs in daughter DNA molecules, and TDG does not alter the frequency of A.T to G.C mutations [44,96,97]. Similarly, TDG does not reduce mutations generated *in vitro* by PCR over bulky adducted bases [44, 96,97].

2.6 Statistical analysis

Mutation frequencies were compared using Fisher’s Exact test or a Chi-square test for linear association. These tests assume that each mutation observed is an independent event. This test has been used previously for similar analyses [97,99].

3. Results and Discussion

3.1. Mutagenesis by BP and metabolites

3.1.1 Papillomas—An earlier study reported that relatively few (23%, 3 out of 13) BP-induced SENCAR mouse skin papillomas contained oncogenic H-*ras* mutations (one GGC^{Gly} to CGC^{Arg} and two GGC^{Gly} to GTC^{Val} at codon 13) [15]. In contrast, similar experiments in the CD-1 mouse showed a high percentage (90%, 9 out of 10) of skin papillomas carry oncogenic H-*ras* mutations, and a greater variety of mutations (two GGA^{Gly} to GTA^{Val} at codon 12, five GGC to GTC at codon 13 and two CAA^{Gln} to CTA^{Leu} at codon 61) [14]. Our previous study in SENCAR mice also showed that a high percentage (69 %, 9 out of 13) of BP-induced skin papillomas carry oncogenic H-*ras* mutations (seven GGC to GTC at codon 13 and two CAA to CTA at codon 61) [16]. In mouse skin, BP forms 2.5-fold more depurinating (34% BP-6-C8G, 10% BP-6-N7G, 2% BPDE-10-N7G, 22% BP-6-N7A, 3% BPDE-10-N7A) than stable DNA adducts (23% BPDE-10-N^2dG, and 6% other adducts, including small amounts of BPDE-10-N^6dA) [22,86]. The distribution of clonal H-*ras* mutations (which, due to their abundance, are detectable by direct sequencing, see Materials and Methods, section 2.4) in the papillomas correlated better with BP-induced depurinating adducts (54% codon 13 G to T mutations with 46% G-depurinating adducts, and 15% codon 61 A to T mutations with 25% A-depurinating adducts) than with the stable adducts. To make a similar correlation with stable adducts, the BPDE-N^6dA stable adducts should be highly mutagenic and account for the codon 61 A to T mutations. However, the BPDE-N^6dA adduct does not appear to be exceptionally mutagenic [82] and forms A to G mutations in codon 61 [69].

To re-examine the correlation between BP-DNA adducts and mutations, and to determine whether other types of oncogenic H-*ras* mutations can be found in BP-induced papillomas, we have analyzed nine additional tumors, bringing the total to 22 (Table 1). Taken together, eleven papillomas (50%) showed the codon 13 GGC to GTC mutation and five papillomas (23%) showed the codon 61 CAA to CTA mutation. This result strengthened the relationship between the clonally-enriched oncogenic mutations in papillomas and the depurinating adducts.

To our knowledge, there are no reports of mutation analysis in BPDE-induced skin papillomas. However, one study reported that *anti*-BPDE forms the H-*ras* codon 12 GGC to GTC mutation in NIH3T3 cells to induce anchorage independence [100]. The solitary BPDE-induced papilloma obtained in our study contained the H-*ras* codon 13 GGC to GTC mutation (Table 1). In mouse skin, *anti*-BPDE forms 100-fold more stable (98% BPDE-10-N^2dG and 0.6% other) than depurinating adducts (0.1% BPDE-10-N7G, 1.2% BPDE-10-N7A) [22,86]. Studies have shown that the BPDE-N^2dG stable adduct mainly forms G to T mutations [49,82,101]. Thus, *anti*-BPDE would be expected to form the codon 13 G to T mutation in papillomas. We did not have enough tumors for this study; therefore, this result should be considered anecdotal.

To obtain greater insight into the role of the diolepoxide-DNA adducts in inducing oncogenic mutations, we examined twelve papillomas induced by the immediate precursor of BPDE, BP-7,8-dihydro-9,10-diol (BPDHD). BPDHD forms more stable adducts (61% BPDE-10-N^2dG, 2% unidentified) than BP and a reverse profile of depurinating adducts (25% BPDE-10-N7A, 12% BPDE-10-N7G) compared to BP (25% Ade- and 46% Gua-depurinating adducts) (Table 1) [22]. Two of the BPDHD-induced papillomas contained the H-*ras* codon 13 GGC to GTC mutation, while another four contained the codon 61 CAA to CTA mutation. The relative proportions of the codon 13 G to T (17%) and codon 61 A to T (33%) mutations in BPDHD-induced papillomas made a strong correlation with depurinating adducts. The relative proportions of the H-*ras* mutations in BP- and BPDHD-induced papillomas followed those of the depurinating adducts, even though these carcinogens induce opposite profiles of these adducts. In contrast, the abundant N^2dG stable adduct did not correlate with the relatively few

codon 13 mutations. These results suggest that the depurinating adducts may make a greater contribution than the stable adducts in inducing the oncogenic H-*ras* mutations. However, these correlations do not exclude the possible role of the BPDE-N^2dG stable adducts in inducing the codon 13 GGC to GTC mutation.

3.1.2 Preneoplastic mutations—Forward mutation assays in the *Hprt* and *Aprt* genes in Chinese hamster cells showed that BPDE induces 65–75% substitution and 26–36% deletion mutations [102–105]. Among the substitution mutations, G.C to T.A/C.G/A.T mutations were abundant (79–86%); however, significant numbers of A.T to G.C/T.A/C.G mutations (14–21%) were also observed. As the BPDE dose was lowered, the relative proportions of the A.T mutations increased [102,106,107]. Both BP and BPDE induced essentially similar spectra of *Aprt* mutations in Chinese hamster ovary cells [108]. This similarity was cited as evidence that BPDE is the chief mutagenic metabolite of BP. However, this similarity has not been explored in reporter gene assays, which score more PAH-induced mutations than other forward mutation assays that score mutations based on survival [109] and may show a somewhat different mutational spectrum. For example, analysis of transfected *SupF* plasmid in monkey cells [108,110] and a transgenic *lacI* in the skin of BB mice [111] shows that BPDE induces essentially a similar pattern of G.C-specific mutations as in the survival-based forward mutation assays, but far fewer (0–7%) A.T-specific mutations. Information about the mutagenic contributions of BP-DNA adducts in preneoplastic mouse skin has been lacking.

To examine the preneoplastic mutations in mouse skin, we have amplified the H-*ras* exon 1-2 region with a high fidelity mixture of DNA polymerases (8:1 combination of Tth and Vent DNA polymerases) [43,44,96,97]. Previous studies showed that this enzyme cocktail induces occasional misincorporations over untreated DNA ($5.5–6.8 \times 10^{-5}$) [43,44,96,97]. To determine PCR-induced spontaneous errors *in vitro*, a plasmid clone (pWT) containing the mouse H-*ras* exon 1-2 segment was PCR amplified, cloned and sequenced (Fig. 1). No mutations in 32 plasmids were observed (mutation frequency <3% of clones or $<5.7 \times 10^{-5}$ sequenced bases).

In addition to spontaneous misincorporations, unrepaired PAH-DNA stable adducts and abasic sites [42] in DNA can induce base incorporation errors during PCR. PCR-induced misincorporations over unrepaired lesions may make it difficult to identify the mutations formed by the cell. To test the nature of PCR-induced misincorporations over depurinated or stably-adducted DNA, pWT was reacted *in vitro* with a depurination buffer (10 mM NaCl, 10 mM sodium citrate, 10 mM Na_3PO_4, pH 2.0) [44] or with *anti*-BPDE (1 μg in 10 μL acetonitrile to 4 μg pWT for 15 min). As observed previously [44], PCR amplification of the depurinated pWT resulted in 0 mutations in 33 plasmids (mutation frequency <3%, $<5.5 \times 10^{-5}$). The lack of mutations from the depurinated DNA could be related to the refractory nature of abasic site-containing DNA to PCR amplification [44,98]. On the other hand, *anti*-BPDE-treated pWT yielded nine mutations in 42 plasmids (mutation frequency = 21.4%, 38.9×10^{-5}), all of which were G.C to T.A transversions. This result was expected because *anti*-BPDE forms principally (98%) the N^2dG stable adduct, which is known to induce these mutations [49]. Therefore, under our experimental conditions, if skin DNA treated with BP or its metabolites contained unrepaired *anti*-BPDE-stable DNA adducts, chiefly G.C to T.A misincorporations would be introduced by the PCR polymerases (8:1 mixture of *Tth* and *Vent* DNA polymerases).

Next, we examined whether acetone (used as solvent for the PAH treatments) induces any mutations in mouse skin DNA (Fig. 1). Analysis of 35 plasmids from acetone (100 μL)-treated DNA showed one A to G mutation (mutation frequency = 2.8% of the clones or, 5.2×10^{-5} of sequenced bases) (Table 2). This result indicates that acetone may form a very low frequency of A.T to G.C mutations in mouse skin.

Treatment of mouse skin with BP resulted in five mutations in 71 plasmids (mutation frequency = 7%, 12.8×10^{-5}) (Table 2), four of which were G.C to T.A transversions and one was an A.T to G.C transition (Fig. 1). Similar treatment with BPDHD induced five mutations in 32 plasmids (mutation frequency = 15.6%, 28.4×10^{-5}) (Table 2), all of which were G.C to T.A transversions. *Anti*-BPDE induced nine mutations in 42 plasmids (21.4%, 38.9×10^{-5}) (Table 2), six of which were G.C to T.A transversions, and one each of G.C to A.T, A.T to G.C and A.T to T.A mutations (Fig. 1).

The increasing mutagenesis by BP < BPDHD < *anti*-BPDE (7% *vs*. 15.6% *vs*. 21.4%, p = 0.026) in mouse skin supports the idea that BP metabolism is an important factor for generating these preneoplastic mutations. None of these three compounds showed either the codon 13 (GGC to GTC) or the codon 61 (CAA to CTA) transforming mutation at 1 d. However, these mutations are found in the papillomas, which suggests that BP and its metabolites induce the transforming mutations in preneoplastic skin. Under our experimental conditions, G.C to T.A mutations were most abundant in the mouse skin spectra induced by BP, BPDHD or *anti*-BPDE. These mutations are similar to that formed by PCR bypass errors over BPDE-N^2dG adducts (Fig. 1). Therefore, the G.C to T.A mutations cannot be readily identified as 'real' (physiologically formed) mutations. On the other hand, skin mutations other than G.C to T.A transversions (G.C to A.T, A.T to G.C and A.T to T.A mutations) could be physiological. Since we have examined preneoplastic mutations in the pre-replication repair period [33–39], these results raise the possibility that excision repair errors in the skin cells contributed to generating some of these mutations.

In summary, we found a strong relationship between depurinating adducts and clonally-expanded papilloma mutations. We are unable to make a correlation between preneoplastic mutations and BP-DNA adducts, because of the difficulties in identifying the abundant G.C to T.A mutations by origin (physiological *vs*. PCR bypass misincorporations). A meaningful relationship should consider only the physiological mutations. The clonally-expanded, oncogenic mutations in the papillomas are physiological, because these mutations are needed to establish the tumors and therefore cannot be PCR artifacts.

3.2. Mutagenesis by DB[a,l]P

3.2.1 Papillomas—We have previously reported that four out of five DB[*a,l*]P-induced papillomas contained the clonal H-*ras* codon 61 (CAA to CTA) mutation, and the fifth (A43) contained an uncharacterized codon 61 clonal mutation [16]. To identify the clonal mutation in A43, we amplified its H-*ras* PCR product, cloned it in pUC18, and sequenced recombinant plasmids from individual colonies (Fig. 2). This papilloma contained the clonal CAA to CTT mutation. Both CTA and CTT code for Leu. In addition, A43 contained a variety of non-clonal mutations. These were mostly transitions (eight A.T→G.C and threeG.C→A.T out of twelve point mutations). In addition, there were two G-deletions (ΔG^{28}, ΔG^{29}). To examine whether similar non-clonal mutations can be found in other papillomas, we examined another DB[*a,l*]P-induced papilloma (B32) (Fig. 2). This papilloma contained the CAA to CTA as its clonal H-*ras* mutation. B32 also showed a similar spectrum of non-clonal mutations (six A.T to G.C transitions in a total of eight point mutations, and a C^{276}-deletion). Except for one plasmid clone from B32 (which contained both the clonal CAA to CTA mutation and a non-clonal A^{431} to G mutation), all plasmid clones from these two papillomas contained only one mutation. The frequencies of clonal codon 61 mutations in the two papillomas [B32, 6 among 26 plasmids (23%) and A43, 11 in 26 plasmids (42%)] are consistent with frequencies of clonal mutations in other DB[*a,l*]P-induced papillomas (14–47%) previously determined by direct quantification [43]. Therefore, mutation frequencies calculated from the spectra match those obtained by direct quantification.

Our previous studies of mutations in DB[*a,l*]P-treated mouse skin suggested that most cells carrying the early mutations (12 h-4 d) are eliminated in the later preneoplastic period (5–7 d) [44,74]. Therefore, we investigated whether promoting treatments with phorbol ester (TPA) could be the origin of the non-clonal mutations in DB[*a,l*]P-induced papillomas. TPA is known to induce infiltration of neutrophils in mouse skin, which release reactive oxygen to form oxidative DNA damage [112]. We verified that 12 h after TPA treatment there was a significant increase in myeloperoxidase activity (a neutrophil marker) [113] and visible neutrophil infiltration (hematoxylin and eosin staining of skin sections) (results not shown). At this time point, TPA induced seven mutations in 48 clones, of which 50% were A.T to G.C mutations (Fig. 2). This result is consistent with the idea that some of the non-clonal mutations in the papillomas may have been generated by the chronic TPA treatment.

Tandem base mutations (GG to TT) in the K-*ras* gene have been reported in lung cancer [114]. Since PAH contribute to lung cancer, the observation of the tandem base mutation in H-*ras* codon 61 (CAA to CTT) in a DB[*a,l*]P-induced papilloma is interesting. To determine how frequently DB[*a,l*]P induces the tandem base codon 61 mutations, we analyzed seven additional DB[*a,l*]P-induced papillomas by direct sequencing of the H-*ras* PCR product (Table 1). Of these, six contained the H-*ras* codon 61 (CAA to CTA) clonal mutation, whereas the seventh tumor did not carry any clonal mutations in the exon 1-2 region. Taken together with previous results [16], it appears that DB[*a,l*]P induces the single-base codon 61 mutations at a much higher frequency (83%, ten out of twelve papillomas) than the tandem base mutation (8%, one out of twelve papillomas).

In mouse skin, DB[*a,l*]P forms 99% depurinating adducts (49% N3A, 14% N7A, 10% C8G and 2% N7G, generated by the DB[*a,l*]P radical cation, and 18% N7A, 6% N7G generated by DB[*a,l*]PDE) and 1% stable adducts (0.5% N^2dG, 0.45% N^6dA, generated by DB[*a,l*]PDE) [23,24]. Taken together, DB[*a,l*]P forms 81% Ade-depurinating adducts, 18% Gua-depurinating adducts and 1% stable (equal amounts of A and G) adducts. Thus, the H-*ras* codon 61 CAA to CTA/CTT mutations in DB[*a,l*]P-induced skin papillomas strongly correlated with the abundant A-depurinating adducts. It is worth noting that a different profile of mutations is found in the DB[*a,l*]P-induced adenomas in the A/J mouse lung. These adenomas carry a variety of K-*ras* mutations (44% carry codon 12 GGT to TGT/GTT/CGT mutations and 56% carry codon 61 CAA to CTA/CGA/CAT/CAC mutations) [115,116]. A correlation of these adenoma mutations with DB[*a,l*]P-induced stable adducts has been suggested [115,116]. To our knowledge, depurinating adduct formation by DB[*a,l*]P in the A/J mouse lung has not been studied.

3.2.2 Preneoplastic mutations—DB[*a,l*]P treatment of mouse skin resulted in 17 mutations in 50 plasmids (mutation frequency = 34%, 61.8×10^{-5}) (Fig. 1 & Table 2). Twelve of these mutations (70%) were A.T to G.C transitions. This result is similar to that obtained in a previous study [44]. The PCR-cloning-sequencing procedure cannot determine whether these mutations are A to G or T to C transitions. However, the T.G-DNA glycosylase (TDG) assay indicates that these mutations are A to G transitions, formed as G.T heteroduplexes (see Section 3.4). If so, the abundant A.T to G.C mutations can be readily correlated with the abundant Ade-depurinating adducts (81% of all adducts).

These mutations do not show a ready correlation with DB[*a,l*]P-induced stable adducts. Our studies show that DB[*a,l*]P forms its stable adducts through its diolepoxides (equal amounts of Ade and Gua adducts) [24]. We had previously observed that *anti*-DB[*a,l*]PDE treatment of mouse skin results in the formation of 53% A.T-specific (16 out of 30) and 47% G.C-specific (14 out of 30) mutations [44]. This spectrum of mutations is correlated with the relative proportions of stable adducts. Fourteen of these A.T-specific mutations were A.T to G.C transitions. Most of the DB[*a,l*]PDE-induced N7Ade-depurinating adducts are contributed by

syn-DB[*a,l*]PDE; the *anti*-isomer produces only 6% of the total. The TDG assay did not identify the *anti*-DB[*a,l*]PDE mutations as G.T heteroduplexes [44]. This is in contrast with mutagenesis by DB[*a,l*]P itself. These results differentiated the A.T to G.C mutations induced by DB[*a,l*]P from those induced by DB[*a,l*]PDE.

It is worth noting that depending on the cell type, DB[*a,l*]P or its metabolites may induce different adduct profiles. For example, in MCF-7 cells, DB[*a,l*]PDE forms predominantly Ade-specific stable adducts [117]. In Chinese hamster V79 cells, *anti*-DB[*a,l*]PDE forms both G.C-specific and A.T-specific mutations in the *Hprt* gene [99]. At lower *anti*-DB[*a,l*]PDE doses, A.T mutations predominated, whereas at higher doses, G.C mutations were more abundant. On the other hand, DB[*a,l*]PDE forms A.T to T.A > G.C to T.A mutations in the *cII* gene of Big Blue mouse cells [118].

3.3 Mutagenesis by DMBA

3.3.1 Papillomas—Like DB[*a,l*]P, DMBA forms mainly (99%) depurinating adducts (79% N7A and 20% N7G from the radical cation) and 1% stable adducts generated by DMBADE [21,61]. Even though the DMBADEs form mainly A-specific stable adducts [61,119], in mouse skin, DMBA itself forms only ~23% more A than G stable adducts [61].

A high percentage (90–100%) of mouse skin papillomas induced by DMBA contain the H-*ras* codon 61 CAA to CTA mutation [16,120,121]. DMBA can also induce other types of mutations in papillomas. For example, H-*ras* codon 61 CAA to CGA and CAA to CAT mutations have been reported in 4% and 2% of DMBA-induced papillomas [122]. Similar results were obtained with transgenic C57BL/6 mice expressing ornithine decarboxylase. The majority (50%) of DMBA-induced papillomas were found to contain the H-*ras* codon 61 CAA to CTA mutation, whereas 4% contained K-*ras* codon 12 GGT to TGT, and another 29% contained K-*ras* codon 61 CAA to CTA mutations [123]. Therefore, in all these studies, A-specific mutations predominated. All six DMBA-induced papillomas newly reported here contained the H-*ras* codon 61 CAA to CTA mutation (Table 1). In a previous study, four DMBA-induced papillomas were found to contain the H-*ras* codon 61 (CAA to CTA) mutation [16]. Taken together with our previous results, ten out of ten DMBA-induced papillomas contained this mutation.

Therefore, DMBA showed a strong stochastic relationship between the major Ade-depurinating adducts and the codon 61 A to T mutations in papillomas (Table 1). Tang *et al*. have suggested that these mutations originate from the stable adducts induced by DMBADE [61]. DMBADE induces 5–9-fold fewer papillomas than DMBA, but 94–96% of these papillomas also contain the codon 61 CAA to CTA mutation [61]. Even though DMBADE forms mainly A-specific stable adducts, its reaction with H-*ras* codon 12 GGA, codon 13 GGC and codon 61 CAA sequences has been demonstrated [124], which makes it difficult to explain why its papillomas only show A-specific mutations. DMBADE also forms depurinating adducts (N7A and N7G) [125], but how much of these adducts are formed in mouse skin is yet to be determined.

Although different mutations in the same oncogenic site are not equally expandable by phorbol ester (cells carrying the CAA to CTA mutation at codon 61 are favored over cells carrying the CAA to CGA or CAA to CAT mutations) [59], it is not very likely that the absence of codon 13 mutations among DMBA papillomas is a result of their inefficient clonal expansion. After all, H-*ras* codon 13 GGC to GTC mutations were found in a majority of BP-induced papillomas in the same species of mouse (Table 1). It is more likely that relatively few codon 13 GGC to GTC mutations are formed in DMBA-treated skin. To address these questions, we analyzed H-*ras* mutations in DMBA-treated preneoplastic skin.

3.3.2 Preneoplastic mutations—Analysis of the H-*ras* gene 1 d after DMBA treatment of SENCAR mouse skin also showed predominantly A.T to G.C mutations (Fig. 1). Of the 21 mutations, six were G.C-specific (two G.C to A.T and four G.C to T.A) and the remaining 15 were A.T-specific (fourteen A.T to G.C and one A.T to T.A). The overall mutation frequency was 53.8% or 97.9×10^{-5}. This result is consistent with earlier observations reporting preneoplastic mutation frequency ($\sim 10^{-4}$) in DMBA-treated mouse skin [58]. However, the DMBA mutation frequency was higher than that observed with DB[*a,l*]P, which is a stronger carcinogen than the former [94]. It is worth noting that at the chosen dose (200 nmol), DB [*a,l*]P shows significant skin toxicity, which makes it difficult to compare mutagenesis by these two PAH.

Earlier studies also reported that DMBA forms mainly A.T-specific mutations. For example, in DMBA-treated Big Blue rats, spleen lymphocytes and mammary glands show predominantly A.T to T.A (42–44%) and some G.C to T.A (11–24%) mutations in the *Hprt* and *lacI* genes [126,127]. Since in mouse skin, DMBA forms 23% more A than G stable adducts of DMBADE [61], in the absence of evidence that DMBADE-G stable adducts should show much weaker mutagenic properties compared to the DMBADE-A stable adducts, it would be difficult to correlate the abundant A.T to G.C mutations with DMBADE-A stable adducts. It is worth noting that DB[*a,l*]PDE also induces similar mutational spectra (A.T to T.A > G.C to T.A) in the *cII* gene of Big Blue mouse cells, and it is difficult to explain the minority of G mutations by the stable adducts, even after exploring factors such as differences in their removal by NER [118]. In contrast, there is better correlation between the BPDE-N^2dG stable adduct and the abundant G.C to A.T/T.A/C.G mutations observed in a shuttle vector assay in monkey cells or in the *lacI* gene of Big Blue mice [110,111]. Our observation that DMBA forms abundant amounts of A.T to G.C mutations in preneoplastic mouse skin (Fig. 1) does permit a straightforward correlation with the abundant A-depurinating adducts (Table 1).

3.4 DB[a,l]P and DMBA form mismatched heteroduplex mutations

DB[*a,l*]P and DMBA preneoplastic mutations were abundantly A.T to G.C transitions, and thus, were suitable for the T.G-DNA glycosylase (TDG) assay to determine whether these mutations are induced as G.T heteroduplexes [44,96,128]. In this assay, skin DNA is incubated with TDG, the DNA re-purified, and subjected to mutation analysis by the PCR-cloning-sequencing protocol. TDG converts G.T heteroduplexes to G.abasic sites. Since abasic site-containing DNA is refractory to PCR amplification, TDG treatment of G.T heteroduplex-containing DNA drastically reduces the frequency of A.T to G.C mutations in the spectra (*see* Section 2.5).

TDG treatment of *anti*-BPDE-treated pWT did not significantly reduce the frequency of mutations in the PCR misincorporation spectra ($p = 0.79$) [nine out 42 plasmids (21.4%, 38.9×10^{-5}) before and nine among 50 plasmids (18%, 32.7×10^{-5}) after TDG treatment] (Figs 1 and 3). Acetone-treated skin DNA showed a background frequency of mutations (one A to G mutation in 35 plasmids) (Fig. 1), whereas TDG treatment of the same DNA did not show any mutations (no mutations in 40 plasmids) (Fig. 3). These results suggest that TDG does not act on BPDE-induced stable adducts, or form new mutagenic lesions. In contrast, TDG treatment of DB[*a,l*]P- or DMBA-treated skin DNAs caused drastic reductions in the frequency of A.T to G.C mutations (Figs. 1 and 3). For example, DB[*a,l*]P formed 17 mutations in 50 plasmids (34%, including 12 A.T to G.C mutations), which were reduced by TDG treatment to five mutations in 35 plasmids (14.3%) ($p = 0.048$) (Table 2). Three of the five mutations in the DB [*a,l*]P-TDG spectrum were the oncogenic codon 61 CAA to CTA (nt 391) mutation (Fig. 3). The reduction of A.T to G.C mutations indicates that the A.T to G.C mutations are formed as G.T heteroduplexes, as would be expected if these mutations are formed by errors in repair. We think that the increased frequency of codon 61 mutations observed after TDG treatment is

due to PCR enrichment of mutations present in DNA that did not contain the A.T to G.C mutations. This result also confirms that DB[*a*,*l*]P forms the tumor-forming codon 61 oncogene mutations (Table 1) in the early preneoplastic repair period. Similarly, DMBA formed 21 mutations in 39 plasmids (53.8%, including 14 A.T to G.C mutations), which were largely eliminated by TDG treatment (2 insertions in 35 plasmids, 5.7%) ($p < 0.001$) (Table 2). Therefore, like DB[*a*,*l*]P, DMBA also forms G.T heteroduplex mutations in the early preneoplastic repair period. Papillomas formed by DMBA contain the codon 61 mutation (Table 1); however, these mutations were not detected in preneoplastic DMBA mutation spectra with or without TDG treatment. This result, however, does not exclude the possibility that DMBA also forms the codon 61 mutations in this period, but targets this codon at lower affinity than DB[*a*,*l*]P. This idea is worth consideration, because multiple studies have not produced evidence that overall mutagenic efficiency is a good indicator of tumor-forming ability. Indeed, we find that compared to BP, overall mutagenesis with equimolar amounts of *anti*-BPDE- or BPDHD-treated skin was greater, which is contrary to their tumor-forming activities.

3.5 Sequence contexts of the A.T to G.C mutations

If the abundant A.T to G.C mutations are formed by errors in BER of abasic sites generated by the de-adenylation of the appropriate depurinating adducts, they should correspond to gap-filling error preferences of BER polymerases. As noted in Section 1, this mutational specificity is consistent with the error preferences of DNA polymerases β and ι [66,77]. If so, analysis of the sequence contexts of these mutations may provide further characteristics of the sequence selectivity for inducing this type of mutation.

Alignment of the sequences of the DMBA- or DB[*a*,*l*]P-induced preneoplastic A.T to G.C mutations revealed that they frequently occurred at putatively conserved sequences (Fig. 4). In both cases, these mutations frequently occurred at adenines flanked by doublets at 5′ or 3′, or both. The sequence preference can be dictated at three levels. First, they reflect the preferences of PAH intercalation [129]. Second, they should be among the hotspots of PAH-DNA reaction [25–32]. Third, they should be among the sequences in which BER polymerases make frequent errors [66,77].

Previous studies with DMBA showed the formation of abundant amounts of A.T-specific mutations, but they were chiefly A.T to T.A transversions [126,127]. These mutations may arise by a different mechanism, as they frequently occurred at 5′CA sites [126]. If these mutations are also generated from PAH-induced abasic sites, bypass errors over unrepaired abasic sites could be a possible mechanism, especially since it would satisfy the A-rule [130, 131]. In our studies, however, A.T to T.A mutations were rare in DMBA- or DB[*a*,*l*]P-treated preneoplastic skin. It would be interesting to examine whether these differences in mutagenesis are a reflection of gene-specific differences in DNA damage repair, such as those known among active and inactive genes in G1 and G2 phase cells [132].

4. Conclusions

Our previous study with DB[*a*,*l*]P was the only one in which preneoplastic skin and papilloma mutations were correlated with DNA adducts [44]. That study showed that depurinating adducts exhibit a strong stochastic relationship with both skin and papilloma mutations in the initiating oncogene. The current study expands on those findings. The results suggest that depurinating adducts induced by DMBA and DB[*a*,*l*]P play a major role in generating the H-*ras* mutations that lead to tumors. These results also suggest that the mutations are generated by errors in the repair of the abasic sites generated by the spontaneous dissociation of the depurinating adducts from DNA. Available biological evidence about the role of quiescent cells in DMBA tumorigenesis supports this idea. Although a strong relationship between BP-

depurinating adducts and H-*ras* mutations in papillomas was found, we were unable to validate this relationship in preneoplastic skin, because the mutation analysis protocol used in this study cannot identify the abundant G.C to T.A mutations as either PCR misincorporation artifacts over BP-stable adducts or as 'real' mutations generated physiologically. Nevertheless, the current study highlights the value of studying PAH-induced mutations in both preneoplastic and tumor tissue. We propose that the role of DNA adducts in forming the mutations that lead to tumors can be described with greater certainty when adducts can be correlated with mutations in both types of studies.

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Abbreviations

BP
: benzo[*a*]pyrene

BPDHD
: benzo[*a*]pyrene-7,8-dihydrodiol

***anti*-BPDE**
: (±)-*anti*- benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide

DB[*a,l*]P
: dibenzo[*a,l*]pyrene

DMBA
: 7,12-dimethylbenz[*a*]anthracene

PAH
: polycyclic aromatic hydrocarbons

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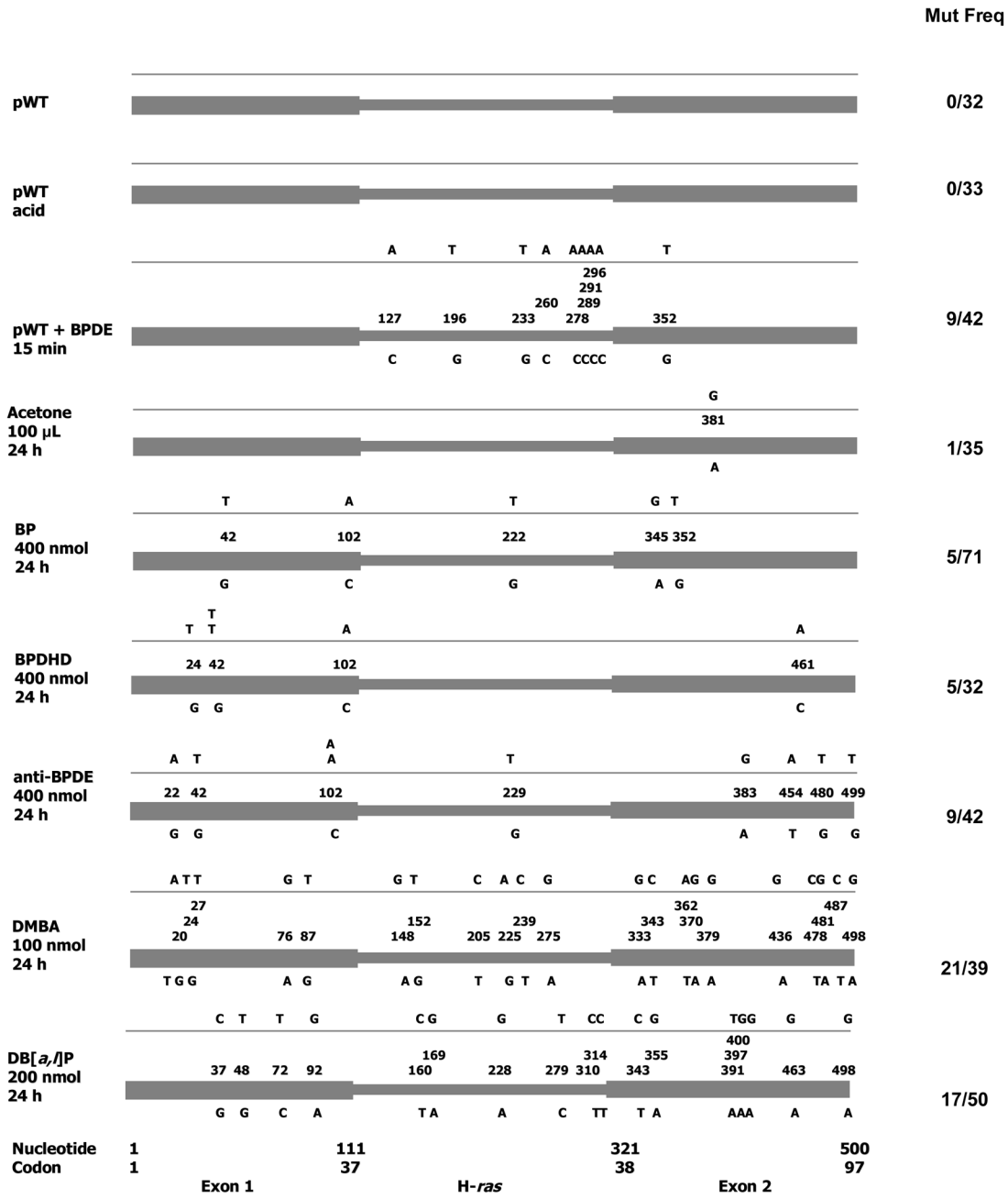

Figure 1. H-*ras* mutations induced in mouse skin 24 h after treatment with various PAH

The nucleotide numbers correspond to GenBank U89950. The cartoons depict the location of the mutations in the H-*ras* gene, the wild-type bases are shown underneath and the mutations above. *In vitro control*: pWT, pWT + acid, pWT (4 µg) + *anti*-BPDE (1 µg in acetonitrile). *Experimental*: acetone, BP, BPDHD, *anti*-BPDE, DMBA, DB[*a*,*l*]P.

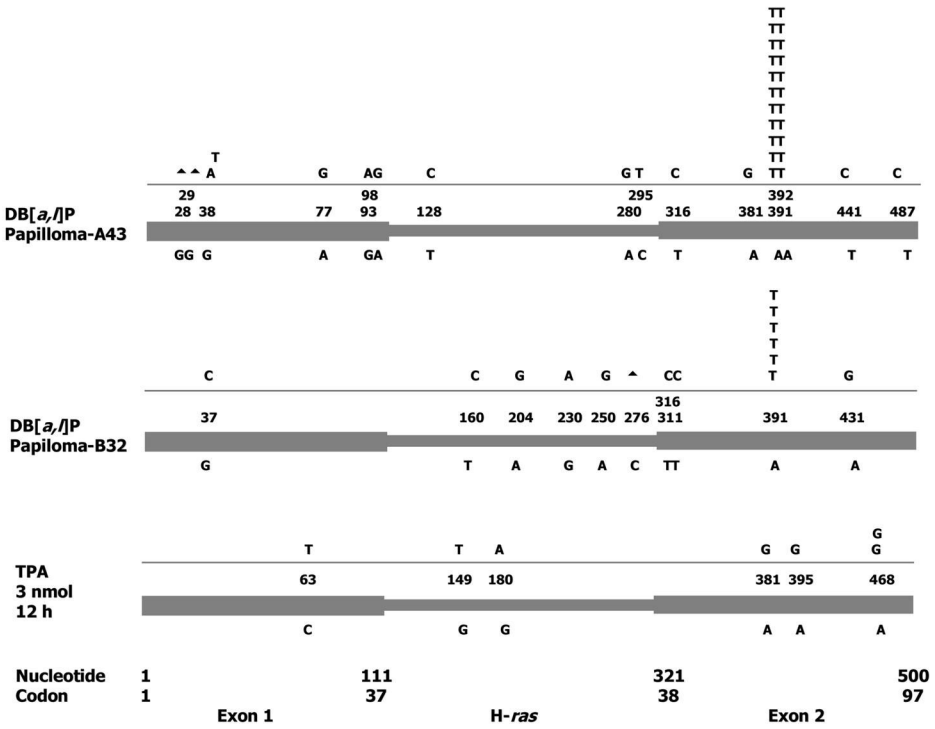

Figure 2. H-*ras* mutations in mouse skin papillomas induced by DB[*a,l*]P

The two papillomas (A43 and B32) were obtained by treating the dorsal skin of SENCAR mice with DB[*a*,*l*]P (200 nmol) and promoting with chronic twice-weekly treatments with 3 nmol phorbol ester. No. of mutations/Total No. of plasmids: 15/17 (B32) and 25/26 (A43). ▲, deletion. *Control*: A single treatment with TPA resulted in seven mutations in fortyeight clones (50% A.T to G.C transitions), suggesting that some of the mutations in the tumors may be due to the chronic TPA treatment.

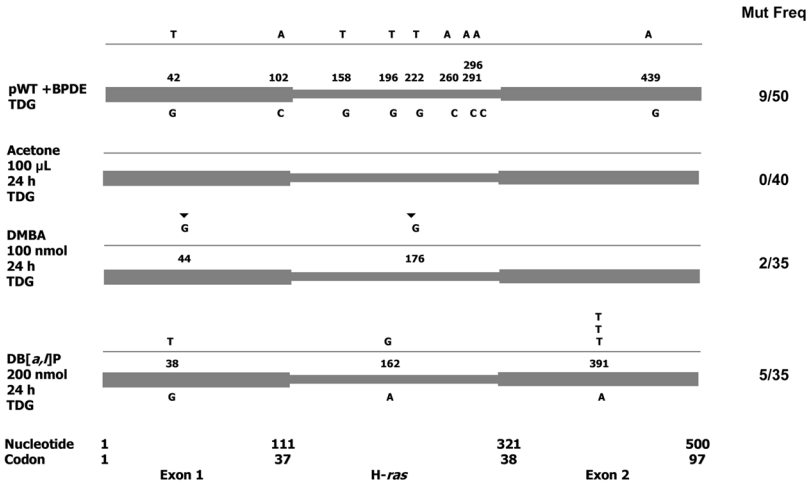

Figure 3. H-*ras* mutations after treatment of mouse skin DNA with T.G-DNA glycosylase

Incubation of PAH-treated skin DNA with this glycosylase (TDG) results in drastic reduction of A.T to G.C mutations present as G.T heteroduplexes. Mutations/total No. of plasmids: *Controls*: pWT + *anti*-BPDE + TDG, acetone-treated skin + TDG. *Experimental*: DMBA + TDG (2 ins/35), DB[*a*,*l*]P + TDG (5/35). ▼, insertion.

Figure 4. Sequence contexts of PAH-induced A.T to G.C mutations

Alignment of the sequences that showed A.T to G.C mutations in mouse skin treated with DMBA or DB[*a,l*]P. Some of the mutations were scored as A to G and others as T to C, the latter are also presented as A to G (italicized). The results show a frequent presence of doublet sequences flanking the mutated Adenines (underlined) either on the 5′, 3′ or both sides.

Table 1

Summary of PAH-DNA adducts and H-*ras* mutations in papillomas induced by PAH initiation and TPA promotion and in PAH-treated preneoplastic skin

PAH (nmol)	Papillomas/ Mouse[a]	H-*ras* mutations in papillomas			DNA adducts in skin (4h after PAH treatment) [c]		Preneoplastic H-*ras* mutations (24 h after PAH treatment)		
		Codon	Sequence	No./ total papillomas*	Stable (% total)	Depur. (% total)	Mutation type	No.of mutations/ total mutations/ total clones*	Distribution
BP (400)	82/24	13 61	GGC to GTC CAA to CTA	11/22[b] 5/22[b]	G (23) Other (6)	G (46) A (25)	G.C to T.A A.T to G.C	4/5/71 1/5/71	G.C (80%) A.T (20%)
BPDHD (400)	62/28	13 61	GGC to GTC CAA to CTA	2/12 4/12	G (61) Other (2)	G (12) A (25)	G.C to T.A	5/5/32 -	G.C (100%)
Anti-BPDE (400)	1/27	13	GGC to GTC	1/1	G (98) Other (0.6)	G (0.1) A (1.2)	G.C to A.T G.C to T.A A.T to G.C A.T to T.A	1/9/42 6/9/42 1/9/42 1/9/42	G.C (78%) A.T (22%)
DMBA (100)	397/24	61	CAA to CTA	10/10[b]	Other (0.9)	G (20) A (79)	G.C to A.T G.C to T.A A.T to G.C A.T to T.A	2/21/39 4/21/39 14/21/39 1/21/39	G.C (6/21) A.T (15/21)
DB[*a,l*]P (200)	129/30	61 61	CAA to CTA CAA to CTT	10/12[b] 1/12[b]	G (0.5) A (0.5)	G (18) A (81)	G.C to A.T G.C to T.A G.C to C.G A.T to G.C A.T to T.A	2/17/50 1/17/50 1/17/50 12/17/50 1/17/50	G.C (4/17) A.T (13/17)

[a] papillomas from previous experiments. The PAH doses are outside of their linear ranges, therefore, the papilloma yields do not reflect their tumorigenic potential. For comparative tumorigenicity, see [91,94].

* new results,

[b] combined with previous results [16].

[c] adduct data from [23,24,134]. The preneoplastic mutation spectra are generated by combining mutations from skin samples of two animals of the experiment.

Table 2

Preneoplastic mutation frequencies in PAH-treated mouse skin

Treatment		Mutation frequency	
		Total mutations/Total no. of plasmids (%)	Mutations/Sequenced bases
Acetone (solvent)	100 μL	2.8	5.2×10^{-5}
Acetone + TDG		0	0
BP	400 nmol	7	12.8×10^{-5}
BPDHD	400 nmol	15.6	28.4×10^{-5}
Anti-BPDE	400 nmol	21.4	38.8×10^{-5}
DMBA	100 nmol	53.8	97.9×10^{-5}
DMBA + TDG		5.7	10.3×10^{-5}
DB[*a*,*l*]P	200 nmol	34	61.8×10^{-5}
DB[*a*,*l*]P + TDG		14.3	25.9×10^{-5}