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Relating aromatic hydrocarbon-induced DNA adducts and c-H-*ras* mutations in mouse skin papillomas: The role of apurinic sites

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ABSTRACT **Mouse skin tumors contain activated c-H-*ras* oncogenes, often caused by point mutations at codons 12 and 13 in exon 1 and codons 59 and 61 in exon 2. Mutagenesis by the noncoding apurinic sites can produce G → T and A → T transversions by DNA misreplication with more frequent insertion of deoxyadenosine opposite the apurinic site. Papillomas were induced in mouse skin by several aromatic hydrocarbons, and mutations in the c-H-*ras* gene were determined to elucidate the relationship among DNA adducts, apurinic sites, and *ras* oncogene mutations. Dibenzo[*a,l*]pyrene (DB[*a,l*]P), DB[*a,l*]P-11,12-dihydrodiol, *anti*-DB[*a,l*]P-11,12-diol-13,14-epoxide, DB[*a,l*]P-8,9-dihydrodiol, 7,12-dimethylbenz[*a*]anthracene (DMBA), and 1,2,3,4-tetrahydro-DMBA consistently induced a CAA → CTA mutation in codon 61 of the c-H-*ras* oncogene. Benzo[*a*]pyrene induced a GGC → GTC mutation in codon 13 in 54% of tumors and a CAA → CTA mutation in codon 61 in 15%. The pattern of mutations induced by each hydrocarbon correlated with its profile of DNA adducts. For example, both DB[*a,l*]P and DMBA primarily form DNA adducts at the N-3 and/or N-7 of deoxyadenosine that are lost from the DNA by depurination, generating apurinic sites. Thus, these results support the hypothesis that misreplication of unrepaired apurinic sites generated by loss of hydrocarbon-DNA adducts is responsible for transforming mutations leading to papillomas in mouse skin.**

The *ras* family of oncogenes includes the closely related H-, Kirsten-, and N-*ras* genes. Activated *ras* oncogenes are involved in tumor initiation (1–4). Many mouse skin tumors have activated H-*ras* oncogenes (3). Activation is frequently caused by point mutations at one of several specific positions, including codons 12 and 13 in exon 1 and codons 59 and 61 in exon 2 of the H-*ras* gene (3).

If these mutations are induced by carcinogen-DNA adducts, the mutations observed in tumors should correlate with the DNA-binding characteristics of the carcinogen. Mutations can arise from both miscoding and noncoding lesions in DNA (5). A miscoding lesion is found in rat mammary carcinomas induced by nitrosomethylurea (1, 4, 6) that contain an activated H-*ras* oncogene bearing a G → A transition at the second position of codon 12. This mutation is associated with O^6-methylguanine that mispairs with thymine during DNA replication to generate a G → A transition (6, 7). In contrast, tumors induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) show an A → T transversion at codon 61 of the H-*ras* gene (2, 8). This mutation may arise from depurination of a DMBA-DNA adduct, followed by misreplication across the unrepaired apurinic site (see below). Mutagenesis by noncoding lesions produces G → T and A → T transversions by DNA misreplication across apurinic sites with most frequent insertion of deoxyadenosine opposite the lesion (9, 10).

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Carcinogen-DNA adducts can be divided into two categories based on their retention in DNA. "Stable" adducts remain intact in DNA, whereas "depurinating" adducts are released from DNA by hydrolysis of the bond between the purine base and deoxyribose. Adducts such as benzo[*a*]pyrene (BP) diol epoxide bound to the 2-amino group of deoxyguanosine are stable and found in DNA (11, 12). Adducts with polycyclic aromatic hydrocarbon(s) [(PAH)] bound to the N-7 of guanine or the N-3 and N-7 of adenine are depurinating adducts (11–14), which are released from DNA to generate apurinic sites.

PAH bind to DNA by two major mechanisms of activation: one-electron oxidation to form radical cations (15) and monooxygenation to form diol epoxides (16). The stable and depurinating adducts of three potent carcinogenic PAH (Fig. 1), BP (11, 12), DMBA (13, 14), and dibenzo[*a,l*]pyrene (DB[*a,l*]P) (17) have been analyzed. Both *in vitro* and in mouse skin, 99% of the DMBA-DNA adducts are depurinating adducts formed by one-electron oxidation, in which the 12-methyl group of DMBA reacts with the N-7 of adenine or guanine in a 4:1 ratio, respectively. Only a fraction of the 1% of stable adducts corresponds to diol epoxide products.

When DB[*a,l*]P is bound to DNA after activation by rat liver microsomes, six depurinating adducts are formed, constituting 84% of all detected adducts (17). The three major adducts (73%) contain DB[*a,l*]P (42%) bound at the N-3 or N-7 of adenine or its diol epoxide (31%) bound at the N-7 of adenine to form depurinating adducts.

For BP, ≈80% of the adducts *in vitro* or in mouse skin are depurinating adducts formed by one-electron oxidation, with binding of BP at C-6 to the N-7 of adenine or guanine or the C-8 of guanine (11, 12). The stable adduct BP diol epoxide bound at the 2-amino group of deoxyguanosine accounts for 22% of the adducts detected.

In this paper, c-H-*ras* oncogene mutations in mouse skin papillomas were examined to determine whether there is a relationship among DNA adducts, apurinic sites, and *ras* oncogene mutations. DB[*a,l*]P, DB[*a,l*]P-11,12-dihydrodiol, DB[*a,l*]P-8,9-dihydrodiol, and *anti*-DB[*a,l*]P diol epoxide (*anti*-DB[*a,l*]PDE) were studied because DB[*a,l*]P is the most potent carcinogenic PAH (18, 19) and these two dihydrodiols are major metabolites formed by rat liver microsomes (20). The 11,12-dihydrodiol is the proximate metabolite in the diol epoxide pathway and is almost as potent as DB[*a,l*]P (19). The 8,9-dihydrodiol is extremely weak (18). One of the putative ultimate carcinogens, *anti*-DB[*a,l*]PDE, is a less potent tumor initiator than DB[*a,l*]P or its 11,12-dihydrodiol (21).

The c-H-*ras* mutations in mouse skin papillomas induced by DMBA were compared to those in papillomas induced by 1,2,3,4-tetrahydro-DMBA (Fig. 1), a potent carcinogen that cannot be activated by the diol epoxide pathway (22). Finally,

Abbreviations: BA, benz[*a*]anthracene; BP, benzo[*a*]pyrene; DB[*a,l*]P, dibenzo[*a,l*]pyrene; *anti*-DB[*a,l*]PDE, *anti*-DB[*a,l*]P diol epoxide or (±)-*r*-11,*t*-12-dihydroxy-*t*-13,14-epoxy-11,12,13,14-tetrahydrodibenzo[*a,l*]pyrene; DMBA, 7,12-dimethylbenz[*a*]anthracene; PAH, polycyclic aromatic hydrocarbon(s).

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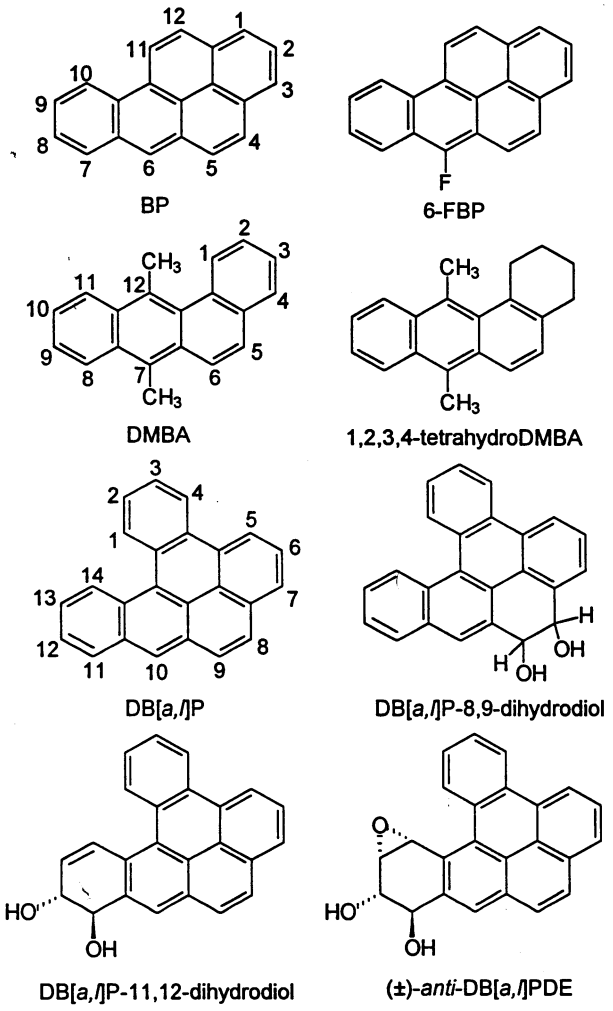

FIG. 1. Structures of PAH studied.

c-H-*ras* mutations in papillomas induced by BP or the weak carcinogen 6-fluoro-BP were studied because these two PAH produce similar profiles of stable adducts (23).

MATERIALS AND METHODS

PAH and Metabolites. BP, 6-fluoro-BP, DMBA, 1,2,3,4-tetrahydro-DMBA, and DB[*a,l*]P were available in our laboratory. DB[*a,l*]P-8,9-dihydrodiol was metabolically prepared (18, 20). DB[*a,l*]P-11,12-dihydrodiol and *anti*-DB[*a,l*]PDE were synthesized (21).

Tumor Induction in Mouse Skin. In initiation-promotion experiments, 8-week-old female SENCAR mice (National Cancer Institute–Frederick Cancer Center, Frederick, MD) were treated on the dorsal skin with the selected PAH (18, 21). Tumors were excised and stored at −80°C until extraction of DNA. Analyses of oncogene mutations were conducted on papillomas, each from a different animal, to determine oncogenic mutations associated with initiation of tumorigenesis.

Amplification and Sequencing of c-H-*ras* Exon 1 and 2 Region. The oligonucleotides used in PCR amplification and *Taq* DNA polymerase-mediated cycle sequencing with fluorescent dye-labeled dideoxynucleotides (DyeDeoxy Terminator; Applied Biosystems) had the following sequences: MRF, 20-mer (5′-CCGCTGTAGAAGCTATGACA) (nt -14–6); MRFD, 22-mer (5′-TGTAGAAGCTATGACAGAATAC) (nt -10–11); MRB, 22-mer (5′-AAGACCTGGGCTGTTTGGTCAT) (nt 183c–162c); MRI, 18-mer (5′-GGTCTGAGGAGAGGTGAG) (nt 256–273); MRR, 20-mer (5′-CCGCTGTAGAAGCTATGACA) (nt 532c–513c); 1251, 21-mer (5′-CACCTGTACTGATGGATGTCC) (nt 500c–480c). The numbers in parentheses correspond to the nucleotide numbers in Brown *et al.* (24); the c indicates the complementary strand. DNA sequences flanking the exon 1 and 2 region (24) were used in PCR amplification of a 546-bp region. High molecular weight chromosomal DNA was extracted from individual tumors by standard methods (25). PCR amplification was normally conducted on either 2 μg of undigested genomic DNA or 30 ng of *Eco*RI (BRL)-digested DNA by using MRF and MRR oligonucleotides. Some template preparations resulted in low yields. To obtain sufficient product in such cases, an additional seminested PCR was conducted with MRF and 1251 oligonucleotides on 1:1000 dilutions of the primary product. In all cases, cycling involved initial denaturation at 95°C for 10 min, followed by 45 cycles of a three-step amplification: a 90-s denaturation at 94°C, a 60-s annealing at 55°C, and a 180-s extension at 72°C. A final 30-min incubation at 72°C concluded the amplification process. The reactions were conducted in a Perkin–Elmer thermal cycler in a total volume of 20 μl, containing 2 pmol of each primer with 1× GeneAmp reaction buffer that contained 50 mM KCl, 10 mM Tris·HCl (pH 8.3), 1.5 mM $MgCl_2$, and 0.001% gelatin. The PCR products were fractionated in a low-melting-point agarose gel and the DNA was extracted by β-agarase digestion according to vendor (Gelase, Epicenter Technologies, Madison, WI).

The purified PCR products (100 ng) were cycle-sequenced with *Taq* DNA polymerase with 0.8 μM end primers (MRF, MRR, or 1251) or internal primers (MRFD, MRB, and MRI), by using an Applied Biosystems sequencing kit containing fluorescent-dye-labeled dideoxynucleotides and 1× TACS buffer [80 mM Tris·HCl, pH 9/2 mM $MgCl_2$/20 mM $(NH_4)_2SO_4$] in a total volume of 20 μl for 25 cycles (96°C, 30 s; 50°C, 15 s; 60°C, 4 min). The reaction products were electrophoresed for 12 h in a 6.5% polyacrylamide gel in 1× TBE buffer containing 7 M urea in an Applied Biosystems model 373A DNA sequencer. Data were interpreted with the ANALYSIS version 1.0.2 sequence analysis program (Applied Biosystems) in the Molecular Biology Core Facility at the Eppley Institute. Sequencing with MRFD for the region of codons 12 and 13 and MRI for the region of codons 59–61 gave the best results. Data from extension with the other primers were primarily used for sequence confirmation.

RESULTS

Mouse skin papillomas were harvested from several initiation-promotion experiments comparing the relative tumor-initiating activities of DB[*a,l*]P, 11,12-dihydrodiol, 8,9-dihydrodiol, and anti-DB[*a,l*]PDE, and mutations in exons 1 and 2 of the c-H-*ras* oncogene were investigated. Each tumor analyzed was harvested from a different mouse.

Mutations were found in codon 61, but not in codon 12, 13, or 59. No other mutations were detected in exon 1 or 2. After DB[*a,l*]P treatment, four of the five papillomas had the mutation CAA → CTA at codon 61 (Table 1). In addition, one papilloma had both the CAA → CTA and CAA → CAT mutations at codon 61. Since the direct cycle sequencing approach does not clone individual sequences, the PCR product was subcloned into pBluescript II KS⁻ at the *Sma* I restriction site, and 10 clones were sequenced. A CTT allele was not found at codon 61. This indicates that some cells contain the CTA mutation and some the CAT.

DB[*a,l*]P-11,12-dihydrodiol also produced CAA → CTA mutations at codon 61 in all seven papillomas studied (Table 1). The same CAA → CTA mutation at codon 61 was observed in tumors induced by *anti*-DB[*a,l*]PDE and the few tumors induced by DB[*a,l*]P-8,9-dihydrodiol. With DB[*a,l*]P and its dihydrodiols, the same mutation was observed at initiating doses ranging from 1.33 to 300 nmol.

Table 1. c-H-*ras* mutations observed in mouse skin papillomas induced by selected PAH

Compound	No. of mutations/ no. of tumors	Mutations observed in c-H-*ras* (Codon) exon 1	(Codon) exon 2
DB[*a,l*]P*	4/5	None	(61) CAA → C(A/T)A†
DB[*a,l*]P-11,12-dihydrodiol‡	7/7	None	(61) CAA → C(A/T)A
DB[*a,l*]P-8,9-dihydrodiol*	3/3	None	(61) CAA → C(A/T)A
anti-DB[*a,l*]PDE‡	5/5	None	(61) CAA → C(A/T)A
DMBA*	4/4	None	(61) CAA → C(A/T)A
1,2,3,4-tetrahydro-DMBA§	5/5	None	(61) CAA → C(A/T)A
BP*	7/13	(13) GGC → G(G/T)C	None
	4/13	None	None
	2/13	None	(61) CAA → C(A/T)A
6-Fluoro-BP	0/4	None	None

*Tumors were obtained from the experiment in Cavalieri *et al.* (18).
†One tumor had the double mutation C(A/T)(A/T).
‡Tumors were obtained from the experiment in Gill *et al.* (21).
§Tumors were initiated on a shaved area of dorsal skin with 100 nmol of 1,2,3,4-tetrahydro-DMBA or 600 nmol of 6-fluoro-BP in 100 μl of acetone. One week later, twice-weekly promotion with 3.24 nmol of phorbol ester in 100 μl of acetone was begun. The experiment was terminated after 12 weeks of promotion and the tumors were harvested.

Mutations in the c-H-*ras* oncogene were also investigated in papillomas induced by DMBA and its carcinogenic derivative 1,2,3,4-tetrahydro-DMBA (Fig. 1). The c-H-*ras* codon 61 mutations induced by DMBA (CAA → CTA; Table 1) confirmed previous findings (2, 8). 1,2,3,4-Tetrahydro-DMBA induced the same CAA → CTA mutation.

The mutations in papillomas induced by 6-fluoro-BP were compared to those induced by BP, because the profile of stable adducts of the weaker 6-fluoro-BP (26) indicates activation to the 6-fluoro-BP diol epoxide (23). Four of the 13 papillomas induced by BP exhibited no mutation in exons 1 and 2 of the c-H-*ras* oncogene (Table 1). Seven (54%) had a GGC → GTC transversion at codon 13, which has been found previously in BP-induced mouse skin papillomas, in ≈30% (27) or 50% of the tumors (28). A CAA → CTA mutation at codon 61 was observed in two papillomas (15%); this mutation was previously found in 20% of papillomas induced by BP (28). The weak carcinogen 6-fluoro-BP caused no mutation in exons 1 and 2.

DISCUSSION AND PERSPECTIVES

DNA Adducts, Oncogene Mutations, and Carcinogenicity. Formation of depurinating adducts by DMBA, DB[*a,l*]P, BP, and some of their derivatives appears to play a critical role in mutation of oncogenes and tumor initiation. Information about carcinogenicity, DNA adducts, and mutation of the H-*ras* gene in tumors leads to this conclusion.

DMBA. Evidence for the predominant metabolic activation of DMBA by one-electron oxidation derives from the tumorigenic activities of benz[*a*]anthracene (BA) and its alkylated derivatives, and the chemical properties of their radical cations (15, 29, 30). BA is a borderline carcinogen (31), but substitution of methyl groups at the 7- and 12-*meso*-anthracenic positions leads to DMBA (Fig. 1), one of the most potent carcinogenic PAH (31). Although less potent than DMBA, 1,2,3,4-tetrahydro-DMBA (Fig. 1) is a strong carcinogen, despite being saturated in the angular ring and thus unable to be activated to the bay-region diol epoxide (22).

Both *in vitro* and in mouse skin, 99% of the DMBA-DNA adducts formed are depurinating adducts in which DMBA specifically binds through its 12-methyl group to the N-7 position of adenine (80%) or guanine (19%) (13, 14). The specificity of binding of DMBA at the 12-methyl group to DNA bases correlates well with results of carcinogenicity experiments. In fact, when the two methyl groups are substituted with ethyl groups, the 7,12-diethyl-BA is noncarcinogenic (32). The inactivity of the ethyl-substituted compound is consistent with the lack of nucleophilic substitution at the benzylic methylene group of an ethyl-PAH radical cation (29). 7-Methyl-12-ethyl-BA is a much weaker carcinogen than DMBA, whereas 7-ethyl-12-methyl-BA shows activity similar to that of DMBA (32). These data suggest that the 12-methyl group plays the major role in the carcinogenic activity of DMBA.

Some evidence supports the bay-region diol epoxide being responsible for the carcinogenic activity of DMBA. DMBA-3,4-dihydrodiol is a more potent tumor initiator than DMBA (33), and several stable DMBA diol epoxide-DNA adducts have been identified (34, 35). DMBA-3,4-dihydrodiol is carcinogenic through its diol epoxide, but the activity of the parent DMBA is initiated by its radical cation, based on the relative formation of depurinating vs. stable adducts and the results of the tumorigenicity experiments described above.

DMBA consistently produces A → T transversions in codon 61 of the c-H-*ras* oncogene in mouse skin tumors (Table 1) (2, 8). Based on the preponderant formation of the N-7 adenine adduct of DMBA (79%; refs. 13 and 14), the A → T transversions can be attributed to loss of the N-7 adenine adducts, generation of apurinic sites, and misreplication at these sites (Table 2 and Fig. 2). In this scheme, the glycosidic bond between the N-7 adenine adduct and deoxyribose is hydrolyzed, leading to loss of the adduct and formation of an apurinic site. In the next round of DNA replication, the most likely base to be inserted opposite the apurinic site is adenine,

Table 2. Correlation of depurinating adducts with H-*ras* mutations

PAH	Major DNA adduct (%)	H-*ras* mutations No. observed/ total no.	Change
DMBA	N-7 Ade (79)	4/4	CAA → C(A/T)A
DB[*a,l*]P	N-7 Ade (45)	4/5	CAA → C(A/T)A
	N-3 Ade (28)		
DB[*a,l*]P-11,12-dihydrodiol	N-7 Ade (18)*	7/7	CAA → C(A/T)A
anti-DB[*a,l*]PDE	N-7 Ade (0.4)†	5/5	CAA → C(A/T)A
BP	C-8 Gua + N-7 Gua (44)	7/13	GGC → G(G/T)C
	N-7 Ade (30)	2/13	CAA → C(A/T)A

*This adduct is 95% of the depurinating adducts formed by DB[*a,l*]P-11,12-dihydrodiol, and its absolute amount is similar to the total adenine depurinating adducts obtained from the parent compound (17).
†This adduct is 80% of the depurinating adducts formed by *anti*-DB[*a,l*]PDE (unpublished results).

as demonstrated with bacterial and some mammalian DNA polymerases (9, 36, 37). When the coding strand of DNA is then replicated, a thymine is inserted opposite the new adenine. This results in the A → T transversion observed in codon 61 of the c-H-*ras* oncogene. When a guanine adduct is lost by depurination, leaving an apurinic site in the DNA, the preferential insertion of adenine in the opposite DNA strand leads to a G → T transversion at the site of the adduct.

The same A → T mutation in codon 61 was observed in papillomas induced by 1,2,3,4-tetrahydro-DMBA (Table 1). The adducts formed biologically by this carcinogen are not identified, but electrochemical oxidation of 1,2,3,4-tetrahydro-DMBA produces depurinating adducts of guanine and adenine (unpublished results) similar to those of DMBA (30). Thus, we anticipate that 1,2,3,4-tetrahydro-DMBA is metabolically activated at the 12-methyl group like DMBA, forming a preponderance of N-7 adenine adducts.

DB[*a,l*]P. The c-H-*ras* mutations in mouse skin papillomas induced by DB[*a,l*]P and its 11,12-dihydrodiol (Table 1) are A → T transversions in four of the five tumors induced by the parent and all seven tumors induced by the metabolite. DB[*a,l*]P is the most potent carcinogenic PAH, and its proximate carcinogenic metabolite, DB[*a,l*]P-11,12-dihydrodiol, is slightly less potent (18, 19). N-7 and N-3 adenine depurinating adducts represent 73% of the DNA adducts of microsome-activated DB[*a,l*]P (17). The major depurinating adduct formed by DB[*a,l*]P-11,12-dihydrodiol is also an N-7 adenine adduct, and its absolute amount is almost that found as N-3 and N-7 adenine adducts of DB[*a,l*]P (17). Apurinic sites generated by adenine depurinating adducts (Table 2) correlate with deficient repair of these sites, and the resulting A → T transversions obtained when these apurinic sites are incorrectly replicated. *Anti*-DB[*a,l*]PDE induces A → T transversions in codon 61 (Tables 1 and 2) and forms the depurinating N-7 adenine adduct (unpublished results).

The very weak carcinogen DB[*a,l*]P-8,9-dihydrodiol (18) also produced an A → T transversion in codon 61 in the three papillomas studied (Table 1). This compound may be metabolized to the 11,12-dihydrodiol, forming DB[*a,l*]P-8,9,11,12-tetraol, which could be further metabolized to its fjord region 13,14-epoxide. A similar mechanism of activation has been proposed for the carcinogen dibenz[*a,h*]anthracene (38).

BP. Approximately 75% of the DNA adducts of BP are formed in mouse skin by one-electron oxidation with binding at the C-8 and N-7 of guanine (44%) and the N-7 of adenine (30%) and rapidly depurinate to generate apurinic sites (12).

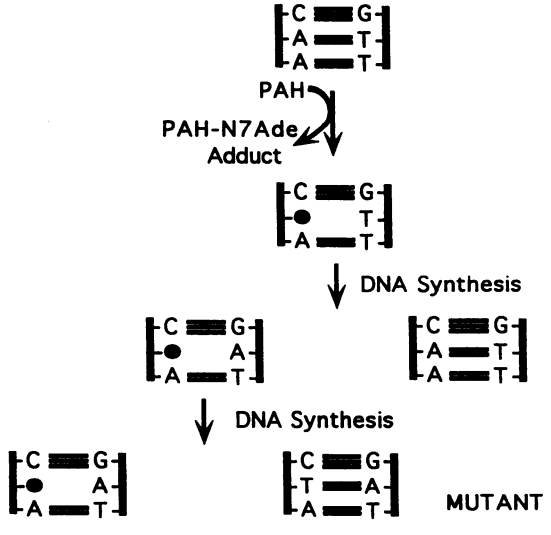

FIG. 2. Possible scheme for inducing A → T transversions from N-7 adenine depurinating adducts.

Two c-H-*ras* mutations were found in mouse skin papillomas induced by BP (Table 1). Seven of 13 papillomas exhibited G → T transversions at codon 13. This mutation has been reported in a smaller (27) or similar (28) percentage of BP-induced tumors. It can be attributed to misreplication of unrepaired apurinic sites generated by loss of N-7 and C-8 guanine adducts (Table 2) (12). A → T transversions at codon 61 were observed in two other papillomas, as reported at a similar low frequency (28). This mutation can be attributed to the N-7 adenine adduct formed in mouse skin.

6-Fluoro-BP is a weaker carcinogen than BP (26, 39). The profile of stable adducts of 6-fluoro-BP formed *in vitro* after activation by rat liver microsomes resembles that of BP (23), but its depurinating adducts have not been analyzed. The four papillomas induced by this weak carcinogen did not exhibit any mutations in exons 1 and 2 of the c-H-*ras* oncogene (Table 1). Further studies are needed to ascertain whether this compound induces mutations in this oncogene.

Aflatoxin B_1. Further evidence that depurinating adducts generate apurinic sites and then transversions derives from studies of the liver carcinogen aflatoxin B_1 (40). The predominant adduct (90%) is derived from reaction of the 2,3-epoxide of aflatoxin B_1 with the N-7 of guanine. This adduct is recovered in DNA, but it is labile, with a half-life of 8 h under physiological conditions (41). Thus, the aflatoxin B_1-N-7 guanine adduct is a mixture of depurinating and stable adduct at any given time. The widely found G → T transversion in codon 249 of the p53 gene in human hepatocellular carcinomas has been attributed to this aflatoxin B_1-N-7 guanine adduct (42, 43). This can be considered the first oncogenic mutation identified that presumably arises from misreplication of an apurinic site generated by loss of a carcinogen-DNA adduct.

Stable Adducts and Carcinogenicity. Until now, mutagenesis by bulky chemical carcinogens has been thought to arise from stable DNA adducts. For example, *N*-acetoxy-2-acetylaminofluorene formed the N-7 guanine depurinating adduct (44), in addition to the known stable adduct formed at C-8 of guanine. Studies of the N-7 guanine adduct have never been pursued, and all subsequent research on arylamines and other bulky carcinogens has focused only on stable adducts.

The (+)-*anti*-BP diol epoxide, considered the major ultimate carcinogenic metabolite in the diol epoxide pathway, predominantly forms a stable adduct with C-10 bound to the 2-amino group of deoxyguanosine. This adduct is mutagenic in an *Escherichia coli* plasmid with site-directed methods (45) and in primer-extension reactions catalyzed by the Klenow fragment of *E. coli* DNA polymerase I (46). However, its carcinogenic activity by repeated application (47) and its tumor-initiating activity (48) in mouse skin are considerably weaker than that of BP. This discrepancy has been rationalized as the reactive BP diol epoxide not reaching DNA targets in mouse skin. This explanation is incorrect, because BP diol epoxide covalently binds to mouse skin DNA more than BP, and the stable diol epoxide adduct accounts for 99% of the adducts (12). These results suggest that the stable adduct plays only a minor role in the initiation of tumorigenesis by BP.

Similarly for DB[*a,l*]P, DB[*a,l*]P-11,12-dihydrodiol is activated by rat liver microsomes to form more stable adducts than DB[*a,l*]P itself (17), but the dihydrodiol is less tumorigenic than the parent compound (18, 19). The diol epoxides of DB[*a,l*]P are much less tumorigenic than the 11,12-dihydrodiol, and the *syn*-isomer is more potent than the *anti*-isomer (21). *Syn*- and *anti*-DB[*a,l*]PDE were reacted with calf thymus DNA for 30 min at 37°C, and the stable and depurinating adducts were analyzed (49). *Syn*-DB[*a,l*]PDE produces 10-fold more depurinating adducts than *anti*-DB[*a,l*]PDE, whereas the *anti*-isomer forms 6-fold more stable adducts than the *syn*-isomer (unpublished results). For *syn*-DB[*a,l*]PDE, ≈20% of the total adducts

depurinate, whereas only 0.5% of *anti*-DB[*a,l*]PDE adducts do. These results are consistent with the hypothesis that depurinating adducts play the major role in tumor initiation.

Conclusions. The profiles of DNA adducts determined for BP, DMBA, DB[*a,l*]P, DB[*a,l*]P-11,12-dihydrodiol, and *anti*-DB[*a,l*]PDE suggest that oncogenic mutations found in mouse skin papillomas induced by PAH are generated by misreplication of unrepaired apurinic sites derived from the loss of depurinating adducts. A decade ago, apurinic sites were postulated to be mutagenic DNA lesions that can initiate carcinogenesis (5), although bulky carcinogens like PAH were not known to form high proportions of depurinating adducts.

More recently, in *Drosophila melanogaster*, methyl methanesulfonate was found to produce A → T and G → T transversions caused by depurination of 3-methyladenine and 7-methylguanine, followed by misreplication at the resulting apurinic sites (50). Mutations in the *lacI* gene of *E. coli* treated with (±)-*anti*-BP diol epoxide have been studied. The predominant G → T transversions were attributed to formation of the depurinating BP diol epoxide-N-7 guanine adduct and misreplication at apurinic sites (51). Furthermore, six stereoisomers of the stable BP diol epoxide-N^6-deoxyadenine adduct were inserted at position 2 of human N-*ras* codon 61 (CAA) in an 11-base oligonucleotide. In *E. coli*, these adducts induced only A → G mutations (52), not the A → T transversions found in codon 61 of mouse skin tumors initiated with BP (Table 1 and ref. 28). This result supports the view that the codon 61 A → T mutation observed in PAH-induced tumors does not derive from a stable adenine adduct.

Presumably, some apurinic sites generated by bulky carcinogens are not repaired. In fact, the amount of depurinating adducts detected in mouse skin treated with BP or DMBA, along with the estimated number of cells in the treated area of skin, provides conservative estimates of apurinic sites of ≈40,000 sites per cell for DMBA and 25,000 sites per cell for BP formed during the 4 h of treatment with the PAH (12, 14). It has been reported that 10,000 apurinic sites spontaneously form per cell per day (53). Hence, depurinating BP or DMBA adducts cause at least 10-fold more apurinic sites than the ≈2000 spontaneously formed in a cell during 4 h.

The nonrepair may be due to excessive apurinic sites beyond the repair capacity of the cells or to the presence of stable adducts interfering with the error-free repair process. The qualitative and quantitative study of adducts generated by various carcinogens and the mutations induced in the *ras* oncogene are expected to yield further insight into the important role of apurinic sites in the tumor initiating process.

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