trans-3,4-Dihydroxy-*anti*-1,2-epoxy-1,2,3,4-tetrahydrodibenz[a,j]acridine Involvement in Dibenz[a,j]acridine DNA Adduct Formation in Mouse Skin Consistent with Ha-*ras* Mutation Patterns in Tumors

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Dibenz[a,j]acridine (DBA), is a *N*-heteropolycyclic aromatic environmental carcinogen found in complex combustion mixtures. The major route of DBA metabolic activation is reportedly through the *trans*-3,4-dihydroxy-3,4-dihydroDBA (DBA-3,4-DHD). The present studies were undertaken to determine the role of *trans*-3,4-dihydroxy-*anti*-1,2-epoxy-1,2,3,4-tetrahydroDBA (DBADE) in DBA activation pathway(s), the DNA bases involved in the binding of DBA to DNA, and whether the adducts produced are consistent with the mutation pattern in the Ha-*ras* gene. DBA (300 μg) or 50 μg synthesized (±)-DBADE was applied to the back of female Hsd:ICR(Br) mice. The mice were sacrificed 48 h later, and skin DNA was isolated, hydrolyzed, and analyzed with ^{32}P-postlabeling. Of the four adducts produced in vivo, adduct **1** was the major adduct for DBA (>50%) and adduct **2** was the major adduct for DBADE (89%). After the reaction of (±)-DBADE with purine nucleotides or calf thymus (CT) DNA in vitro, 100% of the DBADE−2′-dAMP adducts and 94% of DBADE−CT DNA adducts were chromatographically identical on TLC with adduct **2** and 86% of the DBADE−2′-dGMP adducts were chromatographically consistent with adduct **1** by ^{32}P-postlabeling. Papillomas were induced on the backs of mice by a single application of 0.2 μmol of DBA followed by twice-weekly application of 12-*o*-tetra-decanoylphorbol-13-acetate (TPA, 2 μg) for 24−26 weeks. Skin carcinomas were induced by twice weekly applications of DBA (0.1 μmol) on the backs of mice. A to T and G to T transversions were found in codons 12, 13, and 61 of the Ha-*ras* gene in the treated mouse skin carcinoma and papilloma DNA. The mutational spectra in the Ha-*ras* gene are consistent with the DNA binding of DBA to dG or dA in vivo. Thus, this research has indicated that DBADE plays an important role in DBA metabolic activation and DNA binding in mouse skin, and an alternative pathway through a bis-dihydrodiol-epoxide of DBA may also be involved.

Introduction

N-Heteropolycyclic aromatic compounds (NHA)[1] are formed from the incomplete combustion of organic material They are widely distributed in the environment and are found in tobacco smoke condensate, synthetic fuels, automobile engine exhaust, and as effluent from the combustion of coal (*1*, *2*). Many of these NHA are carcinogenic including dibenz[a,j]acridine (DBA, Figure 1), 7H-dibenzo[c,g]carbazole (DBC), dibenz[c,h]acridine, and dibenz[a,h]acridine, etc. (*1*−*5*). DBA has been found to be a lung carcinogen in mice by subcutaneous administration (*6*), a moderate mouse skin tumor initiator (*7*) and a skin carcinogen in mice following topical application (*8*). In comparison with DBC and benzo[*a*]pyrene (BaP) in Hsd:ICR(Br) mice, DBA produced 27 carcinomas in 50 mice relative to 42 and 49 carcinomas for DBC and BaP, respectively (*8*). Similar skin tumor data were obtained for DBA, DBC, and BaP in the C3H mouse (*9*). Like many mutagenic/carcinogenic polycyclic aromatic hydrocarbons (PAH), NHA require metabolic activation to electrophilic species in order to bind covalently with cellular components and to exert their carcinogenic effect. Consistent with the bay-region theory of PAH carcinogenesis (*10*), DBA has been reported to metabolize to a series of dihydrodiols, phenols and epoxides of which the *trans*-3,4-dihydroxy-3,4-dihydrodibenz[a,j]acridine (DBA-3,4-DHD, Figure 1) is the major product (*11*−*13*). These metabolites are further activated, and *trans*-3,4-dihydroxy-*anti*-1,2-epoxy-1,2,3,4-tetra-hydroDBA (DBADE, Figure 1) is predicted to be an ultimate active metabolite (*12*).

In previous studies, DBA and its metabolites were topically applied to the backs of mice (*14*). DNA was isolated and DNA adducts were analyzed by ^{32}P-postlabeling. DBA produced two distinct adducts (**1** and **2**) in

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[1] Abbreviations: NHA, *N*-heteropolycyclic aromatic compounds; DBA, dibenz[a,j]acridine; DBC, dibenzo[c,g]carbazole; PAH, polycyclic aromatic hydrocarbon, BaP, benzo[a]pyrene; DB[a,h]A, dibenz[a,h]-anthracene; DB[a,j]A, dibenz[a,j]anthracene; DB[a,l]P, dibenzo[a,l]-pyrene; DBA-3,4-DHD, *trans*-3,4-dihydroxy-3,4-dihydrodibenz[a,j]-acridine; DBA-5,6-DHD, *trans*-5,6-dihydroxy-5,6-dihydroDBA; DBADE, *trans*-3,4-dihydroxy-*anti*-1,2-epoxy-1,2,3,4-tetrahydro-DBA; (±)-DBADE, synthetic racemic DBADE; DBA-3,4,8,9-bis-DHD-1,2-epoxide, 1,2-epoxy-3,4,8,9-tetrahydroxy-1,2,3,4,8,9-hexahydroDBA; dG, 2′-deoxyguanosine; dA, 2′-deoxyadenosine; TPA, 12-*o*-tetra-decanoylphorbol-13-acetate; EH, epoxide hydrolase; MALDI-TOF-MS, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry.

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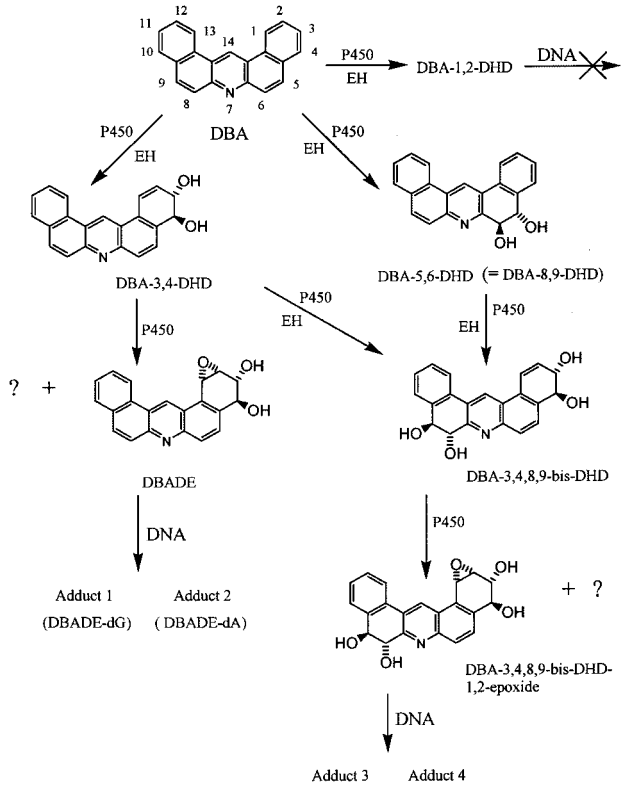

Figure 1. Chemical structure of DBA and metabolites and proposed pathways of DBA metabolicactivation. Only one enantiomer is shown, for each chiral compound.

skin. The same two adducts were seen when DBA-3,4-DHD was topically applied. The total adduct levels elicited by the DBA-3,4-DHD was twice that of DBA. In addition, two different DNA adducts (**3** and **4**) were seen in mouse skin when the *trans*-5,6-dihydroxy-5,6-dihydroDBA (DBA-5,6-DHD, Figure 1) was topically applied. Using more sensitive nuclease P_1 ^{32}P-postlabeling, all four DNA adducts were detected following topical administration of the DBA-3,4-DHD. These data suggested that the route of DBA activation was through the DBA-3,4-DHD and formation of the DBADE and another pathway involving a common secondary metabolite of both DBA-3,4-DHD and DBA-5,6-DHD, bis-DHD-epoxide (Figure 1) similar to dibenz[a,h]anthracene (DB[a,h]A; *15*, *16*), was proposed (*14*). Therefore, we undertook studies to determine (1) the role of DBADE in metabolic activation pathways and DNA binding of DBA, (2) the nucleotide bases involved in binding of DBADE to DNA, and (3) whether the formation of the DNA adducts are consistent with the mutation pattern produced in the Ha-*ras* gene. In this study, the DBADE used is the chemically synthesized racemic mixture, (±)-DBADE.

Experimental Procedures

Materials. DBA (> 99% purity) was synthesized following the published procedures (*17*) and recrystallized from benzene, mp 221−222 °C. The following reagents and enzymes were purchased from Sigma Chemical Co. (St. Louis, MO): urea, dithiothreitol, calf thymus DNA, ethylenediamine tetraacetic acid (EDTA), 2′-deoxyadenosine-3′-monophosphate (3′-dAMP), 2′-deoxyadenosine-5′-monophosphate (5′-dAMP), 2′-deoxyguanosine-3′-monophosphate (3′-dGMP), 2′-deoxyguanosine-5′-monophosphate (5′-dGMP), 12-*o*-tetradecanoyl-phorbol-13-acetate (TPA), phosphatase alkaline (bacteria, type III, 82 units/mg), micrococcal endonuclease (grade VI, 100 units/mg), sodium pyruvate (type II), sodium adenosine diphosphate, ribonuclease (Rnase T1, grade IV, 400 000 units/mL), and RNaseA (type IIIa, 75 units/mg). Boehringer-Mannheim Biochemicals (Indianapolis, IN) supplied: calf spleen exonuclease (phosphodiesterase 2 untis/mg), proteinase K (20 untis/mg), L-glycerol-3-phosphate and β-nicotinamide adenine dinucleotide (NAD grade I, 100%). Poly-ethyleneimine-cellulose (PEI) thin-layer plastic-backed sheets were purchased from Alltech Inc. (Waukeegan, IL). Polynucleotide kinase was obtained from U.S. Biochemical Co. (Cleveland, OH). Carrier-free [^{32}P]H_3PO_4 was obtained from ICN Biochemicals (Irvine, CA). γ-[^{32}P]ATP was synthesized as described previously (*18*). Sep-Pak cartridges were from Waters (Milford, MA). Fisher Scientific (Pittsburgh, PA) was the source of all other chemicals used in this work.

Caution: DBA and metabolites are potentially hazardous and were handled in accordance with NIH Guidelines.

Instrumental Analysis. HPLC was performed on a Waters system equipped with model 501 pumps, a WISP 710B autoinjector and 484 tunable absorbance detector controlled with the Millenium data system. Fluorescence excitation, emission and synchronous spectra in methanol solution were recorded on a Perkin-Elmer MPF-66 Fluorescence Spectrophotometer. ^{1}H NMR spectra were recorded on a Bruker NMR/300 AF spectrometer in deuterated dimethyl sulfoxide, and chemical shifts reported with tetramethylsilane (TMS) as the internal standard (0 ppm). The mass spectral data of synthesized standards were recorded on a Kratos MS-890 high-resolution MS (HR-MS) operated with a DS-90 data system based on Data General Nova/4 eclipse. A matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS was employed to obtain MS data of adducts. An aliquot (0.5 μL) of the matrix solution (α-cyano-4-hydroxy cinnamic acid in 30% acetonitrile/1% trifluouroacetic acid) was mixed with the sample in a 0.5 mL vial, and the resultant mixture was deposited onto the MALDI probe tip. Multiple sample probe tip was then introduced into the MALDI/TOF mass spectrometer, and the solvent was allowed to evaporate under the vacuum conditions. MALDI MS was obtained on a TOF instrument (VG TOF SPEC-SE model) under reflectron mode as well as the linear mode. Background pressure within the instrument was better than 3×10^{-7} Torr as measured by an ion gauge located below the source. The laser used for ionization was nitrogen laser at 337 nm with a pulse width of 6 ns and 180 μJ/pulse. Each laser shot produced a full mass spectrum. The spectra were averages of 20−30 laser shots. The ion acceleration potential was set at 25 kV. Ion detection and signal amplification were achieved using micro channel plate detector-discrete dynode multiplier assembly. The data were processed and stored on a DEC-300 *a*-work station.

Synthesis of (±)-DBADE. Following a known method (*19*) with modifications (±)-DBADE (>95% purity) was synthesized and the synthesis should be performed just prior to use due to the instability of the compound. DBA-3,4-DHD (2.1 mg, previously synthesized in this laboratory according to ref *20*), was dissolved in 3 mL of anhydrous tetrahydrofuran (THF) and 1.2 mg of *m*-chloro-peroxybenzoic acid (mCPBA, Aldrich Chem. Co., Milwaukee, WI) was added. The mixture was stirred at ambient temperature in the dark, under dry nitrogen for 2 h and an additional 1.2 mg of mCPBA was added. The reaction was monitored using HPLC and allowed to run for additional two to 4 h. Then, the reaction mixture was diluted with 10 mL of ethyl acetate and washed with 2% triethylamine/water (2 × 5 mL), saturated sodium chloride aqueous solution (2 × 5 mL), and then dried over sodium carbonate. After evaporation of the solvent, the product was isolated using HPLC on a 7.8 × 300 mm Waters μPorasil column with an isocratic eluting program of 7/3 hexane/mixture of ethyl acetate, methanol and triethylamine (3/1/0.02) at a flow rate of 5 mL/min. Fluorescence spectra are shown in Figure 2; HR-MS: 329.1008, calcd 329.1052 for $C_{21}H_{15}NO_3$; ^{1}H NMR: 3.83(d, 1H, H2), 3.91 (dd, 1H, H3), 4.55 (dd, 1H, H4), 5.50 (d, 1H, H1), 5.76 (d, 1H, O*H*3), 5.97 (d, 1H, O*H*4), 7.8−8.2 (aromatic H), 9.37 (d, 1H, H13), 10.35 (s, 1H, H14).

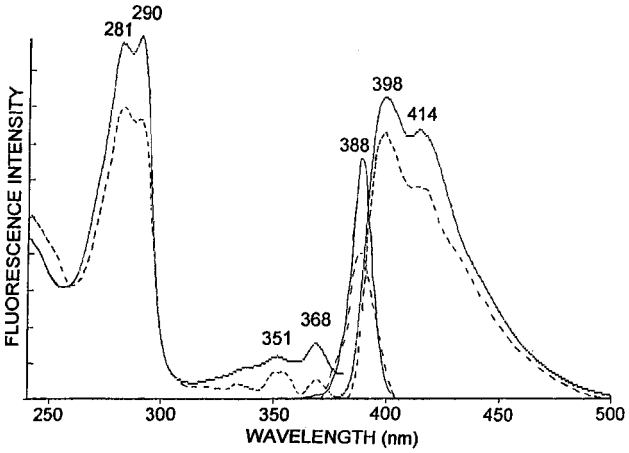

Figure 2. Comparison of fluorescence excitation, emission and synchronous spectra of synthetic (±)-DBADE in methanol (—) and DBADE−dAMP(- - -) made from reaction of (±)-DBADE with 5′-dAMP, in vitro: ex. scan from wavelength 240 to 380 nm with em. set at 398 nm; em. scan from 380 to 500 nm with ex. set at 281 nm; synchronous scan from 300 to 450 nm with the delta wavelength, $\Delta\lambda = 10$ nm, and the peak shown at 388 nm.

Adduction of DBADE with DNA or Nucleotides in Vitro. (±)-DBADE (~0.1 mg) was dissolved in 0.1 mL of THF mixed with a solution of either 5′-dGMP (5 mg), 5′-dAMP (5 mg), 3′-dGMP (1.5 mg), or 3′-dAMP (1.5 mg) in 1 mL of 0.1 M Tris (pH 7.0) or calf thymus DNA (1.5 mg) in 0.3 mL of water and 0.7 mL of 0.1M Tris (pH 7.0) and then incubated at 37 °C in the dark for 8−16 h. DBADE adduct formation with the 3′-phosphates or DNA were analyzed by ^{32}P-postlabeling, while adducts produced by reaction with 5′-phosphates were further isolated and purified for chemical analysis.

Isolation and Analysis of Nucleotide Adducts. Unmodified nucleotides were removed from the 5′-phosphates reaction mixtures through HPLC using a Whatman Partisil 10 ODS-2 column (4.6 × 250 mm) and eluted with 100% water for 12 min and 100% methanol for 18 min with a flow rate of 1 mL/min. Fractions from 20 to 26 min were collected and evaporated to dryness under nitrogen at 30 °C. The residues from DBADE−dAMP adduction were redissolved in 1 mL of 0.1 M Tris (pH, 9.0) and incubated with 0.1 mL of phosphotase alkaline solution (25 units/ml) in 0.02 M Tris (pH, 7.0) at 37 °C for 24 h to convert the nucleotide adducts to nucleoside adducts (*21*, *22*). The digested mixtures were purified on a preconditioned Sep-Pak cartridge as previously described (*23*). DBADE−dA adducts were separated by HPLC using a gradient program: 40% methanol/water for 4 min, increasing to 60% methanol over 15 min, isocratic for 6 min, increasing to 100% methanol over 5 min and maintained for 8 min at 100% methanol, with a flow rate of 1 mL/min. To isolate adducts on HPLC, several runs were repeated and six fractions were collected. After fluorescence spectroscopic analyses, each fraction was evaporated to dryness under nitrogen and then acetylated using acetic anhydride/pyridine procedures described previously (*24*) to give the completely acetylated derivatives. Fractions at t_R 29.2 and 29.8 min were identified as the DBADE-adducts (in their pentaacetyl form) using MALDI-TOF MS, $(M + H)^+$ 791 for $C_{41}H_{38}N_6O_{11}$. For DBADE-dGMP adduction, the reaction mixture, after removal of unmodified nucleotides and evaporation of solvent, was redissolved in 0.3 mL of 30% methanol/water and directly separated on HPLC with the same solvent gradient program and seven fractions were collected for analysis. MALDI-TOF MS showed that the fraction at t_R 32.2 min. was the DBADE-dGMP adduct, $(M + H)^+$ 677 for $C_{31}H_{29}N_6O_{10}P$.

Animal Treatment with DBA or DBADE. Female Hsd: ICR (Br) mice were purchased from Harlan Sprague Dawley (Indianapolis, IN) at 4−6 weeks of age and allowed to acclimatize for two weeks. Animals were housed individually in plastic cages under controlled temperature, humidity and day-night cycle. Animal chow and water were given ad libitum. DBA (300 μg) in 50 μL of acetone or acetone alone were applied to the shaved interscapular dorsal skin. Animals were sacrificed 48 h after dosing. Skin, liver, and lung were removed and were frozen in liquid nitrogen, stored at −80 °C until analyzed. Nine animals were treated once with 50 μg of (±)-DBADE in 50 μl acetone or vehicle alone and three animals as a group were sacrificed at 4, 8, and 24 h as described above.

Extraction of DNA from Tissues and ^{32}P-Postlabeling Analysis. DNA was extracted from the various tissues using techniques described earlier (*25*) and DNA (2 μg at 0.5 μg/μL) was hydrolyzed to 3′-phosphodeoxynucleotides as described earlier (*14*). DBA−DNA adduct levels are maximal when hydrolyzed at pH 6 for 6 h (*26*). DNA adducts were analyzed using a ^{32}P-postlabeling assay as described earlier (*27*−*29*). Nucleotide digests were labeled under adduct intensification conditions employing a limiting amount of [^{32}P]ATP relative to DNA (*25*, *30*). The intensification factor for adducts **1** and **2** under these labeling conditions were 71.7 ± 8.4- and 57.0 ± 7.1-fold, respectively. The intensification factors for adducts **3** and **4** were not determined because the levels were too low for reliable determination. For TLC separation, 2.6 M lithium formate, 6 M urea, pH 3.6, was used as D3 solvent, and the D4 solvent was 0.75 M lithium chloride, 0.45 M Tris, 8.2 M urea, pH 8, as previously described (*14*). Unadducted nucleotides were labeled and analyzed on TLC, and adduct spots and background areas were excised from the TLC plates and the radioactivity in each adduct was determined using Cerenkov counting. The level of adduction and relative adduct labeling (⟨RAL⟩) values were determined as follows:

$$\langle \mathrm{RAL} \rangle = \frac{\mathrm{cpm}_{\mathrm{adducts}}}{\mathrm{cpm}_{\mathrm{unadducted\ nucleotides}}} \times 10^7$$

These values represent the number of adducts in 10^7 unadducted nucleotides under carrier-free conditions and should be divided by the intensification factors to estimate the unbiased adduct levels.

Tumor Induction and Ha-ras Analysis. Papillomas were induced by a single topical application of DBA (0.2 μmol) to the shaved backs of 12 female Hsd:ICR(Br) mice followed by twice weekly applications of TPA (2 μg) for 24−26 weeks. Six TPA and six no treatment animals were used as controls. Carcinomas were induced in eight female Hsd:ICR(Br) mice by twice weekly topical applications of DBA (0.1 μmol) until tumor development. Six acetone control animals and six no treatment animals were used as controls. Twelve papillomas and eight carcinomas were removed and DNA extracted by a nonorganic method (Gentra Systems). The DNA was screened for Ha-*ras* codon 12, 13, and 61 mutations using a modified enriched polymerase chain reaction (EPCR) method (*31*). This method, which eliminates interference of K-*ras*, N-*ras*, and pseudogenes by using an intron based primer on the 3′ end, is a four-step reaction that includes initial amplification, intermediate digestion, secondary amplification and final digestion. The final digestion products were resolved on 3% MetaPhor agarose-1X TBE gels. Positive bands were isolated, reamplified, purified (Wizard PCR Preps, Promega) and reversed sequenced (*fmol* Sequencing System, Promega). Sequencing autoradiograms were visualized on Kodak Xomat AR film.

Results

It has been proposed that DBADE is an ultimate active metabolite of DBA (*12*) responsible for DNA binding and carcinogenicity. In this study, the covalent adducts of (±)-DBADE with dAMP and dGMP were isolated from in vitro reaction and identified using fluorescence spectral and mass spectral analysis. For DBADE−5′-dAMP adducts, the phosphate group was enzymatically hydrolyzed

and the ensuing dA adduct mixtures were separated by HPLC to remove the major byproduct—1,2,3,4-tetrol of DBA. Fluorescence spectra of each fraction were recorded to confirm the DBADE moiety of adducts (Figure 2). To facilitate the chemical stability and vaporizability, the adduct fractions were then acetylated for MS analysis. Due to the low yield of the adduction and the small scale of the reaction, the acetylated adducts were analyzed using MALDI-TOF MS. That confirmed the formation of the adduct(s) similar to other PAH-diol-epoxides (*21*), the binding was through the 1,2-epoxide-ring opening in DBADE and the resultant benzylic carbon, i.e., C1, to link with nucleophilic site(s) of the dA residue to form the covalent bonded adduct(s), most likely, 2,3,4-trihydroxy-1,2,3,4-tetrahydroDBA-1-dA. However, the binding site of Ade and the stereo-preference of the ring opening of the epoxide need to be further characterized by NMR although reportedly, the amino group of Ade and Gua is the site to be linked by dihydrodiol-epoxides for PAHs (*32*). By virtue of the MALDI-TOF MS (*33*), the mass spectral data of the DBADE−5′-dGMP adduct(s) were obtained without hydrolyzing the phosphate group and acetylating the hydroxyls in adducts that would be required by the conventional electron impact MS (*21*). The MALDI-TOF mass spectral data showed that the chemical identity of DBADE−5′-dGMP adduct(s) was similar to the dA adduct(s), through the covalent linkage of C1 in DBADE with dG residue. The reaction rate of DBADE with dG was found to be slower and at lower yield than dA adduction.

To study DNA adducts in vivo, five mice were treated topically with 300 μg of DBA, a 3-fold higher dose than the earlier study (100 μg, ref *14*), and sacrificed 48 h later, and skin DNA was isolated and analyzed using ^{32}P-postlabeling. Four adducts were observed in the skin DNA (Figure 3A), and the relative percentage level of the four adducts is reported in Table 1. At least half of the total adduct level was found in adduct **1**. The DNA adduct patterns of (±)-DBADE in vivo were determined by topically applying 50 μg of (±)-DBADE to nine mice, and three animals each were sacrificed 4, 8, and 24 h later. Then, the DNA was isolated and ^{32}P-postlabled with the same techniques as those used for DBA assays. The adduct data are reported in Table 1 and Figure 3B. The majority of the adduction of (±)-DBADE to DNA was found in adduct **2** (89%), which was different from the results observed for DBA in vivo. The highest level of the adduct **2** was observed at 8 h (Figure 4). To verify the DNA bases involved in binding in vivo, 3′-dAMP, 3′-dGMP, and CT-DNA were incubated in vitro with (±)-DBADE individually, and each reaction mixture was directly analyzed using ^{32}P-postlabeling (Table 1). Chromatographic comparison of the ^{32}P-postlabeling TLC maps with those obtained from in vivo experiments indicated that adduct **1** was DBADE bound to dG (86%), adduct **2** was DBADE bound to dA (100%), and for CT DNA, DBADE was almost exclusively bound to dA (94%) forming adduct **2**. Adducts **3** and **4** were not seen in any of these three DBADE reactions. It would appear from these data that there is another pathway other than DBADE in the activation of DBA to produce metabolite-(s) that bind to DNA to form adducts **3** and **4**.

Mutations in the Ha-*ras* gene of DBA-induced mouse skin tumors produced by two different carcinogenesis protocols were determined using EPCR screening method (*31*, *34*) and confirmed by DNA sequencing analysis

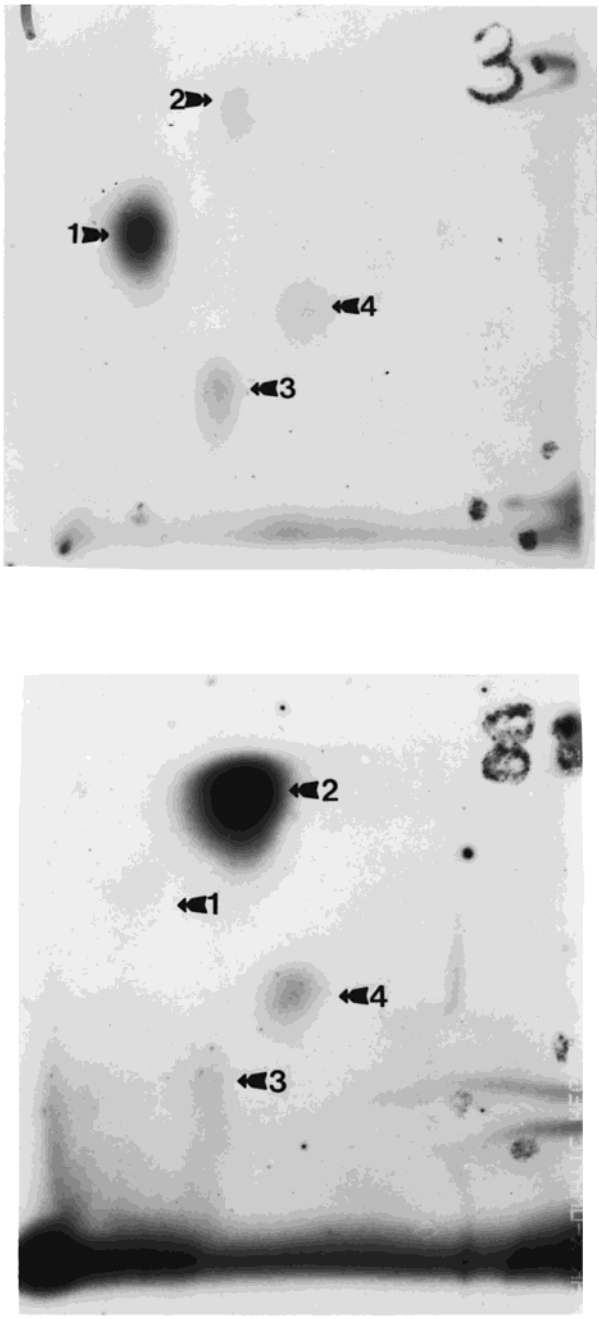

Figure 3. Autoradiogram resulting from ^{32}P-postlabeling of mouse skin DNA adducts formed with a single topical application of (A) 300 μg of DBA and (B) 50 μg of (±)-DBADE.

(Table 2 and Figures 5 and 6). Four of eight carcinomas, produced in eight mice, were found to have mutations in the Ha-*ras* gene. One mutation was an A→ T transversion in the second base of codon 61 similar to DBC and DMBA (*34*, *35*), a second mutation was a G → T transversion in the second base of codon 12 similar to BaP (*36*) and a third mutation was a G → T transversion in the first base of codon 13. These three mutations are in accordance with the DBA−DNA binding studies, which indicate that both dG and dA are involved in binding of DBA. The fourth mutation was a G → T transversion in the first base of codon 13 that was consistent with a spontaneous mutation (*37*). The acetone control had a spontaneous skin carcinoma with a mutation in the first base of the 13th codon, a G → C transversion (*37*). Six of 12 papillomas, produced in 12 mice were found to have

Table 1. Relative Percentage Levels of DBA−DNA Adducts in Vitro and in Vivo

adduction	adduct 1 (dG adduct)	adduct 2 (dA adduct)	adduct 3	adduct 4
DBA in vivo	50.3 ± 13.2	4.7 ± 2.5	24.6 ± 4.1	20.4 ± 11.5
DBADE in vivo[b]	1.7 ± 1.0	89.3 ± 9.5	4.6 ± 4.6	4.4 ± 5.5
DBADE-DNA in vitro[c]	6	94.0 ± 6.7	0	0
DBADE-dGp in vitro[d]	86.0 ± 6.0	14[f]	0	0
DBADE-dAp in vitro[e]	0	100	0	0

[a] DBA, 300 μg applied topically to backs of mice and sacrificed 48 h later. [b] (±)-DBADE, 50 μg applied topically to backs of mice and sacrificed 8 h later. [c] (±)-DBADE incubated with calf thymus DNA for 16 h. [d] (±)-DBADE incubated with 3′-dGMP for 12 h. [e] (±)-DBADE incubated with 3′-dAMP for 12 h, only one adduct seen. [f] May not be chromatographically identical to adduct 2.

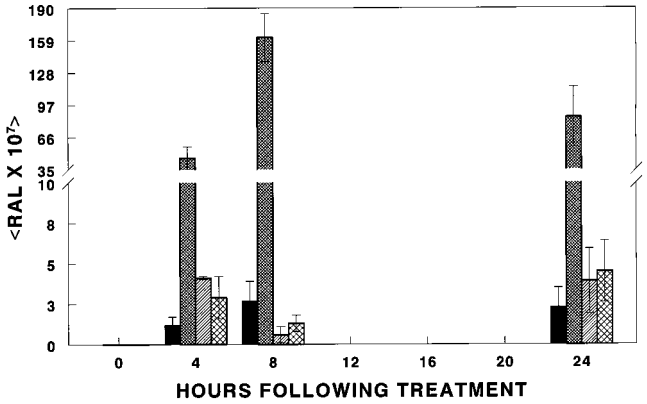

Figure 4. Relative adduct labeling ⟨*RAL*⟩ value of DNA adducts formed over 4, 8, and 24 h, following a single topical application of 50 μg of (±)-DBADE to mouse skin. Black bars, adduct **1**; white-black dots, adduct **2**; slanted lines, adduct **3**; cross-sectioned, adduct **4**.

mutations in the Ha-*ras* gene. One mutation was a G → T transversion in the second base of codon 13 similar to BaP (*36*), and three mutations were A → T transversions in the second base of codon 61 similar to 3-methylcholanthrene (*38*). The remaining two mutations were C → A transversions in the first base of codon 61 which could be due to a G → T transversion on the non-coding strand. These mutations, in accordance with the DBA−DNA binding studies, indicate that both dG and dA are involved in binding of DBA.

Discussion

The formation of covalent DNA adducts in vivo is usually regarded as the initiating step in the carcinogenic process. Identification and characterization of the DNA adducts formed would provide useful information in understanding the activation mechanism of carcinogens. When DBA is applied topically in vivo, four adducts are formed and the major adduct is adduct **1**. Of the four adducts observed, adducts **1** and **2** match chromatographically the dG and dA adducts of DBADE, respectively. On the other hand, (±)-DBADE applied topically in vivo or in vitro with CT DNA, results predominantly in binding to dA (adduct **2**). In addition, chemical reactions of (±)-DBADE with dAMP and dGMP also show the preference to react with dA at a higher yield than dG adduct. Presently, there is no clear understanding of factors that direct dihydrodiol epoxides to dA or dG residues in DNA. Some studies on PAH, however, have indicated that the product distribution between the two purine-nucleosides in DNA was dependent on the configuration of the dihydrodiol epoxides and the planarity of the hydrocarbons (*39*). Therefore, the different steric structures (configurational isomers) of the chemically synthesized racemic DBADE and the enzymatically produced DBADE from DBA in vivo would probably be one of the factors that lead to their distinct relative reactivity with dA and dG. The synthesis and resolution of stereoisomers of DBADE will be important in further investigation of the activation mechanism of DBA.

At lower dose of DBA (100 μg), adducts **1** and **2** were almost exclusively formed (*14*). We now found that higher dose of DBA (300 μg) resulted in increased levels of adducts **3** and **4** (Table 1). It may be that at higher doses, the primary activation pathway to form DBADE−dG (adduct **1**) and DBADE−dA (adduct **2**) becomes saturated leading to an alternative pathway to produce adducts **3** and **4** (see Figure 1). They are most likely the corresponding dG and dA adducts of a common active species from the metabolism of DBA-5,6-DHD and DBA-3,4-DHD, the 1,2-epoxy-3,4,8,9-tetrahydroxy-1,2,3,4,8,9-hexahydroDBA (DBA-3,4,8,9-bis-DHD-1,2-epoxide, Figure 1), because (1) their relative positions on TLC are very similar to adducts **1** and **2** except that they are more polar, (2) they are not formed to an appreciable extent when DBADE is applied in vivo or in vitro, suggesting that most DBADE binding with DNA occurs before the DBA-5,6-epoxide and subsequent dihydrodiol can be formed, and (3) the precursor of the DBA-3,4,8,9-bis-DHD-1,2-epoxide, DBA-3,4,8,9-bis-DHD has been found in DBA metabolism (*12*) and it could be formed either through DBA-3,4-DHD or DBA-5,6-DHD by monooxygenase and epoxide-hydrolase (EH) in P450. These data support the hypothesis that bay-region 1,2-epoxides are important in the activation of DBA to genotoxic species. In addition, a novel mechanism for the formation of NHA−DNA adducts, involving the bis-DHD-epoxides, is consistent with that has been described for some PAHs, such as DB[a,h]A (*15*, *16*). However, definitive proof that a bis-DHD-epoxide of this type derived from DBA binds to DNA should await synthesis and analytical characterization of this bis-DHD-epoxide and its DNA adducts formed.

Mutations in papillomas and carcinomas as a result of exposure to DBA occur in codons 12, 13, and 61 of the Ha-*ras* gene. These transversions occur at the Gua and Ade bases, which are consistent with the formation of DNA adducts at dG and dA residues (Tables 1 and 2). These data are similar to that reported for DB[a,l]P in which lung tumors in the strain A/J mice following exposure to this PAH produced a mixture of both codon 12 and codon 61 Ki-*ras* mutations involving the first and second base of each codon (40). The predominant mutations were G → T transversions in the first base of codon 12, A → G transitions in the second base of codon 12 and A → T transversions in the second and third bases of codon 61 consistent with DNA adduct profiles even though the major adduct was identified as the product of the DB[a,l]P-diol-epoxide with a dA residue (*40*). It

Table 2. Results of Ha-Ras Mutation by EPCR Screening and Sequencing of DBA-Induced/TPA-Promoted Skin Papillomas and DBA-Induced Skin Carcinomas

compd	tumor type		codon	mutations	amino acid
DBA/TPA	papilloma		13	1-GGC → GTC	Gly → Val
			61	3-CAA → CTA	Gln → Leu
			61	2-CAA → AAA	Gln → Lys
acetone/TPA control	none				
no treatment control	none				
	carcinoma	82 weeks	12	GGA → GTA	Gly → Val
		82 weeks	13	GGA → GTA	Gly → Arg
		81 weeks	13	GGC → TGC	Gly → CYS
		75 weeks	61	CAA → CTA	Gln → Leu
acetone control	carcinoma	89 weeks	13	GGC → CGC	Gly → Arg
no treatment control	none				

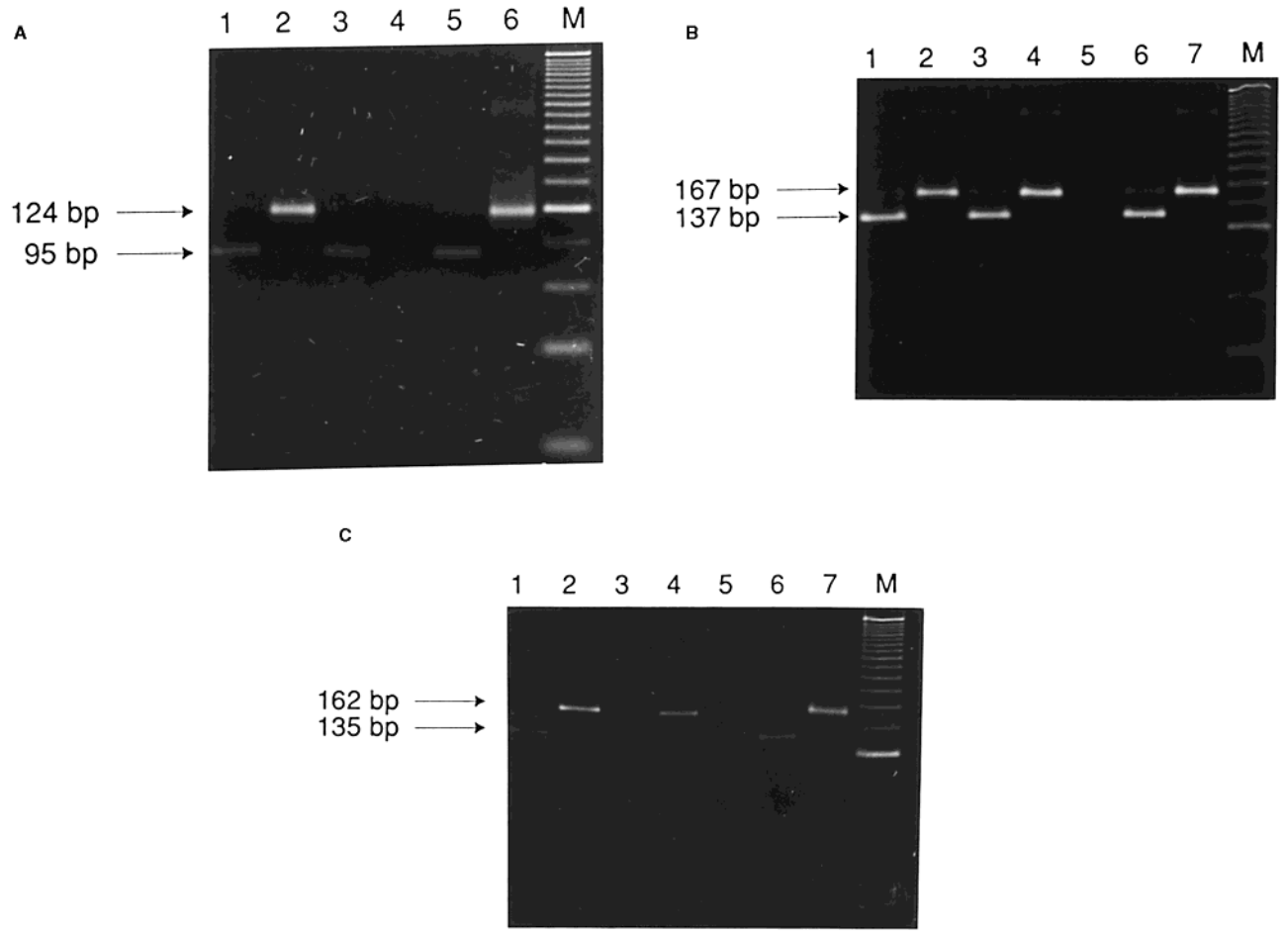

Figure 5. EPCR screening of DBA induced mouse skin tumor DNA for Ha-*ras* codon 12, 13, and 61 mutations. (A) Codon 12: lane 1, negative carcinoma; lane 2, positive carcinoma; 3, negative papilloma; 4, H_2O; 5, negative control; 6, positive control; lane *m*, 25 bp marker. (B) Codon 13: lane 1, negative carcinoma; lane 2, positive carcinoma; lane 3, negative papilloma; lane 4, positive papilloma; lane 5, H_2O; lane 6, negative control; lane 7, positive control; lane *m*, 25 bp marker. (C) Codon 61: lane 1, negative carcinoma; lane 2, positive carcinoma; lane 3, negative papilloma; lane 4, positive papilloma; lane 5, H_2O; lane 6, negative control; lane 7, positive control; lane *m*, 25 bp marker.

also has been shown that BaP-induced papillomas had mostly Ha-*ras* mutations (70%) in codons 12 and 13 and 20% mutations in codon 61. The major adduct was identified as the product of bay-region diol-epoxide with a dG residue (*41*). On the basis of the data for DBA, it would be apparent that NHA or PAH that induce adducts at both bases, induce both types of mutation. In contrast, 7,12-dimethyl-benz[*a*]anthracene induced skin carcinomas had exclusively Ha-*ras* codon 61 mutations with major adduction resulting extensively from the diol-epoxide and both dG and dA residues (*35*, *38*, *42*). In addition, 3,4-dihydroxy-1,2-epoxide-1,2,3,4-tetrahydrobenzo[*c*]phenanthrene induced mouse skin tumors produced significant number of mutations in the codon 61 (*43*), which is consistent with major adduction at dA residues (*44*). In another study, DB[a,j]A, DB[a,j]A-diol-epoxide, and 7,14-dimethyl-DB[a,j]A all produced papillomas with Ha-*ras* mutations in codon 61 in the second base even though adduction of the first two compounds appear to involve both dA and dG residues (*45*). It should be noted, however, that dibenz[c,h]acridine, an NHA, induced papillomas that showed exclusively Ha-*ras* codon 61 mutations although the adduction pattern is not known (*35*). These data indicate that the relationship of induced adducts of both bases with the mutation spectra is compound specific.

This work has shown that the environmental carcinogen DBA is activated and covalently binds to mainly dG

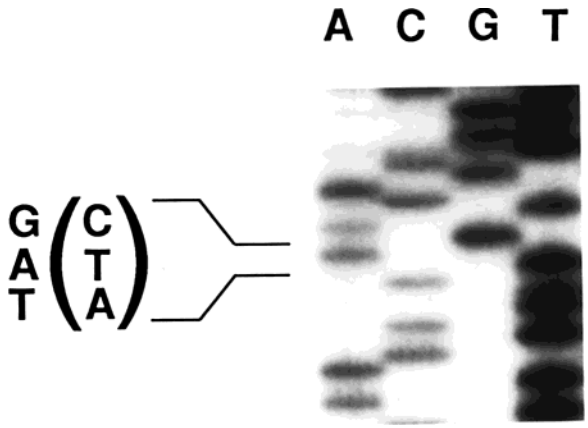

Figure 6. Autoradiogram of reverse cycle sequencing of a Ha-*ras* codon 61 EPCR positive papilloma DNA induced by a single topical application of 0.2 μmol of DBA, showing CAA → CTA.

as well as dA residues in mouse skin DNA via DBADE and an alternative pathway that through DBA-3,4,8,9-bis-DHD-1,2-epoxide to bind to dG and dA, is also involved. The mutational spectra are consistent with the DNA binding of DBA. Further investigation of the metabolic activation of DBA will require the preparation and application of the DBADE stereoisomers and the bis-DHD-epoxide metabolite(s).

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