[CANCER RESEARCH 60, 1849–1856, April 1, 2000]

# Unrepaired Fjord Region Polycyclic Aromatic Hydrocarbon-DNA Adducts in *ras* Codon 61 Mutational Hot Spots[1]

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## ABSTRACT

**The fjord region diol-epoxide metabolites of polycyclic aromatic hydrocarbons display stronger tumorigenic activities in rodent studies than comparable bay region diol-epoxides, but the molecular basis for this difference between fjord and bay region derivatives is not understood. Here we tested whether the variable effects of these genotoxic metabolites of polycyclic aromatic hydrocarbons may result from different DNA repair reactions. In particular, we compared the repairability of DNA adducts formed by bay region benzo[*a*]pyrene (B[*a*]P) diol-epoxides and the structurally similar but significantly more tumorigenic fjord region diol-epoxide metabolites of benzo[*c*]phenanthrene (B[*c*]Ph). For that purpose, we incorporated both types of polycyclic aromatic hydrocarbon adducts into known hot spot sites for carcinogen-induced proto-oncogene activation. Synthetic DNA substrates were assembled using a portion of human N-*ras* or H-*ras* that includes codon 61, and stereospecific B[*a*]P or B[*c*]Ph adducts were synthesized on adenine $N^6$ at the second position of these two *ras* codon 61 sequences. DNA repair was determined by incubating the site-directed substrates in human cell extracts, followed by electrophoretic visualization of radiolabeled oligonucleotide excision products. These cell-free assays showed that all tested bay region B[*a*]P-$N^6$-dA adducts are removed by the human nucleotide excision repair system, although excision efficiency varied with the particular stereochemical configuration of each B[*a*]P residue. In contrast, all fjord region B[*c*]Ph-$N^6$-dA adducts located in the identical sequence context and with exactly the same stereochemical properties as the corresponding B[*a*]P lesions were refractory to the nucleotide excision repair process. These findings indicate that the exceptional tumorigenic potency of B[*c*]Ph or related fjord region diol-epoxides may be attributed, at least in part, to slow repair of the stable base adducts deriving from the reaction of these compounds with DNA.**

## INTRODUCTION

Activating mutations in H-*ras*, K-*ras*, or N-*ras* proto-oncogene sequences are among the most frequent genetic changes associated with cancer. In fact, mutated *ras* genes can be found in adenocarcinomas of the colon, lung, and pancreas, as well as in thyroid tumors and in acute myeloid leukemia (1). In normal tissues, proteins encoded by the *ras* proto-oncogene family function as a molecular switch that regulates cell proliferation in response to external signals. In tumor cells, however, ras proteins are converted to constitutively active oncogene products by single point mutations that lead to a selective growth advantage (2, 3). A large number of physical and chemical carcinogens have been shown to promote such mutagenic processes in both proto-oncogenes and tumor suppressor genes. Polycyclic aromatic hydrocarbons, for example, are environmental air pollutants and food and water contaminants, as well as major genotoxic components of tobacco smoke. These hydrophobic and inert molecules are metabolically activated to diol-epoxide derivatives that react with DNA to form covalent base adducts (4–8). Previous studies have implicated various polycyclic aromatic hydrocarbon-DNA adducts in cell transformation processes mediated by *ras* oncogenes. In particular, exposure of plasmids carrying a human *ras* gene to diol-epoxide derivatives of polycyclic aromatic hydrocarbons generated a transforming oncogene when the damaged DNA was introduced into cultured fibroblasts (9). Subsequent analysis of DNA isolated from transformed cells showed that the resulting *ras* mutations are mainly confined to codon 61 (10). Exactly the same mutations have been found in tumors of mice or rats treated with polycyclic aromatic hydrocarbons, suggesting that *ras* codon 61 is an important molecular target of these ubiquitous carcinogens (11–13).

The metabolically activated polycyclic aromatic hydrocarbons have been classified into bay and fjord region diol-epoxides. This distinction indicates structural differences in the critical area of epoxide formation, involving either a crowded fjord region or a sterically less hindered bay region (Fig. 1). The epoxide group in the fjord region causes a distortion from planarity of the adjacent aromatic rings, whereas, in the case of the bay region compounds, steric hindrance effects are less pronounced, and the aromatic ring system remains planar (6, 7). Both bay and fjord region diol-epoxides can exist in a number of different stereoisomeric configurations that display variable biological activities. Among the bay region B[*a*]P[3] derivatives, the (+)-*anti*-B[*a*]P diol-epoxide shown in Fig. 1 is the most genotoxic isomer in mammalian systems (14–16). The biological activity of fjord region B[*c*]Ph diol-epoxides also depends on their stereochemical configuration, with the (−)-*anti*-B[*c*]Ph diol-epoxide illustrated in Fig. 1 being the most potent isomer in animal tumorigenesis assays (17, 18). Our present study was instigated by previous reports showing that (−)-*anti*-B[*c*]Ph diol-epoxide, as well as analogous metabolites of other fjord region compounds, is up to 10 times more active in inducing tumors in animals than any known bay region diol-epoxide derivative (17–21). This exceptional tumorigenic activity of the metabolites with sterically hindered fjord regions relative to structurally similar compounds with unhindered bay regions is of critical interest for understanding the mechanisms of tumor initiation by chemical carcinogens in detail. No obvious correlation was found between the DNA reactivity of individual diol-epoxides stereoisomers and their known tumorigenic effects (22, 23), suggesting that their variable genotoxic potency is influenced by the distinct molecular properties of the ultimate adducts in DNA. In this study, we tested the hypothesis that the genotoxicity of B[*c*]Ph diol-epoxides may be enhanced by inefficient repair of the fjord region DNA adducts generated by these particular compounds. Because induction of carcinomas in rodents by polycyclic aromatic hydrocarbons has been shown to involve A to T transitions in the second position of *ras* codon 61 (24), we constructed

Received 9/24/99; accepted 2/3/00.

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[1] Supported by Swiss National Science Foundation Grant 3100-050518.97 (to H. N.), NIH/National Cancer Institute Grants CA 20851 (to N. E. G) and 17613 (to S. A.), and Deutsche Forschungsgemeinschaft Grant SFB 302 (to A. S.).

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[3] The abbreviations used are: B[*a*]P, benzo[*a*]pyrene; B[*c*]Ph, benzo[*c*]phenanthrene; HPLC, high-performance liquid chromatography.

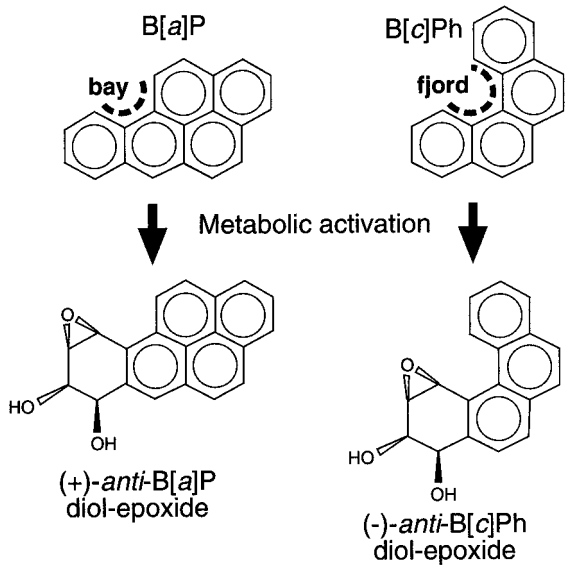


Fig. 1. Stereochemical configuration of the major genotoxic diol-epoxide metabolites of B[*a*]P and B[*c*]Ph. Derivatives with sterically crowded fjord regions display exceptional tumorigenic activities relative to structurally similar polycyclic aromatic hydrocarbons with sterically less hindered bay regions.

stereochemically defined B[*a*]P-dA and B[*c*]Ph-dA adducts at the second base of two distinct *ras* codon 61 contexts and measured excision repair activity elicited by these site-specific lesions. Our results show that human nucleotide excision repair enzymes are indeed able to process a broad range of bay region B[*a*]P-dA lesions with comparable efficiencies. However, we found that the same excision repair system fails to remove the major fjord region B[*c*]Ph-dA adducts from the tested N-*ras* and H-*ras* codon 61 sequences.

## MATERIALS AND METHODS

**Enzymes and Cell Extracts.** T4 polynucleotide kinase and T4 DNA ligase were purchased from Life Technologies, Inc. Creatine phosphokinase, RNase A, and proteinase K were from Boehringer Mannheim. Nucleotide excision repair-proficient cell extracts were prepared from HeLa cells and the mouse embryonic fibroblast cell line F20 as described previously (25).

**Modified Oligonucleotides.** Site-directed B[*a*]P-$N^2$-dG lesions were obtained by reacting the 11-mer oligonucleotide 5′-CCATCGCTACC-3′ or the 14-mer oligonucleotide 5′-ATACCCGGGACATC-3′ with (±)-7$\beta$,8$\alpha$-dihydroxy-9$\alpha$,10$\alpha$-epoxy-7,8,9,10-tetrahydro-benzo(*a*)pyrene (racemic anti-B[*a*]P diol-epoxide) as described elsewhere (26, 27). The modified oligonucleotides were purified by reverse-phase HPLC chromatography using octyldecyl silane Hypersyl columns. To establish the stereochemical identity and purity of the chromatographic fractions, the samples were enzymatically digested to the nucleoside level. The stereochemistry of each modified 2′-deoxyguanosine species was established by HPLC chromatography and circular dichroism using appropriate anti-B[*a*]P-$N^2$-dG standards (28, 29).

Site-directed (+)- or (−)-*trans-anti*-B[*a*]P-$N^6$-dA adducts in the N-*ras* oligonucleotide 5′-CGGACA∗AGAAG-3′ or in the unrelated sequences 5′-GGTCA∗CGAG-3′ and 5′-CTCTCA∗CTTCC, as well as site-directed (+)- or (−)-*trans-anti*-B[*c*]Ph-$N^6$-dA adducts in the N-*ras* oligonucleotide 5′-CCGGACA∗AGAAGC-3′ (the modified adenosine is indicated by the *asterisk*), were obtained by incorporating appropriate phosphoramidites in the automated DNA synthesizer technique (30–32). After DNA synthesis, the modified oligonucleotides were deprotected and purified by reverse-phase HPLC (32). The identity of individual adducts was assessed after enzymatic degradation as described previously (29, 32) and confirmed by negative ion mode electrospray mass spectrometry (33). Similarly, the H-*ras* oligonucleotides 5′-GGCCA∗GGAGGAGTACAGC-3′ containing a (+)-*trans-anti*-B[*a*]P-$N^6$-dA, (−)-*trans-anti*-B[*a*]P-$N^6$-dA, (+)-*trans-syn*-B[*a*]P-$N^6$-dA, (−)-*trans-syn*-B[*a*]P-$N^6$-dA, (+)-*trans-anti*-B[*c*]Ph-$N^6$-dA, or (−)-*trans-anti*-B[*c*]Ph-$N^6$-dA adduct (indicated by the *asterisk*) were synthesized using the appropriate phosphoramidites. After oligonucleotide synthesis and purification, the presence of individual adducts was established by fluorescence emission spectroscopy (30–32). The stereochemistry of each adduct was confirmed by comparing circular dichroism spectra of the respective mononucleosides with those documented in the literature (34, 35).

**Excision Repair Substrates.** To obtain internally labeled DNA duplexes of 139–146 bp, the modified oligonucleotides or their unmodified controls (70 pmol) were 5′ end-labeled with [$\gamma$-$^{32}$P]ATP (7000 Ci/mmol; ICN Pharmaceuticals) and mixed with five other partially overlapping oligonucleotides (100 pmol) that were phosphorylated with cold ATP. The oligonucleotides were annealed and ligated in the presence of T4 DNA ligase, followed by electrophoretic purification of the full-length fragments as described previously (36, 37).

**Excision Assay.** Oligonucleotide excision reactions (36–38) contained (in 25 $\mu$l) 35 mM HEPES-KOH (pH 7.9); 60 mM KCl; 40 mM NaCl; 5.6 mM $MgCl_2$; 2 mM ATP; 80 $\mu$M each of dATP, dCTP, dGTP, and TTP; 0.8 mM DTT; 0.4 mM EDTA; 3.4% (v/v) glycerol; 5 $\mu$g of BSA, 5 fmol (75,000 dpm) of radiolabeled DNA substrate; and 50 $\mu$g (in protein equivalents) of HeLa cell extract. After the indicated incubation times at 30°C, reactions were stopped by the addition of SDS (0.3% w/v) and proteinase K (200 $\mu$g/ml), followed by proteinase K digestion for 15 min at 37°C. DNA was purified by phenol-chloroform extraction and resolved by electrophoresis in 10% polyacrylamide denaturing gels, after which the radiolabeled excision products were visualized by autoradiography. The relative levels of excision were determined by densitometric analysis of oligonucleotides in the 24- to 32-mer size range on appropriately exposed X-ray films (using a Molecular Dynamics computing densitometer with ImageQuant software). The linearity of each densitometric quantification was confirmed by counting Cerenkov radiations of the corresponding gel slices.

## RESULTS

**Excision of the (−)-*cis-anti*-B[*a*]P-$N^2$-dG Standard.** The diol-epoxide metabolites of polycyclic aromatic hydrocarbons react with double-stranded DNA by either *trans* or, less frequently, *cis* opening of the epoxide ring, generating base adducts mainly at position $N^2$ of guanine or position $N^6$ of adenine (4–8). Nucleotide excision repair is the only DNA repair mechanism that is able to eliminate the stable carcinogen adducts resulting from this genotoxic reaction (39–41). In human cells, the nucleotide excision mode of DNA repair is accomplished by cleavage of damaged strands on either side of the lesion, thus releasing oligomeric products of 24–32 residues in length (42–45). To measure this excision repair activity, we constructed DNA substrates of 139–146 bp carrying a site-directed polycyclic aromatic hydrocarbon adduct on a single deoxyguanosine or deoxyadenosine residue (Fig. 2*A*). Before substrate assembly, the central oligonucleotides were labeled with [$\gamma$-$^{32}$P]ATP at the 5′ ends such that the linear duplexes contained an internal radiolabel in the vicinity of the damaged site (Fig. 2*A*). After purification, these double-stranded fragments were incubated in a standard HeLa cell extract that is proficient in nucleotide excision repair activity when supplemented with ATP and deoxyribonucleoside triphosphates (25, 36–38). In this assay, human excision repair enzymes generated radioactive oligonucleotide products that were separated from substrate DNA by denaturing gel electrophoresis and visualized by autoradiography. A typical reaction in human cell extracts is illustrated in the autoradiogram of Fig. 2*B*, where we assessed excision of B[*a*]P-$N^2$-dG adducts in the artificial sequence 5′-TCGCT-3′. As reported previously (41), this comparison revealed considerable differences in repair efficiency, but all tested stereochemical variants of the B[*a*]P-$N^2$-dG lesion were processed by the human nucleotide excision repair system, yielding characteristic excision products of 24–32 nucleotides (Fig. 2*B*, *Lanes 2–5*). No excision products were released from undamaged control substrate (Fig. 2*B*, *Lane 1*), although intact full-length substrate as well as radioactive bands generated by nonspecific nuclease activity can be

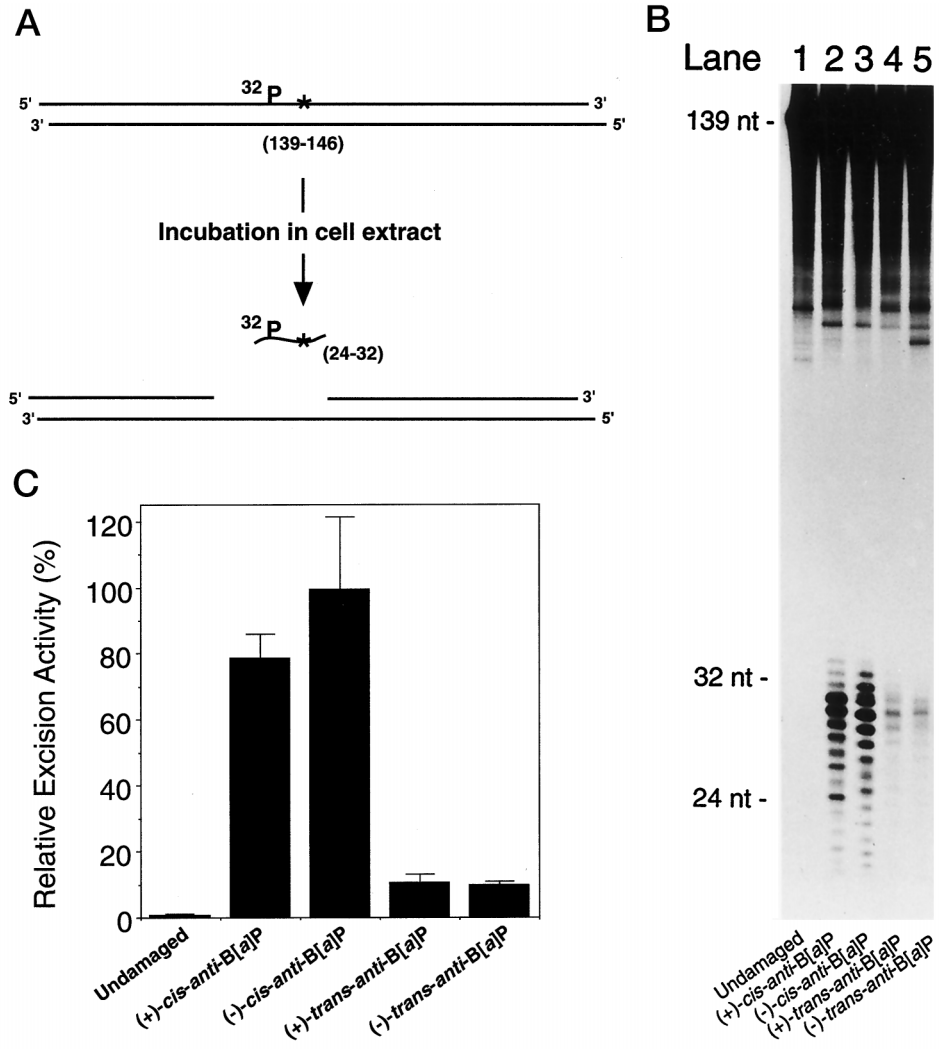


Fig. 2. Determination of human nucleotide excision repair activity. *A*, general scheme illustrating the oligonucleotide excision assay in cell extracts. The adducted base is indicated by the *asterisk*. *B*, autoradiogram of a representative polyacrylamide gel showing excision of B[*a*]P-dG standards in HeLa cell extract. All reactions contained exactly the same amount of radioactive substrate (75,000 dpm). *C*, quantification of excision products in the 24–32-nucleotide range by laser scanning densitometry. All values represent the percentage of excision obtained in response to the (−)-*cis-anti*-B[*a*]P-$N^2$-dG adduct.

observed at similar levels at the top of the gel. The repair efficiency observed during 40-min incubations in cell extract (<10%) is comparable with the rate of global repair in intact human cells, in which moderately distorting DNA adducts such as cyclobutane pyrimidine dimers have been shown to be removed with a half-life of up to 12 h (42). In any case, quantitative analysis of specific 24–32-nucleotide-long products by laser scanning densitometry showed that the rare (−)-*cis-anti*-B[*a*]P-$N^2$-dG lesion rather than the quantitatively more frequent (+)-*trans-anti*-B[*a*]P-$N^2$-dG isomer constitutes a preferred excision repair substrate (Fig. 2*C*). Therefore, this (−)-*cis-anti*-B[*a*]P-$N^2$-dG adduct was used as a positive standard in subsequent repair reactions.

**Excision of Polycyclic Aromatic Hydrocarbon Adducts from N-*ras* Codon 61.** All B[*c*]Ph diol-epoxide stereoisomers react with DNA preferentially at the $N^6$ residue of adenine, in most cases by *trans* opening of the epoxide ring (23). Also, induction of carcinomas in rodents by polycyclic aromatic hydrocarbons involves mutations of *ras* genes mainly at adenine in the second position of codon 61, indicating that DNA lesions at this site are particularly prone to activate *ras* proto-oncogenes (24). These previous findings prompted us to test the repair of (+)- and (−)-*trans* adducts on adenine at the second position of two different *ras* codon 61 sequences. A first series of substrates contained the sequence for codons 60–62 of the human N-*ras* gene, and a total synthesis method was used to generate (+)- or (−)-*trans*-B[*a*]P-$N^6$-dA as well as (+)- or (−)-*trans*-B[*c*]Ph-$N^6$-dA adducts at the second position of codon 61. In these constructs, the site-specific adducts were located in the context 5′-ACA∗AG-3′, where the modified adenosine in N-*ras* codon 61 (CA∗A) is indicated by the *asterisk*. Repair incubations in HeLa cell extract demonstrated that both (+)- and (−)-*trans*-anti-B[*a*]P-$N^6$-dA adducts were able to elicit excision repair activity because, in both cases, we noted characteristic excision products within the typical range of 24–32 nucleotides (Fig. 3*A*, *Lanes 2* and *3*). In contrast to these DNA lesions derived from B[*a*]P, we found that (+)- and (−)-*trans-anti*-B[*c*]Ph-$N^6$-dA adducts located at the same second position of N-*ras* codon 61 failed to stimulate oligonucleotide excision (Fig. 3*A*, *Lanes 5* and *6*). We obtained identical results, *i.e.*, moderate excision of B[*a*]P-$N^6$-dA adducts but no excision of the corresponding B[*c*]Ph-$N^6$-dA adducts, when the same experiment was repeated using independent substrate and HeLa cell extract preparations (gel not shown). As expected, no oligonucleotide excision was observed after incubation of undamaged control substrates in the HeLa cell extracts (Fig. 3*A*, *Lane 4*).

On quantitative analysis by laser scanning densitometry, the repair activity induced by (+)- or (−)-*trans-anti*-B[*a*]P-$N^6$-dA was 20–30% of that observed with the (−)-*cis-anti*-B[*a*]P-$N^2$-dG standard (Fig. 3*B*). Thus, we found that (+)- or (−)-*trans-anti*-B[*a*]P-$N^6$-dA adducts were processed with an efficiency similar to that of the corresponding (+)- or (−)-*trans-anti*-B[*a*]P-$N^2$-dG adducts of Fig. 2. Also, quantitative evaluation of three independent experiments confirmed the complete absence of oligonucleotide excision in response to B[*c*]Ph

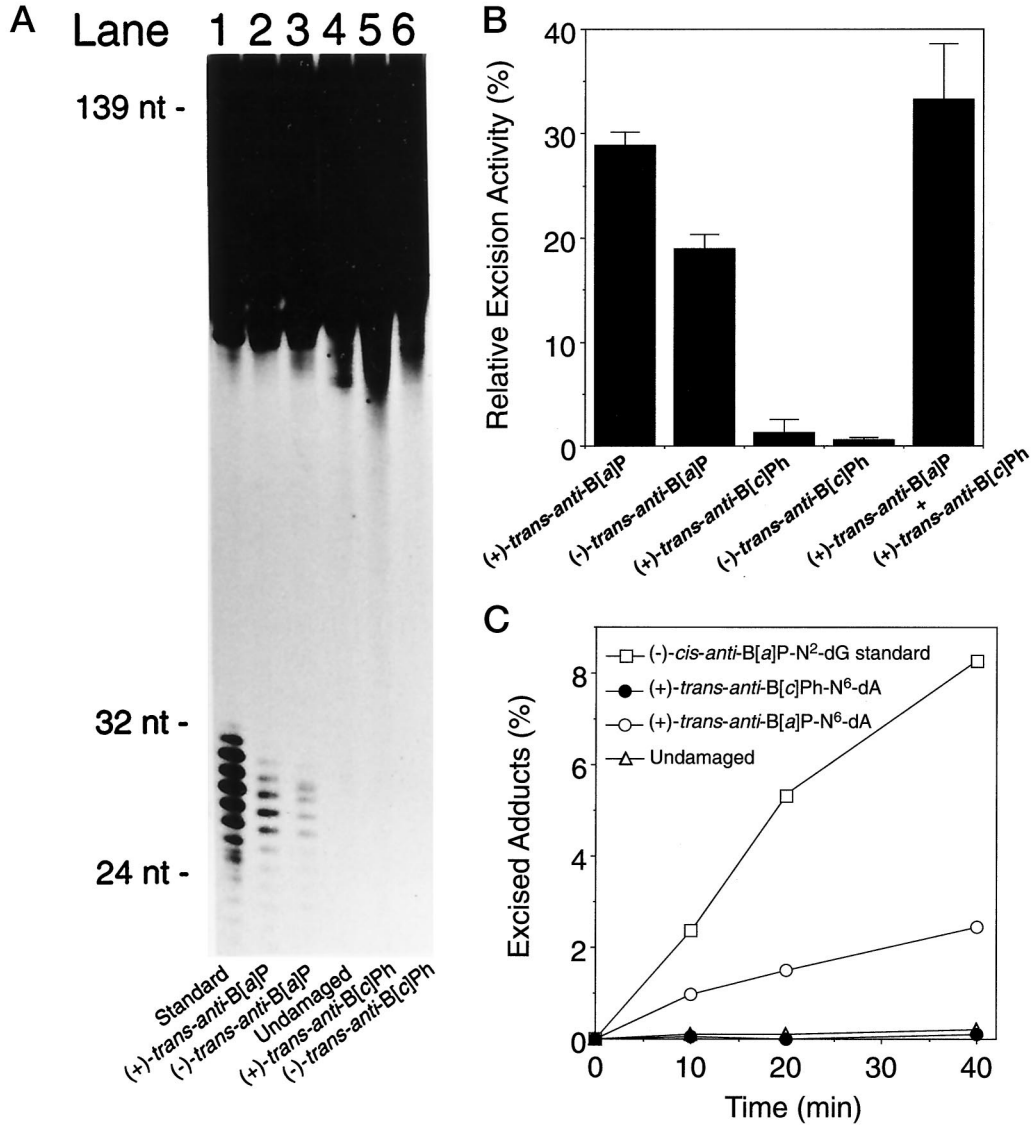


Fig. 3. Excision of polycyclic aromatic hydrocarbon adducts from the N-*ras* codon 61 context [ACA∗AG (the *asterisk* denotes the damaged base)]. *A*, polyacrylamide gel showing the differential excision of B[*a*]P-$N^6$-dA and B[*c*]Ph-$N^6$-dA adducts. All incubations contained the same amount of radioactive substrate (75,000 dpm). A reaction with the (−)-*cis-anti*-B[*a*]P-$N^2$-dG standard is shown in *Lane 1*. *B*, quantification of excision products in the 24–32-nucleotide range by laser scanning densitometry. All values represent the percentage of excision obtained in response to the (−)-*cis-anti*-B[*a*]P-$N^2$-dG standard. *C*, time course of oligonucleotide release in human cell extract.

adducts at the second position of N-*ras* codon 61 (Fig. 3*B*). To rule out the possibility that the DNA preparations with B[*c*]Ph-$N^6$-dA adducts contained a chemical contaminant that may inhibit DNA repair in a nonspecific manner, we performed coincubation reactions in which the actively repaired (+)-*trans-anti*-B[*a*]P-$N^6$-dA adduct was mixed with equal amounts of substrate containing the unrepaired (+)-*trans-anti*-B[*c*]Ph-$N^6$-dA adduct. These coincubation experiments yielded the expected amounts of B[*a*]P excision, thereby excluding the presence of a repair inhibitor in the fractions of B[*c*]Ph-damaged DNA (Fig. 3*B*). Also, time course experiments showed that differential repair of B[*a*]P and B[*c*]Ph adducts in N-*ras* codon 61 is already visible during early stages of the reaction (Fig. 3*C*). Finally, experiments performed with mouse instead of human cell extract as a source of repair factors revealed identical excision preferences. In fact, the mouse nucleotide excision repair enzymes were able to remove B[*a*]P-$N^6$-dA adducts located in the N-*ras* codon 61 sequence but, as observed with the human system, there was marginal or no repair of the corresponding B[*c*]Ph adducts (gels not shown).

**Comparison between B[*a*]P-DNA Adducts Targeted to Different Sequence Environments.** Effective excision of B[*a*]P-$N^6$-dA adducts was not limited to the N-*ras* codon 61 sequence context 5′-ACA∗AG-3′ (the *asterisk* denotes the modified adenosine). A similar level of excision was observed when the same (+)- and (−)-*trans-anti*-B[*a*]P-$N^6$-dA adducts were located in the unrelated sequences 5′-TCA∗CG-3′ (Fig. 4, *Lanes 5* and *6*) or 5′-TCA∗CT-3′ (data not shown). Interestingly, all these (+)- and (−)-*trans-anti*-B[*a*]P-$N^6$-dA adducts were processed nearly as efficiently as a series of single (+)-*trans-anti*-B[*a*]P adducts covalently linked to position $N^2$ of guanine in the sequence 5′-CGGGA-3′ (Fig. 4, *Lanes 1–3*), which, because of its run of contiguous guanines, often represents a hot spot for B[*a*]P-induced mutagenesis (46). Thus, we observed excision of B[*a*]P-$N^6$-dA or B[*a*]P-$N^2$-dG adducts regardless of the particular nucleotide sequence environment; therefore, the ability to remove these adducts (although with variable efficiency) seems to be a general property of the human nucleotide excision repair system.

**Excision of Polycyclic Aromatic Hydrocarbon Adducts from H-*ras* Codon 61.** The lack of B[*c*]Ph-dA excision from N-*ras* codon 61 led us to test the susceptibility to excision repair of the same adducts in a comparable portion of the human H-*ras* proto-oncogene. In this case, DNA substrates contained the sequence for codons 60–65 of human H-*ras*, and a total synthesis method was again used to generate B[*a*]P-$N^6$-dA and B[*c*]Ph-$N^6$-dA adducts at the second position of codon 61. Consistent with the results obtained in all

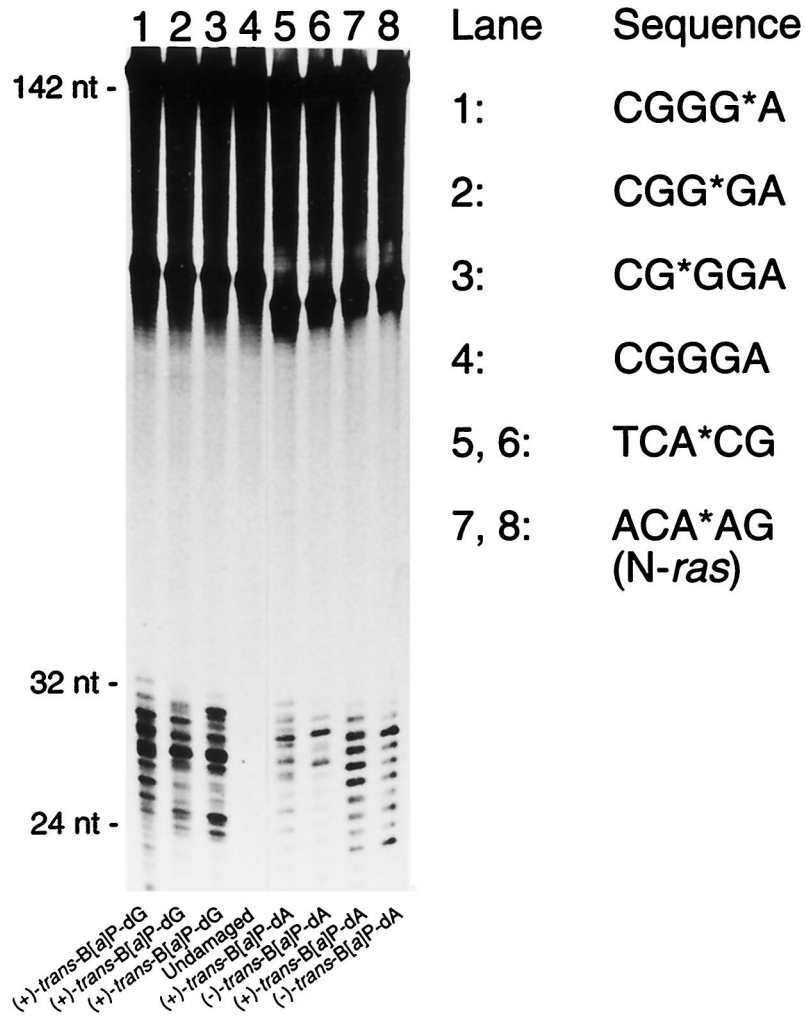


Fig. 4. Nucleotide excision repair activity in response to B[*a*]P-dG and B[*a*]P-dA adducts. The polyacrylamide gel demonstrates that all tested bay region B[*a*]P adducts are processed by the human nucleotide excision repair system. *Lanes 1–3*, reactions with substrates containing a site-directed B[*a*]P-$N^2$-dG adduct; *Lane 4*, control incubation with undamaged DNA; *Lanes 5–8*, reactions with substrates containing a site-directed B[*a*]P-$N^6$-dA adduct. The sequence environments are indicated, with the *asterisk* denoting the adducted base.

previous experiments, we found that both (+)- and (−)-*trans-anti*-B[*a*]P-$N^6$-dA adducts are removed from the H-*ras* codon 61 sequence 5′-CCA∗GG-3′ (Fig. 5*A*, *Lanes 1* and *2*). The specific excision products are evidenced in the longer autoradiographic exposure shown in Fig. 5*B* (*Lanes 1* and *2*). A direct comparison between *Lanes 1* and *7* (Fig. 5*B*) suggests that the (+)-*trans-anti*-B[*a*]P-$N^6$-dA adduct is excised more efficiently from the N-*ras* than from the H-*ras* sequence. Interestingly, we also found comparable levels of oligonucleotide excision when the configuration of these B[*a*]P-$N^6$-dA adducts was changed from *anti* to *syn* (Fig. 5, *A* and *B*, *Lanes 3* and *4*). However, excision repair of adenine lesions in the identical sequence context was abolished when the bay region B[*a*]P adduct was replaced by the fjord region B[*c*]Ph residue. In fact, (+)- and (−)-*trans-anti*-B[*c*]Ph-$N^6$-dA adducts located at the same second position of H-*ras* codon 61 were unable to stimulate any observable excision of the modified sequences (Fig. 5, *A* and *B*, *Lanes 5* and *6*). Laser scanning densitometry of 24–32-nucleotide-long excision products from three different assays, each performed with independent substrate and cell extract preparations, support our conclusion that the tested B[*c*]Ph-$N^6$-dA adducts are resistant to removal by human excision repair enzymes (Fig. 5*C*).

## DISCUSSION

In this report, we used *ras* codon 61 sequences as the main DNA targets to compare the removal of bay region B[*a*]P and fjord region B[*c*]Ph adducts by the human nucleotide excision repair system. These experiments were prompted by rodent carcinogenesis studies demonstrating that the diol-epoxide metabolites of fjord region polycyclic aromatic hydrocarbons are significantly more potent tumorigens than comparable bay region compounds (17–21). The terms “bay” and “fjord” indicate different degrees of steric hindrance introduced into these molecules on their conversion to reactive diol-epoxide derivatives (Fig. 1). Using a standard assay in cell extracts, we found that human nucleotide excision repair enzymes process a broad range of bay region B[*a*]P lesions, including the (+)- or (−)-*trans-anti*-B[*a*]P-dA adducts that are formed relatively inefficiently when B[*a*]P diol-epoxides react with native DNA (29). However, we observed that the same excision repair system fails to remove the major fjord region (+)- or (−)-*trans-anti*-B[*c*]Ph-$N^6$-dA adducts resulting from the reaction of (+)- or (−)-*anti*-B[*c*]Ph diol-epoxide with DNA. This lack of B[*c*]Ph excision was established using the codon 60–62 context of both human N-*ras* (GGACA∗AGAA) and H-*ras* (GGCCA∗GGAG) proto-oncogene sequences, in which the *asterisks* denote the covalently modified adenosine at the second position of codon 61.

The differential repair activity in response to B[*a*]P- and B[*c*]Ph-$N^6$-dA adducts is related to the distinct conformational properties that these lesions impose on the DNA double helix. For example, nuclear magnetic resonance analysis showed that the bay region (−)-*trans-anti*-B[*a*]P-$N^6$-dA adduct is inserted into the Watson-Crick double

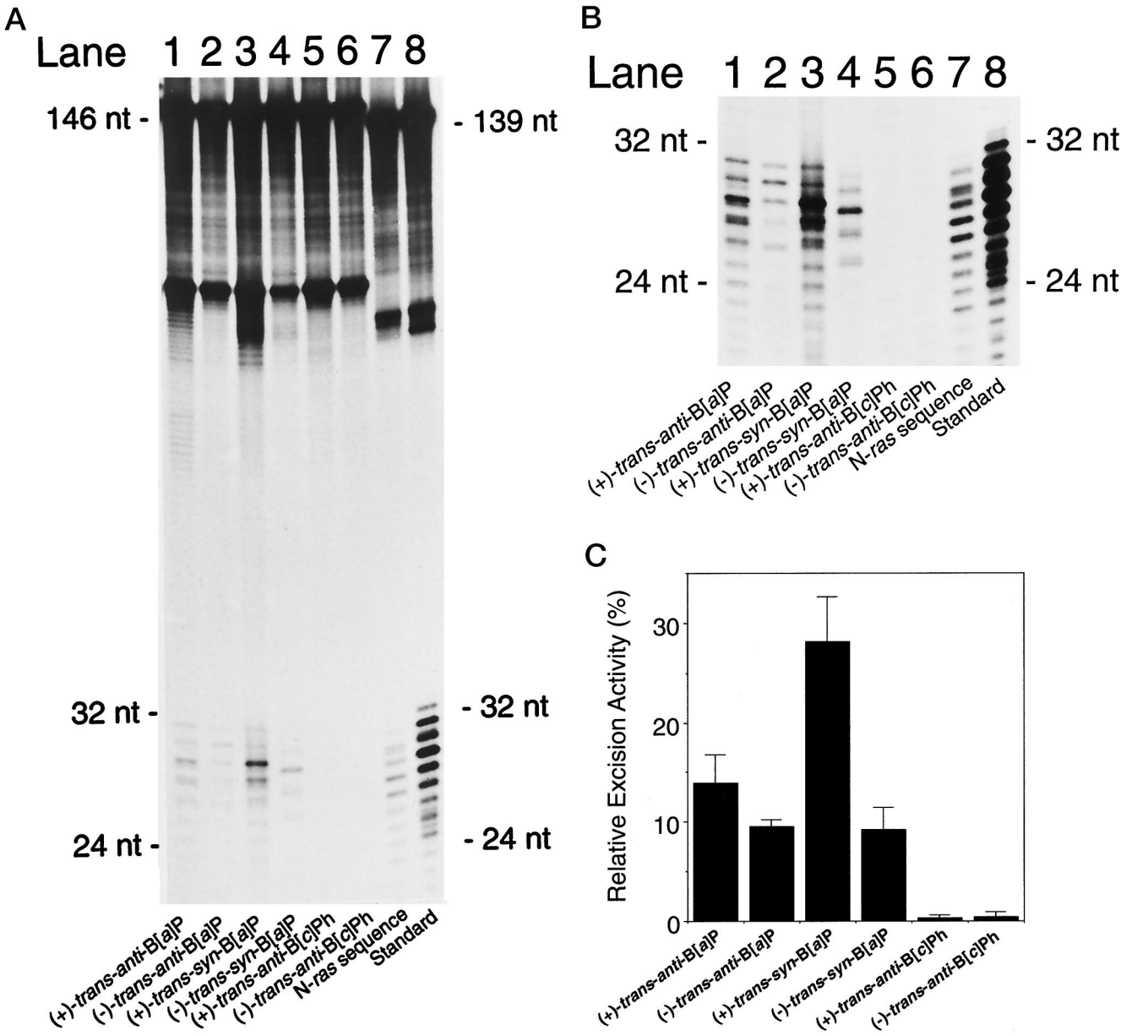


Fig. 5. Excision of polycyclic aromatic hydrocarbon adducts from the H-*ras* codon 61 context (CCA∗GG). *A*, polyacrylamide gel showing the differential excision of B[*a*]P-$N^6$-dA and B[*c*]Ph-$N^6$-dA adducts from H-*ras* codon 61. For comparison, *Lane 7* shows the excision of (+)-*trans-anti*-B[*a*]P-$N^6$-dA from the corresponding codon 61 context of N-*ras* (ACA∗AG). *Lane 8* contains a reaction with the (−)-*cis-anti*-B[*a*]P-$N^2$-dG standard. *B*, longer autoradiographic exposure of the bottom part of the gel containing the specific excision products in the size range of 24–32 nucleotides. This section of the gels was used for quantitative analysis. *C*, quantification of excision products in the 24–32-nucleotide range by laser scanning densitometry. All values represent the percentage of excision obtained with the (−)-*cis-anti*-B[*a*]P-$N^2$-dG adduct.

helix, thereby inducing a modest but detectable distortion of base pairing interactions at the site of covalent modification (47). In contrast, the corresponding fjord region (+)- and (−)-*trans-anti*-B[*c*]Ph-$N^6$-dA adducts are incorporated into the double helix by an intercalative mode that retains normal Watson-Crick base pairing throughout the modified duplexes (48, 49). These nuclear magnetic resonance models are supported by differences in the thermodynamic stability of short double-stranded fragments containing a single bay region or fjord region polycyclic aromatic hydrocarbon adduct. In fact, the DNA duplex melting point is significantly lowered by (+)- or (−)-*trans-anti*-B[*a*]P-$N^6$-dA lesions (50), indicating a weakening of Watson-Crick hydrogen bonds, whereas the melting point is unchanged in the presence of the corresponding B[*c*]Ph-$N^6$-dA lesions (32). On the basis of these biophysical studies, the poor repairability of B[*c*]Ph-$N^6$-dA lesions confirms recent reports from one of our laboratories in which the use of artificial DNA substrates led to the conclusion that destabilization of local base pairing constitutes an essential molecular signal for recruitment of the human nucleotide excision repair system (38, 51). We also found that this specific conformational requirement for nucleotide excision is mediated by the extraordinary affinity of the xeroderma pigmentosum group A protein-replication protein A complex for sites of defective base pairing (51). Thus, nondistorting carcinogen-DNA adducts that maintain normal Watson-Crick base pairing, such as, for example, (+)- or (−)-*trans*-anti-B[*c*]Ph-$N^6$-dA, fail to attract the initial recognition subunits of the excision repair system and, as a consequence, remain unrepaired. Interestingly, we found that poor repairability is not limited to the B[*c*]Ph-$N^6$-dA lesions. In fact, similar experiments in HeLa cell extract showed that at least four other representatives with the fjord structural motif, *i.e.*, (+)- and (−)-*trans-anti*-benzo[*g*]chrysene-$N^6$-

dA (containing the same number of aromatic ring systems as B[*a*]P) or (+)- and (−)-*trans*-*anti*-dibenzo[*a*,*l*]pyrene-$N^6$-dA (containing an additional aromatic ring compared to B[*a*]P) are equally unable to induce excision repair reactions.[4] Taken together, these findings raise the possibility that stable adenine adducts of fjord region polycyclic aromatic hydrocarbons might be generally incompatible with recognition by the human nucleotide excision repair system.

In summary, our report reveals that the exceptional tumorigenic activity of B[*c*]Ph diol-epoxides or other similar fjord region metabolites correlates with the formation of deoxyadenosine adducts that, at least in some crucial target sequences such as H-*ras* or N-*ras* codon 61, are resistant to removal by DNA repair enzymes. These results are consistent with the expectation that inefficient excision constitutes an important determinant of tumorigenic potency. As a consequence, even minor polycyclic aromatic hydrocarbon lesions that are formed with only low efficiency may exert disproportionately large genotoxic effects because of extremely slow repair. This report supports the notion that improved risk assessment procedures, particularly in the problematic low-dose range, require a more complete knowledge of the efficiency with which relevant carcinogen adducts are removed from critical mutational hot spots. Future experiments will be devoted to testing whether excision from short DNA fragments in cell extract accurately represents how the nucleotide excision repair system recognizes carcinogen-DNA adducts formed in oncogene or tumor suppressor gene sequences in intact cells.

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