

## Repair of DNA lesions induced by polycyclic aromatic hydrocarbons in human cell-free extracts: involvement of two excision repair mechanisms *in vitro*

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**Polycyclic aromatic hydrocarbons (PAHs) are significant environmental pollutants representing an important risk factor in human cancers. DNA adducts formed by the ultimate carcinogens of PAHs are potentially toxic, mutagenic and carcinogenic. DNA repair represents an important defense system against these genotoxic insults. Using a human cell-free system we have examined repair of DNA lesions induced by several PAH dihydrodiol epoxides, including *anti*-(±)-benzo[*a*]pyrene-*trans*-7,8-dihydrodiol-9,10-epoxide, *anti*-(±)-benz[*a*]anthracene-*trans*-3,4-dihydrodiol-1,2-epoxide, *anti*-(±)-benz[*a*]anthracene-*trans*-8,9-dihydrodiol-10,11-epoxide, *anti*-(±)-benzo[*b*]fluoranthene-*trans*-9,10-dihydrodiol-11,12-epoxide and *anti*-(±)-chrysene-*trans*-1,2-dihydrodiol-3,4-epoxide. Effective repair of DNA damage induced by these five PAH metabolites was detected. Two distinct mechanisms of excision repair were observed. The major repair mechanism is nucleotide excision repair (NER). The other mechanism is independent of NER and correlated with the presence of apurinic/aprimidinic sites in the damaged DNA, thus presumably reflecting base excision repair (BER). However, the contribution of BER to different PAH lesions varied *in vitro*. These results suggest the possibility that BER may also play an important role in repair of certain PAH-induced DNA lesions.**

### Introduction

Polycyclic aromatic hydrocarbons (PAHs\*) are a group of significant environmental pollutants. They are produced by incomplete combustion of organic materials and are commonly found in tobacco smoke and automobile exhaust. These compounds are metabolically activated in cells to yield highly reactive bay region dihydrodiol epoxide derivatives (1,2). Dihydrodiol epoxides are electrophilic and can effectively attack DNA, forming covalently linked bulky adducts on DNA bases (2). These adducts cause structural changes in DNA, thus leading to disruption of normal cellular functions, such as transcription and replication (3). Furthermore, if not repaired, damaged nucleotides can result in mutations during replication (4–7). Mutations that activate or inactivate critical genetic targets, such as proto-oncogenes and tumor suppressor genes

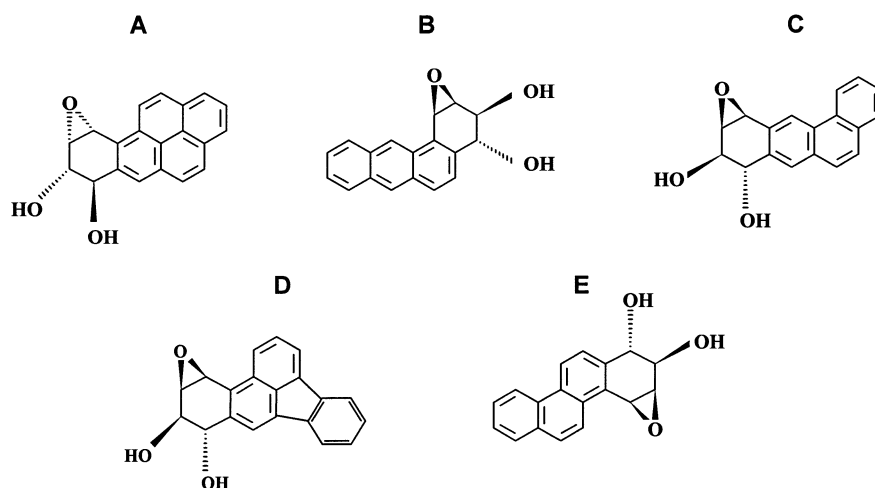
\***Abbreviations:** AP, apurinic/aprimidinic; BADE-I, (±)benz[*a*]anthracene-*trans*-3,4-dihydrodiol-1,2-epoxide; BADE-II, (±)benz[*a*]anthracene-*trans*-8,9-dihydrodiol-10,11-epoxide; BER, base excision repair; BFDE, (±)benzo[*b*]fluoranthene-*trans*-9,10-dihydrodiol-11,12-epoxide; BPDE, (±)benzo[*a*]pyrene-*trans*-7,8-dihydrodiol-9,10-epoxide; CDE, (±)chrysene-*trans*-1,2-dihydrodiol-3,4-epoxide; DTT, dithiothreitol; NER, nucleotide excision repair; PAHs, polycyclic aromatic hydrocarbons; XP, xeroderma pigmentosum.

respectively, provide a plausible mechanistic basis for carcinogenic transformation by PAHs.

Excision repair is a major cellular response to DNA damage that corrects genomic lesions. Two excision repair pathways are known in mammalian cells: nucleotide excision repair (NER) and base excision repair (BER) (3). NER involves damage recognition, dual endonucleolytic incisions flanking the damage, excision of an oligonucleotide containing the damage, DNA repair synthesis and DNA ligation (3). This pathway is biochemically complex, requiring >20 different proteins (8). NER is unique in that it is able to repair a large spectrum of very different damage (3). BER is initiated by a DNA glycosylase, which catalyzes hydrolysis of the *N*-glycosyl bond between the base and the sugar-phosphate backbone, releasing the damaged base and leaving an apurinic/aprimidinic (AP) site in the DNA. Incision at the AP site is mediated by an AP endonuclease, followed by cleavage of the 5' deoxyribose phosphate moiety. DNA repair synthesis fills in one or a few nucleotides and DNA ligation completes the repair pathway (9,10). Several DNA glycosylases have been identified and each has its specific substrates (3). In addition to certain damaged and inappropriate bases, AP sites are also efficiently repaired by BER.

Considerable information has been obtained on the fundamental mechanism of NER and BER using model repair substrates. However, the repair of DNA lesions induced by various PAHs (with the exception of benzo[*a*]pyrene) is not well understood. In *Escherichia coli*, benzo[*a*]pyrene adducts can be repaired by NER (11). In mammals these adducts are also repaired by NER both *in vitro* and *in vivo* (12–15). However, it is not known whether other PAH lesions are similarly repaired by NER, although this is generally assumed to be the case. Moreover, it is not clear whether NER constitutes the sole repair mechanism for PAH lesions. A previous study suggests that an additional repair mechanism independent of NER also plays a role in the repair of benzo[*a*]pyrene-treated CHO cells (16).

We hypothesize that DNA repair is an important determinant in the carcinogenic potential of a PAH. Thus, understanding the repair of DNA damage by various PAHs may shed light on PAH-induced carcinogenesis. In this report we examine the overall repair of DNA lesions induced by five ultimate carcinogens of four PAH compounds using a human cell-free repair assay. The ultimate carcinogens studied are benzo[*a*]pyrene-*trans*-7,8-dihydrodiol-9,10-epoxide (BPDE), benz[*a*]anthracene-*trans*-3,4-dihydrodiol-1,2-epoxide (BADE-I), benz[*a*]anthracene-*trans*-8,9-dihydrodiol-10,11-epoxide (BADE-II), benzo[*b*]fluoranthene-*trans*-9,10-dihydrodiol-11,12-epoxide (BFDE) and chrysene-*trans*-1,2-dihydrodiol-3,4-epoxide (CDE) (Figure 1). These are the metabolites of benzo[*a*]pyrene, benz[*a*]anthracene, benzo[*b*]fluoranthene and chrysene respectively, which are representative PAH compounds present in tobacco smoke (17). Additionally, we determined the *in vitro* repair mechanism for these PAH-induced DNA lesions.



**Fig. 1.** Structures of PAH dihydrodiol epoxides used. (A) Benzo[a]pyrene-*trans*-7,8-dihydrodiol-9,10-epoxide (BPDE); (B) benz[a]anthracene-*trans*-3,4-dihydrodiol-1,2-epoxide (BADE-I); (C) benz[a]anthracene-*trans*-8,9-dihydrodiol-10,11-epoxide (BADE-II); (D) benzo[b]fluoranthene-*trans*-9,10-dihydrodiol-11,12-epoxide (BFDE); (E) chrysene-*trans*-1,2-dihydrodiol-3,4-epoxide (CDE). Only one enantiomer for each dihydrodiol epoxide is shown, but racemic mixtures ( $\pm$ -anti enantiomers) were used.

## Materials and methods

The human cell lines used were HeLa, XPA, XPD and XPF. HeLa is a fibroblast cell line of human cervical carcinoma origin. The XPA (GM4312), XPD (GM8207) and XPF (GM8437) cell lines are SV40-transformed fibroblasts derived from xeroderma pigmentosum (XP) patients belonging to complementation groups A, D and F respectively. Cells were grown as a monolayer in minimum essential medium (BRL) supplemented with 10% fetal calf serum. PAH dihydrodiol epoxide compounds BPDE, BFDE, BADE-I, BADE-II and CDE with purities of 98, 98, 99, 95 and 96% respectively were purchased from the National Cancer Institute Chemical Carcinogen Reference Standard Repository. Each compound was dissolved in dimethylsulfoxide to 10 mM and stored at  $-80^{\circ}\text{C}$ .

### Preparation of damaged DNA

Plasmid pUC18 DNA was propagated in *E. coli* DH5 $\alpha$ , isolated by the alkaline lysis method and purified by cesium chloride/ethidium bromide gradient centrifugation (18). Damaged plasmid DNA was obtained by incubating 50  $\mu\text{g}$  pUC18 DNA in a 500  $\mu\text{l}$  reaction mixture containing TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), 20% ethanol and various concentrations of the PAH compounds (0.3, 1.0, 3.0 and 10.0  $\mu\text{M}$ ). After incubation at  $37^{\circ}\text{C}$  for 3 h in the dark the modified DNA was purified by 5–20% sucrose gradient centrifugation as described by Wang *et al.* (18). Fractions of 0.5 ml each were collected from the bottom of the gradient and 5  $\mu\text{l}$  aliquots were loaded on a 1% agarose gel. Fractions containing supercoiled DNA were pooled, precipitated in ethanol and dissolved in TE buffer.

### DNA damage quantitation

DNA lesions were estimated by quantitative PCR, using a procedure modified from those reported previously (19,20). After linearization with *Eco*RI and *Hind*III restriction endonucleases a 415 bp DNA fragment of damaged or undamaged plasmid pUC18 was amplified by PCR with two primers, 5'-CTCTTACTGTCATGCCATCCGTAAGATG-3' and 5'-GTGCGCGGAACCCTATTTG-3'. PCR reactions (50  $\mu\text{l}$ ) contained 1 ng DNA, 1  $\mu\text{M}$  each primer, 1.5 mM MgCl<sub>2</sub>, 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 200  $\mu\text{M}$  each dATP, dTTP and dGTP, 40  $\mu\text{M}$  dCTP, 1  $\mu\text{Ci}$  [<sup>32</sup>P]dCTP (3000 Ci/mmol) and 1 U Taq DNA polymerase. After heating at  $94^{\circ}\text{C}$  for 2 min 10 cycles of amplification were performed under the following conditions: 45 s denaturation at  $94^{\circ}\text{C}$ ; 45 s annealing at  $65^{\circ}\text{C}$ ; 45 s extension at  $72^{\circ}\text{C}$ . Taq DNA polymerase was added before primer extension during the first cycle. At the end of the cycles an additional incubation was allowed for 10 min at  $72^{\circ}\text{C}$ . Radiolabeled PCR products were separated by electrophoresis on a 10% non-denaturing polyacrylamide gel. After drying the gel, amplified DNA bands were quantitated with a Packard InstantImager. At high temperatures accelerated  $\beta$ -elimination occurs at AP sites, leading to DNA strand breaks. Thus, the majority of AP sites present in some PAH-damaged DNA were expected to be detectable by quantitative PCR under the conditions used.

Assuming a random distribution of lesions (a Poisson distribution), the average lesions per 415 nt DNA strand was calculated by the equation  $n = -\ln(A_d/A_0)$ , where  $A_d$  is c.p.m. for the damaged template and  $A_0$  c.p.m. for the undamaged template. Average lesions per pUC18 DNA molecule were then derived from these calculations as shown in Table I.

**Table I.** Estimation of PAH-induced DNA lesions

PAH	3.0 $\mu\text{M}$		10.0 $\mu\text{M}$	
	PCR (%) <sup>a</sup>	Lesions/pUC18 <sup>b</sup>	PCR (%)	Lesions/pUC18
BPDE	76 $\pm$ 5.8	3.6 $\pm$ 1.0	33 $\pm$ 0.2	14 $\pm$ 0.1
BFDE	65 $\pm$ 1.1	5.6 $\pm$ 0.2	37 $\pm$ 0.9	13 $\pm$ 0.3
BADE-I	67 $\pm$ 5.1	5.1 $\pm$ 1.0	21 $\pm$ 2.8	20 $\pm$ 1.8
BADE-II	62 $\pm$ 5.2	6.2 $\pm$ 1.1	51 $\pm$ 10	8.7 $\pm$ 2.6
CDE	56 $\pm$ 8.4	7.5 $\pm$ 2.0	10 $\pm$ 1.1	30 $\pm$ 1.4

Plasmid pUC18 DNA was modified with the indicated compounds at 3.0 or 10  $\mu\text{M}$  for 3 h and purified by centrifugation in a linear 5–20% sucrose gradient. DNA lesions were estimated by quantitative PCR as described in Materials and methods.

<sup>a</sup>Ratio of amplification by PCR,  $A_d/A_0$ , where  $A_d$  is amplification of damaged DNA and  $A_0$  is amplification of undamaged DNA. A 415 nt DNA fragment of pUC18 was amplified.

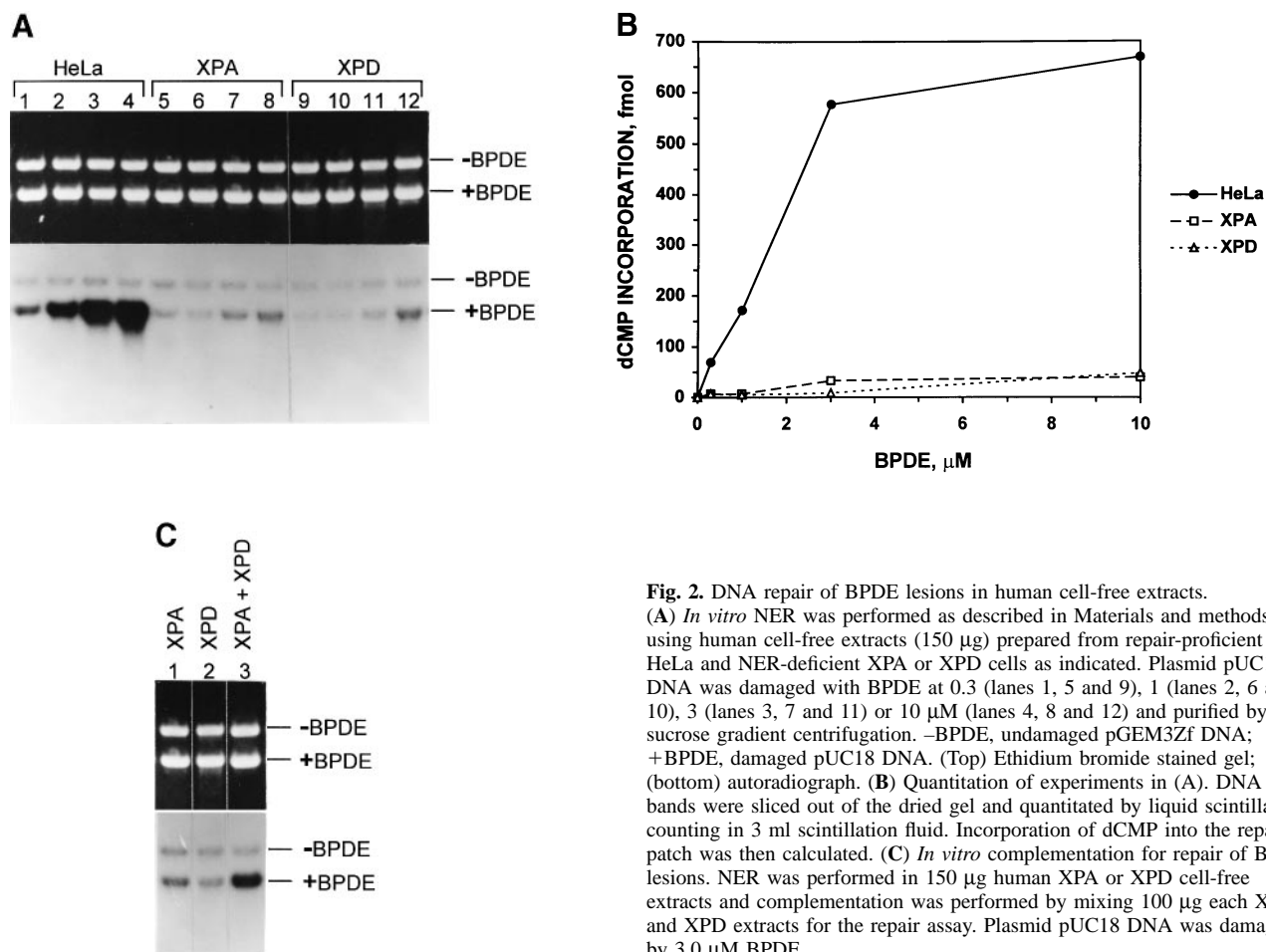
<sup>b</sup>Average number of DNA lesions per pUC18 DNA molecule (2.7 kb) was calculated from the quantitative PCR results.

### Preparation of human cell-free extracts

Whole cell extracts for *in vitro* DNA repair were prepared as previously described (21,22). Briefly, cultured cells were harvested and resuspended in a hypotonic buffer containing 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5 mM dithiothreitol (DTT). After incubation on ice for 20 min cells were disrupted in a Dounce homogenizer. A solution containing 50 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 2 mM DTT, 25% sucrose, 50% glycerol was added, followed by addition of ammonium sulfate to 10% saturation. After centrifugation proteins in the supernatant were precipitated with ammonium sulfate (0.33 g/ml) and dissolved in dialysis buffer. The extracts were then dialyzed against 25 mM HEPES-KOH, pH 7.9, 0.1 M KCl, 12 mM MgCl<sub>2</sub>, 1 mM EDTA, 2 mM DTT, 17% glycerol. Precipitates formed after dialysis were removed by centrifugation and the resulting supernatant was used as soluble extract for repair studies.

### *In vitro* DNA repair in human cell-free extracts

DNA repair assays were performed as previously described (21,22). Briefly, the repair mixture (50  $\mu\text{l}$ ) contained 200 ng each damaged pUC18 DNA and undamaged pGEM3Zf DNA (internal control), 45 mM HEPES-KOH, pH 7.8, 7.4 mM MgCl<sub>2</sub>, 0.9 mM DTT, 0.4 mM EDTA, 2 mM ATP, 20  $\mu\text{M}$  each dATP, dGTP and dTTP, 4  $\mu\text{M}$  dCTP, 1  $\mu\text{Ci}$  [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol), 40 mM phosphocreatine (disodium salt), 2.5  $\mu\text{g}$  creatine phosphokinase, 4% glycerol, 100  $\mu\text{g/ml}$  bovine serum albumin, 150  $\mu\text{g}$  cell-free extract. After incubation at  $30^{\circ}\text{C}$  for 2 h the reaction was stopped by adding EDTA to 20 mM and RNase A to 20  $\mu\text{g/ml}$ . After incubation at  $37^{\circ}\text{C}$  for 10 min SDS and proteinase K were added to 0.5% and 200  $\mu\text{g/ml}$  respectively, followed



**Fig. 2.** DNA repair of BPDE lesions in human cell-free extracts.

(A) *In vitro* NER was performed as described in Materials and methods using human cell-free extracts (150 μg) prepared from repair-proficient HeLa and NER-deficient XPA or XPD cells as indicated. Plasmid pUC18 DNA was damaged with BPDE at 0.3 (lanes 1, 5 and 9), 1 (lanes 2, 6 and 10), 3 (lanes 3, 7 and 11) or 10 μM (lanes 4, 8 and 12) and purified by sucrose gradient centrifugation. -BPDE, undamaged pGEM3Zf DNA; +BPDE, damaged pUC18 DNA. (Top) Ethidium bromide stained gel; (bottom) autoradiograph. (B) Quantitation of experiments in (A). DNA bands were sliced out of the dried gel and quantitated by liquid scintillation counting in 3 ml scintillation fluid. Incorporation of dCMP into the repair patch was then calculated. (C) *In vitro* complementation for repair of BPDE lesions. NER was performed in 150 μg human XPA or XPD cell-free extracts and complementation was performed by mixing 100 μg each XPA and XPD extracts for the repair assay. Plasmid pUC18 DNA was damaged by 3.0 μM BPDE.

by incubation at 37°C for 30 min. DNA was then extracted with phenol/chloroform and precipitated in ethanol. Repair products were digested with *Hind*III and separated by electrophoresis on a 1% agarose gel containing 0.5% ethidium bromide. Repair synthesis was visualized by autoradiography of the dried gel. Repair activity was quantitated by liquid scintillation counting after slicing the DNA bands out of the dried gel.

#### Endonuclease III digestion

Endonuclease III digestion was performed by mixing 400 ng supercoiled plasmid DNA and 500 or 800 ng *E. coli* endonuclease III in buffer containing 45 mM HEPES-KOH, pH 7.8, 7.4 mM MgCl<sub>2</sub>, 0.9 mM DTT, 0.4 mM EDTA, 40 mM phosphocreatine (disodium salt), 4% glycerol, 5 μg bovine serum albumin. After incubation at 37°C for 15 min the products were separated by electrophoresis on a 1% agarose gel containing 0.5% ethidium bromide. Cleavage of plasmid DNA by endonuclease III converts the supercoiled molecule to the nicked circular form, which migrates slower on the gel.

## Results

### Repair of DNA lesions induced by benzo[a]pyrene-*trans*-7,8-dihydrodiol-9,10-epoxide (BPDE)-damaged DNA

A human cell-free system for DNA repair has been developed by Wood *et al.* (21) that faithfully reflects *in vivo* repair properties. We employed this *in vitro* system to study the repair of PAH-induced DNA lesions and examined the repair mechanism involved. Plasmid pUC18 DNA was damaged by five PAH dihydrodiol epoxides (Figure 1) *in vitro*, purified on a 5–20% sucrose gradient and used to examine repair by human cell-free extracts. Excision repair was measured by incorporation of radiolabeled nucleotides into damaged plasmid DNA during repair synthesis catalyzed by human extracts. In the same repair reaction a larger undamaged pGEM3Zf plasmid

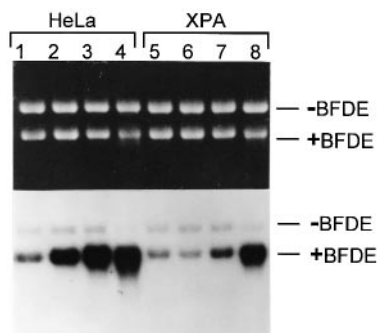
DNA was also included as an internal control for monitoring background levels of non-specific DNA synthesis.

BPDE is a metabolite of benzo[a]pyrene, representing an ultimate carcinogen of the parent compound. BPDE readily attacks DNA forming covalently linked adducts at the N<sup>2</sup> position of guanine (80–90% of the total adducts produced) (23). Minor adducts include N<sup>7</sup> of guanine, N<sup>6</sup> of adenine and N<sup>3</sup> of cytosine (24). BPDE-damaged plasmid pUC18 DNA was examined for repair in human cell-free extracts. After incubation in human HeLa extracts, repair was readily detected in BPDE-damaged DNA, as evidenced by the strong [<sup>32</sup>P]dCMP labeling in the damaged DNA band compared with the undamaged control DNA band (Figure 2A, lanes 1–4). Repair activity increased with increasing BPDE DNA lesions (Figure 2A, lanes 1–4, and B).

To determine the mechanism responsible for *in vitro* repair of BPDE-damaged DNA identical repair experiments were performed in cell-free extracts prepared from XPA and XPD cells. These cells lack the indispensable NER proteins XPA and XPD respectively and thus are specifically deficient in NER (3). In contrast with HeLa cell extracts, repair of BPDE-damaged DNA was abolished in XPA and XPD extracts (Figure 2A). Defective repair was observed at all BPDE doses examined from 0.3 to 10 μM (Figure 2A and B). Since mixing XPA and XPD extracts led to *in vitro* complementation for proficient repair (Figure 2C), these mutant extracts reflected true defects in the NER pathway rather than failed extract preparations. These results show that benzo[a]pyrene DNA adducts are repaired by the NER pathway in human extracts.

*Repair of DNA lesions induced by benzo[b]fluoranthene-trans-9,10-dihydrodiol-11,12-epoxide (BFDE)*

BFDE is a carcinogenic metabolite of benzo[b]fluoranthene. Repair of BFDE-damaged plasmid DNA in HeLa cell-free extracts was readily detected (Figure 3, lanes 1–4). Repair activity increased with increasing BFDE damage to a level similar to that of BPDE repair (Figure 3, lanes 1–4). The contribution of NER to repair of BFDE DNA lesions was then examined by performing identical repair assays in XPA mutant extracts. At low doses of BFDE (up to 1  $\mu$ M), damaged DNA was not repaired in XPA mutant extracts (Figure 3, lanes 5 and 6), indicating that repair in normal cell extracts is mediated by the NER pathway. However, at higher doses of BFDE, repair was detected in XPA mutant extracts (Figure 3, lanes 7 and 8). When the plasmid DNA was damaged by 10  $\mu$ M BFDE, DNA repair synthesis in XPA mutant extract was similar to that in the HeLa extract (Figure 3, compare lanes 4 and 8). These results show that when DNA is extensively damaged by high concentrations of BFDE another excision repair mechanism independent of NER also contributes to repair of BFDE lesions *in vitro*.



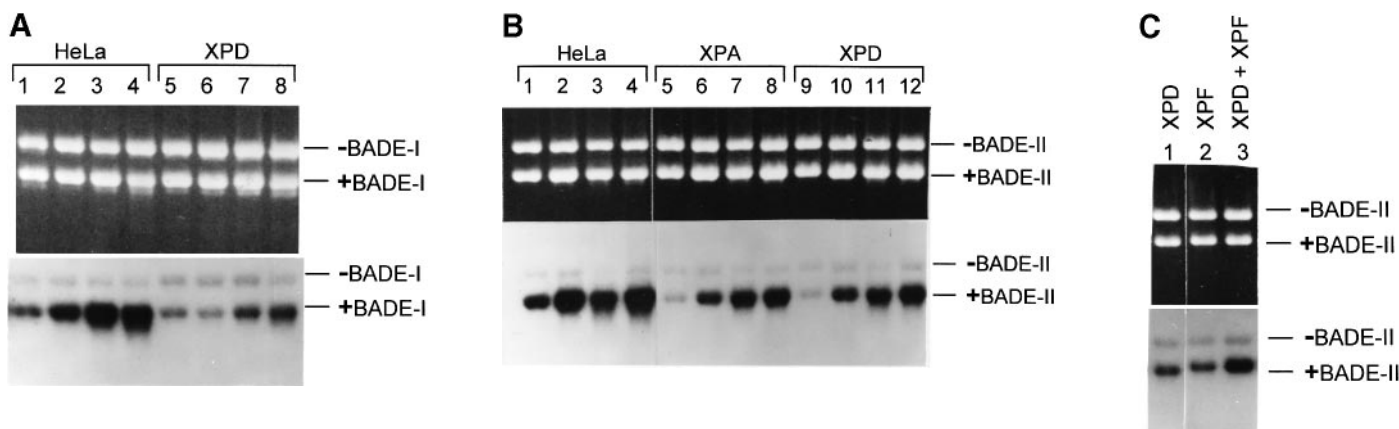
**Fig. 3.** DNA repair of BFDE lesions in human cell-free extracts. *In vitro* NER was performed in human cell-free extracts (150  $\mu$ g) prepared from repair-proficient (HeLa) and NER-deficient (XPA) cells. Plasmid pUC18 DNA was damaged with BFDE at 0.3 (lanes 1 and 5), 1 (lanes 2 and 6), 3 (lanes 3 and 7) or 10  $\mu$ M (lanes 4 and 8). –BFDE, undamaged pGEM3Zf DNA; +BFDE, damaged pUC18 DNA. (Top) Ethidium bromide stained gel; (bottom) autoradiograph.

*Repair of DNA lesions induced by benz[a]anthracene-trans-3,4-dihydrodiol-1,2-epoxide (BADE-I) and benz[a]anthracene-trans-8,9-dihydrodiol-10,11-epoxide (BADE-II)*

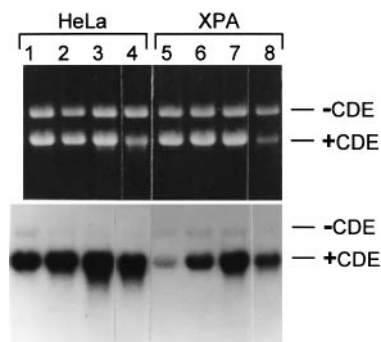
BADE-I and BADE-II are two carcinogenic metabolites of benz[a]anthracene. BADE-I appears to be principally responsible for the tumorigenic properties of benz[a]anthracene. BADE-II is much less mutagenic and carcinogenic (25–27). Thus, it is of especial interest to examine repair of DNA lesions induced by these two structurally similar compounds in human extracts.

In HeLa cell-free extracts, repair of BADE-I damaged DNA was readily detected and the repair activity increased with increasing concentrations of BADE-I to a level similar to that of BPDE repair (Figure 4A). Below 1  $\mu$ M BADE-I, repair was abolished in NER-deficient XPD mutant extract (Figure 4A, lanes 5 and 6). At higher concentrations of BADE-I damage, some repair was also detected in XPD mutant extracts (Figure 4A, lanes 7 and 8), indicating involvement of an NER-independent excision repair mechanism. However, repair synthesis in XPD mutant extracts was significantly lower than that in the HeLa extracts (Figure 4A, compare lanes 3 and 7 and lanes 4 and 8). Thus, BADE-I lesions are mainly repaired by the NER pathway, although an additional excision repair mechanism is evident when DNA is extensively damaged by BADE-I.

When BADE-II-damaged DNA was used as the substrate for repair in HeLa extracts substantial repair synthesis was observed at the lowest dose examined (0.3  $\mu$ M BADE-II) (Figure 4B, lane 1). Furthermore, repair synthesis did not increase in a dose-dependent manner from 1 to 10  $\mu$ M BADE-II (Figure 4B, lanes 2–4). Presumably this may result from saturation of the repair system(s) by BADE-II lesions at 1  $\mu$ M. At 10  $\mu$ M BADE-II, repair synthesis was similar to that of BPDE repair at 10  $\mu$ M (Figure 4B, lane 4). In XPA and XPD mutant extracts repair was not detected at 0.3  $\mu$ M BADE-II (Figure 4B, lanes 5 and 9), but significant repair synthesis was observed at 1  $\mu$ M, although at a lower level compared with that in the HeLa extracts (Figure 4B, compare lanes 6 and 10 with lane 2). These results are indicative of the involvement of NER in repair of BADE-II lesions. Supporting this conclusion,



**Fig. 4.** DNA repair of benz[a]anthracene lesions in human cell-free extracts. *In vitro* NER was performed in repair-proficient (HeLa) or NER-deficient (XPA and XPD) cell extracts (150  $\mu$ g). Plasmid pUC18 DNA was damaged with the benz[a]anthracene metabolites (BADE-I and BADE-II) at 0.3 (lanes 1, 5 and 9), 1 (lanes 2, 6 and 10), 3 (lanes 3, 7 and 11) or 10  $\mu$ M (lanes 4, 8 and 12). (A) DNA repair of BADE-I lesions. (B) DNA repair of BADE-II lesions. (C) *In vitro* complementation for repair of BADE-II lesions. NER was performed in 150  $\mu$ g human XPD (lane 1) or XPF (lane 2) mutant cell extracts and complementation (lane 3) was performed by mixing 100  $\mu$ g each XPD and XPF extracts for repair assay. Plasmid pUC18 DNA was damaged by 1.0  $\mu$ M BADE-II. –BADE-I and –BADE-II, undamaged pGEM3Zf DNA; +BADE-I and +BADE-II, damaged pUC18 DNA. (Top) Ethidium bromide stained gel; (bottom) autoradiograph.



**Fig. 5.** DNA repair of CDE lesions in human cell-free extracts. *In vitro* NER was performed in repair-proficient (HeLa) or NER-deficient (XPA) cell extracts (150  $\mu$ g). Plasmid pUC18 DNA was damaged with CDE at 0.3 (lanes 1 and 5), 1 (lanes 2 and 6), 3 (lanes 3 and 7) or 10  $\mu$ M (lanes 4 and 8). -CDE, undamaged pGEM3Zf DNA; +CDE, damaged pUC18 DNA. (Top) Ethidium bromide stained gel; (bottom) autoradiograph.

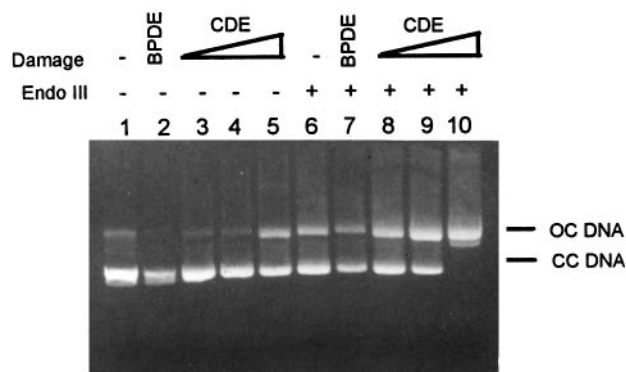
repair at the dose of 1  $\mu$ M in XPD mutant extract was significantly stimulated by adding XPF mutant extract, achieving *in vitro* complementation for NER (Figure 4C). Such complementation was expected for the NER mechanism, since mixing XPD (lacking XPD protein) and XPF (lacking XPF protein) mutant extracts yielded a complete set of functional NER proteins, thus restoring NER activity. With increasing concentrations of BADE-II more repair was observed in XPA and XPD mutant extracts, eventually approaching the levels in HeLa cell extract (Figure 4B, lanes 7, 8, 11 and 12). Moreover, the level of DNA repair was dose-dependent in both mutant extracts from 0.3 to 3  $\mu$ M BADE-II (Figure 4B, lanes 5–7 and 9–11). These results suggest that in addition to NER another excision repair mechanism plays a role in repair of BADE-II lesions.

#### Repair of DNA lesions induced by chrysene-*trans*-1,2-dihydrodiol-3,4-epoxide (CDE)

Chrysene is a relatively weak carcinogen (1,28). CDE is an ultimate carcinogen of chrysene and can covalently modify DNA (29). Strong repair synthesis was observed in HeLa cell extracts at 0.3  $\mu$ M CDE (Figure 5, lane 1). At this dose of DNA damage, repair in XPA mutant extracts was clearly deficient (Figure 5, compare lane 1 with 5), indicating that repair is mainly mediated by NER. With increasing concentrations of CDE repair in XPA extracts became very significant, approaching the level of HeLa extract at 3  $\mu$ M CDE (Figure 5, lane 7). At even higher concentrations of CDE (10  $\mu$ M) the damaged DNA became unstable during storage at  $-20^{\circ}\text{C}$ . After 48 h at  $-20^{\circ}\text{C}$  >50% of the damaged plasmid DNA was fragmented (data not shown). The remaining full-length DNA containing CDE lesions served as a good substrate for repair in both HeLa and XPA extracts (Figure 5, lanes 4 and 8). NER-independent repair in XPA mutant extracts showed a clear dose-dependent response from 0.3 to 3  $\mu$ M CDE (Figure 5, lanes 5–7), i.e. with increasing DNA damage the NER-independent repair mechanism increasingly contributes to overall repair of CDE lesions. These results suggest that in addition to NER another excision repair mechanism also plays a role in repair of CDE lesions. Thus, CDE lesions appear to be repaired similarly to BADE-II lesions.

#### Role of base excision repair for certain PAH-induced DNA lesions

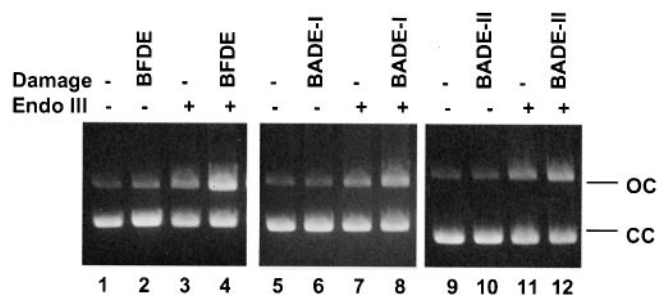
In mammalian cells, in addition to NER, another well-characterized excision repair mechanism is BER. Among the various



**Fig. 6.** Detection of AP sites in PAH-damaged DNA used for *in vitro* repair. CDE- and BPDE-damaged plasmid pUC18 was purified by sucrose gradient centrifugation and subsequently examined for AP sites in DNA. Damaged DNA (400 ng) was incubated with 200 ng *E.coli* AP endonuclease III at  $37^{\circ}\text{C}$  for 15 min (lanes 6–10). Reaction products were separated by electrophoresis on a 1% agarose gel and stained with ethidium bromide. Control experiments without endonuclease III were performed identically (lanes 1–5). PAH treatments are: lanes 1 and 6, none; lanes 2 and 7, 3  $\mu$ M BPDE; lanes 3 and 8, 0.3  $\mu$ M CDE; lanes 4 and 9, 3  $\mu$ M CDE; lanes 5 and 10, 10  $\mu$ M CDE. CC DNA, closed circular DNA; OC DNA, opened circular DNA.

DNA lesions recognized by BER are AP sites (3). Covalent modification of DNA bases often renders the damaged base unstable, promoting base loss and leaving AP sites in the DNA (3). Some PAH adducts in DNA may be unstable and may undergo depurination, producing AP sites in DNA. Thus, we suspected that at least some of the observed repair of PAH-damaged DNA in XP mutant extracts (NER-independent repair) may reflect BER of AP sites following depurination of adducted bases. To examine this possibility we directly measured AP sites in damaged DNA with an AP site-specific enzyme, *E.coli* endonuclease III (30). Cleavage of AP sites by endonuclease III converts damaged plasmid DNA from the supercoiled form to the nicked circular form, which can be separated on an agarose gel in the presence of ethidium bromide. Prior to endonuclease III treatment damaged DNA was purified by sucrose gradient centrifugation to remove DNA containing rapidly formed AP sites which readily led to DNA strand breaks. As shown in Figure 6, DNA damaged by CDE was cleaved by endonuclease III (compare lanes 8–10 with 6), whereas BPDE-damaged DNA was not significantly cleaved (compare lane 7 with 6). This result suggests that after removing rapidly formed AP sites the remaining BPDE adducts are relatively stable, whereas CDE adducts are less stable, leading to slow accumulation of AP sites in DNA. Above a 3  $\mu$ M PAH concentration used for DNA treatment endonuclease III cleavage of BADE-II-damaged DNA was also observed, although not as dramatic as for CDE-damaged DNA (data not shown). At 10  $\mu$ M some BFDE- and BADE-I-damaged DNA was additionally cleaved by endonuclease III (data not shown). These observations are consistent with the repair results in NER-deficient cell extracts. Hence, the NER-independent repair mechanism observed in repair of BFDE, BADE-II and CDE lesions is likely BER of induced AP sites.

It is possible that some PAH adducts are especially unstable, leading to rapid depurination and AP site formation, which would not be detected by the experiments discussed above due to sucrose gradient purification. To test this possibility we examined AP sites by the endonuclease III cleavage assay immediately after damaging DNA, thus avoiding sucrose



**Fig. 7.** Detection of AP sites immediately after DNA damage. Plasmid pUC18 DNA (2 µg) was damaged with 3.0 µM BFDE, BADE-I or BADE-II as indicated in TE buffer containing 20% ethanol (20 µl). After incubation at 37°C for 3 h in the dark 20 µl TE buffer were added to the mixture and 8 µl aliquots were used for endonuclease III assays. Endonuclease III assays were performed by incubating undamaged (lanes 3, 7 and 11) or damaged (lanes 4, 8 and 12) DNA (400 ng) with 500 ng *E. coli* AP endonuclease III under buffer conditions described in Materials and methods. After 15 min at 37°C the products were separated by electrophoresis on a 1% agarose gel containing 0.5% ethidium bromide. Control experiments were identically performed in the absence of endonuclease III as indicated. CC, closed circular DNA; OC, opened circular DNA.

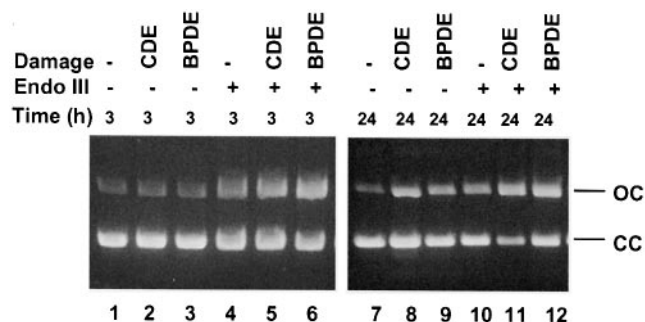
gradient purification. As shown in Figures 7 (compare lanes 4 and 3, lanes 8 and 7 and lanes 12 and 11) and 8 (compare lanes 5 and 6 with lane 4), we detected AP sites in DNA damaged by all five dihydrodiol epoxides.

Comparing these results with those shown in Figure 6, it is apparent that some BPDE adducts are especially labile, resulting in rapid AP sites in DNA, while the remaining BPDE adducts are very stable. In contrast, CDE adducts appear to undergo slow depurination and gradual AP site accumulation over time. To confirm this conclusion further, DNA adducts of BPDE and CDE were left undisturbed for 24 h at 37°C without sucrose gradient purification. AP sites were then assayed by endonuclease III cleavage. Consistent with our conclusion, ~50% DNA was cleaved by endonuclease III whether assayed after treatment with BPDE or 24 h later (Figure 8, compare lane 12 with 6). In contrast, ~30% DNA was cleaved immediately after CDE treatment, but endonuclease III-cleavable DNA increased to ~70% after 24 h (Figure 8, compare lane 11 and 5).

## Discussion

The ability of PAHs to induce tumors are attributable to their ability to damage DNA. Such damage results from electrophilic attack on DNA by metabolically activated forms of the inert parent PAH compounds, mostly the dihydrodiol epoxide derivatives (2). Reactions between DNA and dihydrodiol epoxides of PAHs form covalently linked DNA adducts, notably on guanine residues. If not effectively repaired, these DNA adducts could result in mutations during DNA replication and may eventually lead to neoplastic transformation. Thus, the steady-state level of PAH adducts in DNA may be an important contributor to the carcinogenic effects of PAHs. Conceptually, the persistence of PAH adducts in DNA is a dynamic equilibrium between their formation and removal.

We have examined repair of DNA lesions induced by several PAHs using a human cell-free system. It is generally assumed that bulky DNA adducts such as those induced by PAHs are repaired by the NER pathway (3,11). Our results show directly that NER is indeed an important mechanism in repair of PAH-induced DNA lesions. DNA damage by all five ultimate



**Fig. 8.** Formation of AP sites in DNA after BPDE and CDE treatments are kinetically different. Plasmid pUC18 DNA was incubated with 3.0 µM BPDE or CDE as indicated in TE buffer containing 20% ethanol (20 µl). After 3 h at 37°C DNA was diluted to 50 ng/µl with TE buffer and divided into 8 µl aliquots. Some aliquots were immediately used for endonuclease III assays (lanes 1–6). The remaining aliquots were incubated at 37°C for 24 h before endonuclease III assays were performed (lanes 7–12). Endonuclease III assays were performed by incubating DNA (400 ng) with 500 ng *E. coli* AP endonuclease III under buffer conditions described in Materials and methods. After 15 min at 37°C the products were separated by electrophoresis on a 1% agarose gel containing 0.5% ethidium bromide. Control experiments were performed identically in the absence of endonuclease III as indicated. CC, closed circular DNA; OC, opened circular DNA.

carcinogens of PAHs tested, BPDE, BFDE, BADE-I, BADE-II and CDE, elicited repair in human cell-free extracts and a contribution by NER was detected in every case. Additionally, the contribution of a NER-independent excision repair mechanism was readily detected in BADE-II- and CDE-modified DNA. When DNA was extensively damaged this alternative form of repair was also detected in BADE-I- and BFDE-treated DNA. Hence, depending on the particular PAH compound and the dose, the contribution of the NER-independent repair pathway can differ significantly.

Apparently, the NER-independent repair mechanism detected in our human cell-free system is the BER pathway. Supporting this conclusion, we detected AP sites in BADE-II- and CDE-damaged DNA and DNA damaged extensively by BFDE and BADE-I in sucrose gradient purified preparations. AP sites are known to be repaired mainly by the BER pathway (3,30). While N<sup>2</sup> guanine adducts are relatively stable, PAH modifications at other positions may be less stable, especially adducts at the N<sup>7</sup> position of guanine and N<sup>3</sup> position of adenine (31,32). These unstable adducts promote depurination, leaving AP sites in DNA. Indeed, PAH-promoted AP site formation has been reported (32,33).

Among the five PAH dihydrodiol epoxides in our study the extent of AP site induction varied widely. This probably reflects differences in the adduct spectrum induced and the stability of individual adducts due to structural differences between the PAH compounds. Furthermore, the kinetics of AP site formation also differed dramatically, as illustrated after BPDE and CDE damage. After BPDE treatment some adducts induce rapid formation of AP sites. After sucrose gradient purification DNA containing rapidly formed AP sites was removed due to AP-induced DNA strand breakage. The remaining BPDE adducts are stable even after storage at -20°C for several months, as indicated by the absence of AP sites in the purified BPDE-DNA. In contrast, immediately after CDE damage, AP sites are marginally detected in the damaged DNA. However, AP sites slowly accumulate over 24 h. After longer storage at -20°C (a few weeks) the majority of DNA adducts were depurinated, leading to fragmentation

of the damaged DNA. These observations suggest that a small fraction of BPDE adducts cause rapid depurination, whereas the majority of CDE adducts lead to gradual accumulation of AP sites. Sites of PAH adduct-induced depurination could then be repaired by the BER pathway. Consistent with this notion, Day *et al.* (34) and van Houten *et al.* (35) reported that a fraction of BPDE adducts were removed from cellular DNA *in vivo* shortly after treating XPA cells with BPDE, presumably by a NER-independent mechanism.

DNA repair by the NER pathway is generally a slow process, while repair by the BER pathway is normally fast and efficient. Efficient repair would reduce the mutagenic and carcinogenic potential of a lesion, whereas inefficient repair would enhance the mutagenic and carcinogenic potential of a lesion. Hence, if the DNA adducts induced by a PAH are stable and thus repaired mainly by NER, some adducts may not be efficiently removed. This PAH is predicted to be a strong carcinogen. In contrast, if the DNA adducts induced by a PAH are unstable, the adducts would be more efficiently removed due to spontaneous depurination in addition to repair by NER. The resulting AP sites left in DNA should be effectively repaired by BER. This PAH is predicted to be a weak carcinogen. Our observations that BER contributes to a greater extent to the repair of CDE and BADE-II lesions *in vitro* are consistent with reports that both PAHs are weak carcinogens (25–28). In comparison, the major BPDE adducts are stable and repaired by NER. Consistently, it is a strong carcinogen (36). Such an interpretation may also be applied to BADE-I and BADE-II. While the latter is more actively formed by metabolism of benz[*a*]anthracene, it is significantly less carcinogenic than the former compound (25–27). Thus, a greater contribution of BER to repair of BADE-II lesions observed in our *in vitro* studies may help to explain the carcinogenic differences between these structurally similar compounds.

Due to low repair efficiencies in human cell-free extracts, high levels of DNA damage were used for *in vitro* repair, which could lead to lethality *in vivo*. Thus, *in vivo* evidence for a physiological role of BER at carcinogenic doses of PAHs remains to be established.

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