Published in final edited form as:

Environ Mol Mutagen. 2013 October ; 54(8): 652–658. doi:10.1002/em.21806.

Sequence Context Modulation of Polycyclic Aromatic Hydrocarbon-Induced Mutagenesis

Parvathi Chary¹, Michael P. Stone², and R. Stephen Lloyd^{1,3,*}

¹Center for Research on Occupational and Environmental Toxicology, Oregon Health & Science University, Portland, Oregon

²Department of Chemistry, Rm SC 7300 Stevenson Science Center, Vanderbilt University, Nashville, Tennessee

³Department of Molecular and Medical Genetics, Oregon Health & Science University, Portland, Oregon

Abstract

DNA structural perturbations that are induced by site specifically and stereospecifically defined benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) adducts are directly correlated with mutagenesis, leading to cellular transformation. Although previous investigations had established that replication of DNAs containing *N*⁶-BPDE dA adducts at the second position in the *N-ras* codon 61(CAA) (61²) resulted exclusively in A to G transitions, NMR analyses not only established the structural basis for this transition mutation, but also predicted that if the adduct were positioned at the third position in the same codon, an expanded spectra of mutations was possible. To test this prediction, replication of DNAs containing C₁₀*S*-BPDE and C₁₀*R*-BPDE lesions linked through the *N*⁶ position of adenine in the sequence context *N-ras* codon 61, position 3 (C₁₀*S*-BPDE and C₁₀*R*-BPDE at 61³) was carried out in *Escherichia coli*, and these data revealed a wide mutation spectrum. In addition to A to G transitions produced by replication of both lesions, replication of the C₁₀*S*-BPDE and C₁₀*R*-BPDE adducts also yielded A to C and A to T transversions, respectively. Analyses of single nucleotide incorporation using Sequenase 2.0 and exonuclease-deficient *E. coli* Klenow fragment and pol II not only revealed high fidelity synthesis, but also demonstrated the same hierarchy of preference opposite a particular lesion, independent of the sequence context. Primer extension assays with the two lesions at *N-ras* 61³ resulted in truncated products, with the C₁₀*S*-BPDE adducts being more blocking than C₁₀*R*-BPDE lesions, and termination of synthesis was more pronounced at position 61³ than at 61² for each of the lesions.

© 2013 Wiley Periodicals, Inc.

*Correspondence to: R. Stephen Lloyd; Department of Molecular and Medical Genetics, Oregon Health and Science University, 3181 SW Sam Jackson Park Rd, Portland, OR 97239. lloydst@ohsu.edu.

AUTHOR CONTRIBUTIONS

All authors (P.C., M.P.S., and R.S.L.) meet the stated *EMM* requirements for appropriate authorship in all the following categories: (1) substantial contributions to the conception and design and data analyses and interpretation; (2) drafting and revising the manuscript; and (3) final approval for submission.

Keywords

BPDE; DNA polymerases; mutagenesis

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are formed as a by product of incomplete combustion of fuel and of various smoked food products (Alexandrov et al., 2010; Diggs et al., 2011). In addition, these bulky adducts are found in high concentrations in cigarette, cigar, and hookah smoke and are causally linked to cancer of the lungs, head, neck, larynx, tongue, oesophagus, pancreas, breast, cheek, bladder, pancreas, kidney, liver, and cervix (Hecht, 2012; IARC, 2012; Thun et al., 2013). In addition, PAHs pass through the epithelial mucosa and reach the colon and rectum, resulting in cellular transformation, and can manifest in the formation of polyps. Although the overt disease manifestation as colorectal cancer may take as long as four decades, the impact of repeated PAH exposures on human health cannot be underestimated (Diggs et al., 2011).

Following metabolic activation to an epoxide, PAHs bind DNA at exocyclic amino groups in both the major and minor grooves, such that PAHs including benzo[*a*]7,8 pyrene 9,10 diol-epoxide (BPDE) are predominantly attached through either *N*⁶ of adenine or *N*² of guanine. Repair of these bulky adducts primarily occurs via the nucleotide excision repair pathway, but mismatch repair (MMR) has also been specifically implicated in PAH-triggered apoptosis and suppression of PAH-induced carcinogenesis (Zienolddiny et al., 2006). However, if these lesions are not repaired prior to replication, after translesion synthesis, these adducts can be manifested as point mutations (Klarer and McGregor, 2011). Specifically, error-prone translesion synthesis has been documented to lead to point mutations, especially in the p53 tumor suppressor gene and in specific oncogenes such as *K-ras* and *N-ras* (Miyaki et al., 2002). In addition, based upon the site and stereospecificity of these PAHs, a range of mutations have been reported (Klarer and McGregor, 2011).

Previously, we had characterized the mutagenic frequency and spectra of two major groove-linked stereoisomers of BPDE in a sequence context of codon 61(CAA) in the human *N-ras* proto-oncogene, where the bolded **A** was the site of attachment (61²) (Chary et al., 1995). These analyses revealed exclusively A to G transitions, demonstrating the misincorporation of C opposite the adducted site. To understand the structural basis for both the error-free and error-prone syntheses, NMR solution structural analyses of these adducts linked through this same site were determined, with both BPDE-dA paired with a dT and a dC, respectively (Zegar et al., 1998). Specifically, the C₁₀R-BPDE lesion at 61² was refined as a single structure, in which the B[*a*]P moiety intercalated above the 5' face of the modified adenine, resulting in the buckling of the modified A:T base pair. The base-step between the adducted base pair and its 5' neighbor increased to accommodate the intercalated B[*a*]P, concomitant with unwinding of the helix (Zegar et al., 1996).

These NMR analyses were extended to analyze the BPDE isomer C₁₀R at the third position of the *N-ras* codon 61 (61³), and these data revealed a mixture of two conformations of the deoxyribose sugar puckering of the 5' dA when the adjacent nucleotide differed. Since the

chirality of the adducted molecules is also known to play an important role in influencing biological processes such as exonucleolytic activity and polymerase functioning (Chary and Lloyd, 1995, 1994; Chary et al., 1997), site- and stereospecific BPDE-adducts at N-*ras* codon 61³ were utilized in this study to understand their impact on *in vivo* mutagenesis and *in vitro* replication bypass fidelity using three exonuclease-deficient DNA polymerases (*Escherichia coli* exonuclease-deficient DNA polymerase II, *E. coli* exonuclease-deficient polymerase I, Klenow fragment, the exonuclease-deficient T7 DNA polymerase, Sequenase). The choice of these polymerases was driven by the fact that all are deficient in exonuclease activity, which would allow for an accurate evaluation of the nucleotide incorporation step opposite the adducted nucleotide in the absence of further processing. Overall, these data reveal that the NMR analyses provided reliable hypotheses for mutagenic outcomes.

MATERIALS AND METHODS

Construction of Oligodeoxynucleotides Containing Site- and Stereospecific BPDE Adducts at N-*ras* Codon 61³ and *In Vivo* Mutagenesis

Adducted 11-mer oligodeoxynucleotides bearing either (+)-(7*R*, 8*S*, 9*R*, 10*S*)-anti-*trans*-BPDE dA (designated as C₁₀*S*-BPDE) or (–)-(7*S*, 8*R*, 9*S*, 10*R*)-anti-*trans*-BPDE dA lesions (designated as C₁₀*R*-BPDE) at either the second or third position in the N-*ras* codon 61 (61², 5'-CGGACAAGAAG-3' or 61³, 5'-CGGACAAGAAG-3', respectively) were synthesized and purified as described (Harris et al., 1991; Kim et al., 1995). The structures of these lesions are shown in Figure 1. The purities of the BPDE-adducted 11-mer oligodeoxynucleotides that were used for biological analyses were determined by HPLC, polyacrylamide gel electrophoresis, capillary gel electrophoresis, and enzymatic digestion. The final evaluation of the purity of the 11-mers was demonstrated by 5' end labeling and separation on 15% polyacrylamide gels (data not shown). The purified 11-mers were inserted into single stranded (ss) M13mp7L2 vectors as previously described (Chary et al., 1995). All mutagenesis experiments were carried out a minimum of three independent times. DNA repair-deficient *E. coli* AB2480 (*uvrA6* and *recA13*) cells were obtained from Dr. A. Ganesan (Stanford University).

Construction of Oligodeoxynucleotides-Containing BPDE Adducts for *In Vitro* Replication Studies

Both unadducted and adducted 11-mer oligodeoxynucleotides were engineered into 73-mers by ligation at their 5' and 3' ends with 30- and 32-mers, respectively, using a centrally located 46-mer scaffold. All the 11- and 32-mers were phosphorylated using T4 DNA kinase (New England BioLabs) and ATP. The 30-mer was 5' end labeled with 1:100 mixture of ³²P γ-ATP: ATP for detection of 73-mer following ligation, and purification through a 10% SDS-PAGE. The final sequence of the 73-mer sequence was (5' GACTTAGGCTAGAATGTGGAAGATACTGTGCGACAAGA AGAATGGTCT GGGCAATGTCGTGACTGGGAAAAC 3'), in which the A represents unadducted, C₁₀*S*-BPDE, and C₁₀*R*-BPDE at 61³. These DNAs were used to determine catalytic efficiencies of nucleotide incorporation opposite 61³ (A) for exonuclease-deficient DNA polymerase II, exonuclease-deficient Klenow fragment, and Sequenase. The primer that was used was a

complementary 20-mer, which positioned the 3'-OH one nucleotide from the lesion site when annealed to the template. The 73-mer templates were also used for primer extension assays using a 20-mer primer whose 3' OH end was at Position 5 relative to CAA on the template strand.

For primer extension assays involving an A:C mismatch at the adducted site, 33-mer oligodeoxynucleotides were constructed with the following sequence: 5' CGGACAAAGAAGAATTCGTCGTCCTGGG AAAAC 3' in which the (AA) indicates the sites of 61² and 61³, respectively. The 33-mer templates (unadducted and 61²R- and 61³R-BPDE adducted) were utilized to study the impact of an A•C mismatch opposite the C₁₀R-BPDE adducted positions of adenine at 61² and 61³. In each of these cases, an appropriate 20-mer primer was synthesized such that its 3' OH nucleotide was a C. The enzymes utilized for the mismatch extension studies were exonuclease-deficient Klenow fragment and Sequenase 2.0.

DNA Polymerase Arrest Assays

Exonuclease-deficient *E. coli* DNA pol II (pol II *exo*⁻) and exonuclease-deficient *E. coli* DNA pol I, Klenow fragment (KF *exo*⁻) were obtained from Dr. M.F. Goodman, University of Southern California. T7 DNA polymerase (Sequenase 2.0) was purchased from US Biochemicals, (Cleveland, OH). KF *exo*⁻ (6 U/μL) was purchased from Life Technologies (Bethesda, MD). The oligodeoxynucleotide primers (20-mers) were 5' end labeled with (γ³²P)-ATP (6,000 Ci/mmol; DuPont-New England Nuclear) using T4 DNA kinase. Primer extension reactions with the 20-mer primers were performed at 37°C for 30 min, with template:primer ratio of 5:1 (250 fmol:50 fmol) in all cases. The substrate to enzyme ratio was also 5:1 (10 fmol of the polymerase). The reaction mixture contained 0.1 mg/mL BSA, 1 mM DTT, 1.25 mM ATP, 0.3 mM dNTPs (when used), 33 mM TrisOAc (pH 7.8), 66 mM KOAc, and 10 mM Mg(OAc)₂. Primer extensions were terminated by the addition of 5 μL of stop buffer, 95% (vol/vol) formamide, 20 mM EDTA, 0.05% (wt/vol) bromophenol blue, 0.05% (wt/vol) xylene cyanol/10 μL reaction mixture. Samples were separated on sequencing gels (15% polyacrylamide and 8.3M urea) by electrophoresis at 2,000 V for 3 hr and quantitated by the phosphorimager analyses (Molecular Dynamics, Sunnyvale, CA).

Incorporation rates of dNTPs were found to be linearly dependent over a wide range of dNTP concentrations up to 3 mM and the catalytic efficiencies (k_{cat}/K_m M⁻¹ min⁻¹) calculated. The insertion efficiency allows for comparison between the polymerase's incorporation of a specific nucleotide opposite the adducted sites and nucleotide addition at the control site. Consequently, the catalytic efficiency values corresponding to the control template were normalized to 1. Since each polymerase had different insertion efficiency values for a specific nucleotide and adducted template, the polymerase with the lowest insertion efficiency value was calculated and normalized to the value of 1. The discrimination factor compares the catalytic efficiency of wrong versus right nucleotide (dTTP opposite N⁶ adenine) incorporation opposite control or C₁₀S-BPDE or C₁₀R-BPDE adducted sites, having normalized the dTTP value for a particular template to 1.

RESULTS

In Vivo Mutagenesis of N-ras 61³ BPDE-Adducted DNA Compared With Those at N-ras 61²

Previous NMR analyses of the BPDE isomer C₁₀R-BPDE at 61³ revealed that there was a mixture of two conformations of the deoxyribose puckering of the adjacent 5'-dA nucleotide (Zegar et al., 1998). These NMR studies suggested a structure-based model for modulating the mutagenic spectrum of this lesion. Since in one conformation, the deoxyribose of this adenosine was in the C2'-endo geometry characteristic of a B-DNA duplex, whereas, in the other conformation, the deoxyribose was in the C3'-endo geometry, it was hypothesized that these two conformations could expand the mutagenic spectrum following replication since the C3'-endo geometry might base-pair with another dA (Zegar et al., 1998).

Therefore, the present study was carried out to analyze the mutagenic potential of BPDE-dA adducts when located at the third position in N-ras codon 61 (designated 61³) (Fig. 1, Table I). In order to determine the relative replication efficiency and mutagenic potential of the major groove-linked N⁶-dA BPDE adducts in the N-ras 61³ codon sequence context, site- and stereospecific oligodeoxynucleotides were engineered into a ss M13mp7L2 vector at a unique *EcoRI* site. The relative plaque forming abilities of the control unadducted and adducted DNAs were measured in repair-deficient AB2480 *E. coli* cells and normalized based on the percentage of input closed circular DNA molecules (Table I). To establish accurate mutation frequencies ~2,000 plaques were screened for each of the adducted vectors and more than 20,000 control plaques were assayed for background spontaneous mutagenesis. Previously, we had reported data for mutagenesis induced by BPDE-adducted N-ras 61² templates, and these are included for comparative purposes (Table I) (Chary et al., 1995).

The replication efficiencies of DNAs containing either the C₁₀R-BPDE or C₁₀S-BPDE adduct in codon 61³ or 61² generated ~10% of the total number of plaques than were formed by replication of the same vector containing an undamaged insert. Transformation of the *EcoRI* cut M13mp7L2 DNA yielded plaques at a greatly reduced frequency (0.2%). The mutations frequencies and spectra were measured for all plaques using differential hybridization. The C₁₀S-BPDE adducted templates at 61³ exhibited both A to G and A to C mutations, 0.5% and 0.6% respectively, whereas, the C₁₀R-BPDE adducted templates at 61³ exhibited A to G and A to T mutations, 0.2% and 0.3%, respectively. Thus, in contrast to data from previous studies in which the mutations were exclusively A to G at 61² and ranged from 0.26% to 0.48% for the C₁₀S-BPDE and C₁₀R-BPDE adducts, respectively (Chary et al., 1995), changing the location of these identical adducts by just one position produced an expanded mutation spectra and frequency for both stereoisomers.

Comparative Analyses of Nucleotide Incorporation Opposite the BPDE-Adducted Site at N-ras Codon 61² and 61³

Previously, we have shown that four different stereoisomers of another PAH adduct, benz[a]anthracene-dA in the same 61² context were severely blocking to replication using *E. coli* DNA polymerase III, but that the limited incorporation was error free (McNees et al., 1997). These data suggested that it was likely that other polymerases were responsible for

the *in vivo* bypass of these adducts. Comparable studies had not been previously performed in the 61³ context.

In our current study, the catalytic efficiencies of replication bypass and fidelity of incorporation were analyzed for incorporation opposite a control, unadducted dA and the C₁₀R-BPDE or C₁₀S-BPDE adducts by pol II *exo*⁻, KF *exo*⁻ and Sequenase 2.0 (Table II). Similar to what was previously shown for *E. coli* DNA pol III, in which the polymerase was severely blocked by *N*⁶-benz[*a*]anthracene-dA, *E. coli* pol II was also severely blocked, with bypass efficiencies reduced by several orders of magnitude. However, this polymerase preferentially mis-incorporated a dT opposite both the adducted sites, followed by poor incorporation of the other dNTPs (Table II). Thus, although *in vitro* bypass efficiencies were very low, these could significantly account for a portion of the error-free bypass that was measured in the *in vivo* replication studies described above.

To gain insight into which polymerase might contribute to the low mutation frequency, the catalytic efficiency and fidelity of KF *exo*⁻ was examined (Table II). Since the mutation spectra for the C₁₀R-BPDE was A to T and A to G, these data suggested that dA and dC were misincorporated opposite this adduct and extended. Analyses of the fidelity of KF *exo*⁻ revealed the same pattern of misincorporation with dA and dC/dG being preferentially incorporated. Correspondingly, *in vivo* replication of the C₁₀S-BPDE containing template had yielded A to G and A to C mutations which would predict preferential misincorporation of dC and dG, respectively. However, KF *exo*⁻ misincorporated dA and dG preferentially over dC. Furthermore, we also examined the T7 DNA polymerase (Sequenase 2.0) on these substrates (Table II), and these data were similar to those obtained by the, KF *exo*⁻ (Table II).

Since the KF *exo*⁻ data reflected only incorporation without the possibility of subsequent editing, qualitative nucleotide incorporation was analyzed using KF *exo*⁺ on both 61² and 61³ BPDE-adducted dA substrates. Our observations determined a strong preferential incorporation of dT opposite control and C₁₀R-BPDE, but no incorporation opposite the C₁₀S-BPDE adduct (data not shown). Additionally, KF *exo*⁺ could not incorporate opposite either of the adduct when positioned at 61². Thus, we consider it unlikely that *E. coli* pol I significantly contributed to the bypass of these lesions.

Differential Primer Extension Analysis From an A:C Mismatch Base Pairing Opposite the C₁₀R BPDE-Adducted Site at N-ras Codon 61² or 61³

Based on the preferential incorporation of dC opposite the 61²C₁₀R-BPDE adducted template, primer extension reactions were performed with KF *exo*⁻ and Sequenase 2.0 in which a mismatched dC was positioned opposite a control (C) or either the 61² and 61³ C₁₀R-BPDE adducted template. As shown in Figure 2, when a 20-mer primer was annealed to these 33-mer templates, both KF *exo*⁻ and Sequenase 2.0 could extend the primers to full length with a C positioned opposite the control dA (Lanes 1 and 4, respectively) and the 61² C₁₀R-BPDE adduct (Lanes 2 and 5, respectively). However, neither of the polymerases was able to extend the primer with a C opposite the C₁₀R-BPDE-adducted nucleotide at 61³. These data correlate well with the NMR data.

DISCUSSION

The fidelity of DNA synthesis depends not only on the identity of the specific polymerase, but also on primer-template geometries that affect interactions that can alter the accuracy of nucleotide incorporation at various locations along the template:primer duplex. A direct *in vivo* consequence of the differential interactions is the appearance of mutational hot and cold spots. Factors that significantly influence the polymerase interaction with the template:primer duplex are base stacking interactions, base context effects including template:primer slippage, polymerase active site constraints, and their accessory factors. Therefore, in order to understand these events more closely on encountering an adducted template, both *in vivo* and *in vitro* studies needed to be examined. The final mutagenic potential of PAH-induced DNA adducts reflect the interactions and interdependences of nucleotide excision repair, bypass by various translesion DNA polymerases, and MMR. To elucidate the mechanism of replication bypass and mutagenesis of DNAs containing BPDE adducts, our previous studies involved six stereospecific BPDE adducts on *N*⁶ adenine of the *N-ras* gene at codon 61². *In vivo* replication studies revealed exclusively A to G transitions at frequencies ranging from 0.26% to 1.20% (Chary et al., 1995). Corresponding *in vitro* studies using several DNA polymerases showed that the adduct stereochemistry was the dominant feature that modulated polymerase progression, while the specifics of misincorporation were intrinsic to each enzyme active site (Chary et al., 1995, 1997; Chary and Lloyd, 1996; Zegar et al., 1998). Taken together, these studies demonstrated distinctive patterns of replication blockage that were dependent on both the stereochemistry of the *R* and *S* configurations of the C₁₀ pyrenyl ring attached to the adenine, as well as the polymerase used. Specifically for KF *exo*⁺ or *exo*⁻, Sequenase, polymerase α , and HIV-1 RT, the three C₁₀*S*-BPDE configurations blocked replication one nucleotide 3' to the adduct site, while the three C₁₀*R*-BPDE configurations allowed replication opposite the lesion. In contrast, polymerase β displayed exactly the opposite blockage pattern. When these polymerases were forced to replicate past these lesions, distinctive patterns of misincorporation were also observed (Chary and Lloyd, 1995).

Previously, NMR analyses had shown that DNA containing the C₁₀*R*-BPDE lesion at 61² could be represented as a single conformation, in which the B[*a*]P moiety intercalated above the 5' face of the modified adenine. This conformation resulted in the buckling of the modified A:T base pair such that the base-step between the adduct and its 5' neighbor increased to accommodate the intercalated B[*a*]P, with a concomitant unwinding of the helix (Zegar et al., 1996). In contrast to this single solution conformation, NMR analyses of the C₁₀*R*-BPDE isomer at the third position of the *N-ras* codon 61 revealed a mixture of two conformations of the deoxyribose puckering of the 5' dA when the adjacent nucleotide differed. In one conformation, the deoxyribose of this adenosine was in the C2'-endo geometry, characteristic of a B-DNA duplex, whereas in the other conformation, the deoxyribose was in the C3'-endo geometry. It was hypothesized that these two conformations could expand the mutagenic spectrum following replication. The predictions made based on these NMR structures were tested in the current investigation and were corroborated with both our *in vivo* and *in vitro* studies. In addition to the anticipated A to G mutations that were detected at 61³, additional mutations of A to C were measured

following replication of the C₁₀S-BPDE adduct and A to T transversions were detected in the progeny from synthesis past C₁₀R-BPDE adducted sites. Thus, examination of the refined structures of C₁₀R-BPDE at N-ras 61² and 61³ suggested a structural basis by which the *in vivo* mutagenesis results might be explained. It is possible that the A to T mutations, observed only opposite the C₁₀R-BPDE-adducted site at 61³, were dependent on the C3'-endo conformer of this adduct. In this case, the incoming adenine opposite the adducted site would be shifted toward the minor groove, forming a hydrogen bonding interaction between N⁶ amino group of the incoming adenine and N1 of the modified nucleotide.

The sequence effect observed for the C₁₀R-BPDE adducted sites at 61² and 61³ provided a striking example of the importance of DNA sequence context in understanding adduct properties. Conformational equilibria are a feature of a number of DNA adducts, including propanodeoxyguanosine (Singh et al., 1993), aminofluorene (Cho et al., 1994; Eckel and Krugh, 1994a, 1994b), styrene oxide (Feng et al., 1995, 1996), and benzo[a]pyrene (Jelinsky et al., 1995; Rodriguez and Loechler, 1995). Interconversion between two adduct conformations has been described as a potential "mutagenic switch" (Eckel and Krugh, 1994b), in which each of the two conformations might lead to a different biological outcome. The present data suggest that the C₁₀R-BPDE lesion at 61³ provides an example of such a "switch," in which one conformation leads to low levels of cytosine misinsertion and ultimately, A to G mutations, while the second conformation allows either correct incorporation of thymidine opposite the modified adenine or possibly incorrect incorporation of adenine, resulting in an A to T mutation.

The importance of chirality has been previously exemplified by studying the replication patterns using primers that are five bases 3' to the site of the lesion (Chary and Lloyd, 1995, 1996; Chary et al., 1995, 1997). In comparison with our earlier studies with BPDE adducted DNA at N-ras 61² (Chary and Lloyd, 1995) in which full-length extension products were readily detected, when either the C₁₀S-BPDE 61³ or the C₁₀R-BPDE 61³ adduct was replicated using the exonuclease-deficient Klenow fragment, these adducts (61³) were totally blocking to *in vitro* replication, such that no full-length products were produced. This can be attributed potentially to the extent of local adduct-induced disturbances based on NMR studies. Unlike the C₁₀R-BPDE at 61², where the DNA duplex adopts a homogeneous conformation with local adduct-induced disturbances extending only one base-pair flanking the site of adduction, with C₁₀R-BPDE at 61³, broad DNA resonances were observed as far as two base pairs away from the lesion site.

Furthermore, for the 61² C₁₀R-BPDE adducts, the DNA duplex is in a B form with the sugar rings of the inner bases being in the C2'-endo conformation. Codon 61³ C₁₀R-BPDE adducts exhibit two conformations: the C3'-endo conformation leading to an A form wherein A⁶ was shifted toward the major groove and the C2'-endo conformation wherein A⁶ shifts toward the minor groove. Modeling studies revealed that the C2'-endo base pairing resembled an A:C mismatch, whereas the C3'-endo pairing resembled an A:T base pair or possibly an A:A mismatch. This differential base-pairing data elucidate the fact that even *in vitro* replication by different polymerases could be significantly altered due to local structural perturbations caused by the same adduct on neighboring bases.

In addition to PAHs being the substrates for translesion bypass, they are also potential substrates for MMR and defects in this pathway lead to significant increases in human cancer risk. MMR corrects DNA replication errors that could potentially generate base substitutions, insertions, or deletions by (i) recognizing DNA mismatches, (ii) identifying and excising nascent DNA, and (iii) directing subsequent replacement synthesis (Shah et al., 2010; Pena-Diaz and Jiricny, 2012). Thus, in MMR-deficient eukaryotic cells, mutation rates can increase 10–1,000-fold. Inherited defects in MMR genes cause Lynch syndrome that is associated with a 80–90% lifetime risk for colorectal, endometrial, or other internal cancers due to heterozygous defect, mostly in MLH1 or MSH2 (Poulogiannis et al., 2010). MMR responds to PAH adducts and other DNA lesions by either suppressing lesion-induced mutagenesis or triggering cell cycle checkpoints and apoptosis (Stojic et al., 2004). Therefore, MMR deficiency simultaneously increases mutation and decreases apoptosis.

In addition, recent studies have shown that mutations in pol β are associated with colorectal cancer (Donigan et al., 2012; Nemec et al., 2012). Sweasy and coworkers demonstrated that a variety of mutations in the coding region for pol β from these cancers affect DNA positioning, nucleotide binding, and rates of conformational changes that are important for accurate DNA synthesis. Furthermore, they suggest that the variants compromise base excision repair and lead to the induction of mutations and that base excision repair itself functions as a tumor suppressor (Kidane et al., 2010; Stachelek et al., 2010; Murphy et al., 2011).

In summary, our studies have shown that *in vivo* and *in vitro* replication is influenced not only by the stereochemistry of the BPDE molecule at the C₁₀ position, but also by sequence context. Not only do all the polymerases examined show a differential pattern of replication between C₁₀S-BPDE and C₁₀R-BPDE adducted template at N-*ras* codon 61³ but also these enzymes show distinct behavior differences on encountering C₁₀R-BPDE at 61³ versus 61².

Acknowledgments

We are grateful to Dr. T.M. Harris and Dr. C.M. Harris for the syntheses and gift of the BPDE-adducted templates. We appreciate the assistance of Dan Austin and Dr. Aaron Jacobs in compiling and creating the figures. Special thanks go to Dr. Amanda K. McCullough for insightful comments on the manuscript and to Vijay Chary for assistance in manuscript preparation.

Grant sponsor: National Institute of Health; Grant numbers: ES05355, CA160032.

REFERENCES

- Alexandrov K, Rojas M, Satarug S. The critical DNA damage by benzo(a)pyrene in lung tissues of smokers and approaches to preventing its formation. *Toxicol Lett.* 2010; 198:63–68. [PubMed: 20399842]
- Chary P, Harris CM, Harris TM, Lloyd RS. Differential tolerance to DNA polymerization by HIV-1 reverse transcriptase on N6 adenine C10R and C10S benzo[a]pyrene-7,8-dihydrodiol 9,10-epoxide-adducted templates. *J Biol Chem.* 1997; 272:5805–5813. [PubMed: 9038195]
- Chary P, Latham GJ, Robberson DL, Kim SJ, Han S, Harris CM, Harris TM, Lloyd RS. In vivo and in vitro replication consequences of stereoisomeric benzo[a]pyrene-7,8-dihydrodiol 9,10-epoxide adducts on adenine N6 at the second position of N-ras codon 61. *J Biol Chem.* 1995; 270:4990–5000. [PubMed: 7890605]

- Chary P, Lloyd RS. In vitro replication by prokaryotic and eukaryotic polymerases on DNA templates containing site-specific and stereospecific benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide adducts. *Nucleic Acids Res.* 1995; 23:1398–1405. [PubMed: 7753632]
- Chary P, Lloyd RS. Impact of the stereochemistry of benzo[a]pyrene 7,8-dihydrodiol 9,10-epoxide-deoxyadenosine adducts on resistance to digestion by phosphodiesterases I and II and translesion synthesis with HIV-1 reverse transcriptase. *Chem Res Toxicol.* 1996; 9:409–417. [PubMed: 8839043]
- Cho BP, Beland FA, Marques MM. NMR structural studies of a 15-mer DNA duplex from a ras protooncogene modified with the carcinogen 2-aminofluorene: conformational heterogeneity. *Biochemistry.* 1994; 33:1373–1384. [PubMed: 8312255]
- Diggs DL, Huderson AC, Harris KL, Myers JN, Banks LD, Rekhadevi PV, Niaz MS, Ramesh A. Polycyclic aromatic hydrocarbons and digestive tract cancers: a perspective. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev.* 2011; 29:324–357. [PubMed: 22107166]
- Donigan KA, Sun KW, Nemec AA, Murphy DL, Cong X, Northrup V, Zeltermann D, Sweasy JB. Human POLB gene is mutated in high percentage of colorectal tumors. *J Biol Chem.* 2012; 287:23830–23839. [PubMed: 22577134]
- Eckel LM, Krugh TR. 2-Aminofluorene modified DNA duplex exists in two interchangeable conformations. *Nat Struct Biol.* 1994a; 1:89–94. [PubMed: 7656023]
- Eckel LM, Krugh TR. Structural characterization of two interchangeable conformations of a 2-aminofluorene-modified DNA oligomer by NMR and energy minimization. *Biochemistry.* 1994b; 33:13611–13624. [PubMed: 7947770]
- Feng B, Voehler M, Zhou L, Passarelli M, Harris CM, Harris TM, Stone MP. Major groove (S)-alpha-(N6-adenyl)styrene oxide adducts in an oligodeoxynucleotide containing the human N-ras codon 61 sequence: conformations of the S(61,2) and S(61,3) sequence isomers from 1H NMR. *Biochemistry.* 1996; 35:7316–7329. [PubMed: 8652508]
- Feng B, Zhou L, Passarelli M, Harris CM, Harris TM, Stone MP. Major groove (R)-alpha-(N6-adenyl)styrene oxide adducts in an oligodeoxynucleotide containing the human N-ras codon 61 sequence: conformations of the R(61,2) and R(61,3) sequence isomers from 1H NMR. *Biochemistry.* 1995; 34:14021–14036. [PubMed: 7578000]
- Harris CM, Zhou L, Strand E, Harris TM. New strategy for the synthesis of oligodeoxynucleotides bearing adducts at exocyclic amino sites of purine nucleosides. *J Am Chem Soc.* 1991; 113:4328–4329.
- Hecht SS. Lung carcinogenesis by tobacco smoke. *Int J Cancer.* 2012; 131:2724–2732. [PubMed: 22945513]
- IARC. Personal habits and indoor combustions. Volume 100 E. A review of human carcinogens. IARC Monogr Eval Carcinog Risks Hum. 2012; 100(Pt E):1–538.
- Jelinsky SA, Liu T, Geacintov NE, Loechler EL. The major, N2-Gua adduct of the (+)-anti-benzo[a]pyrene diol epoxide is capable of inducing G→A and G→C, in addition to G→T, mutations. *Biochemistry.* 1995; 34:13545–13553. [PubMed: 7577943]
- Kidane D, Jonason AS, Gorton TS, Mihaylov I, Pan J, Keeney S, de Rooij DG, Ashley T, Keh A, Liu Y, Banerjee U, Zeltermann D, Sweasy JB. DNA polymerase beta is critical for mouse meiotic synapsis. *EMBO J.* 2010; 29:410–423. [PubMed: 20019666]
- Kim SJ, Jajoo HK, Kim HY, Zhou L, Horton P, Harris CM, Harris TM. An efficient route to N6 deoxyadenosine adducts of diol epoxides of carcinogenic polycyclic aromatic hydrocarbons. *Bioorg Med Chem.* 1995; 3:811–822. [PubMed: 7582958]
- Klarer AC, McGregor W. Replication of damaged genomes. *Crit Rev Eukaryot Gene Expr.* 2011; 21:323–336. [PubMed: 22181702]
- McNees AG, O'Donnell M, Horton PH, Kim HY, Kim SJ, Harris CM, Harris TM, Lloyd RS. Lack of correlation between in vitro and in vivo replication of precisely defined benz-a-anthracene adducted DNAs. *J Biol Chem.* 1997; 272:33211–33219. [PubMed: 9407110]
- Miyaki M, Iijima T, Ishii R, Kita Y, Koike M, Kuroki T, Mori T. Increased frequency of p53 mutation in sporadic colorectal cancer from cigarette smokers. *Jpn J Clin Oncol.* 2002; 32:196–201. [PubMed: 12110635]

- Murphy DL, Jaeger J, Sweasy JB. A triad interaction in the fingers subdomain of DNA polymerase beta controls polymerase activity. *J Am Chem Soc.* 2011; 133:6279–6287. [PubMed: 21452873]
- Nemec AA, Donigan KA, Murphy DL, Jaeger J, Sweasy JB. Colon cancer-associated DNA polymerase beta variant induces genomic instability and cellular transformation. *J Biol Chem.* 2012; 287:23840–23849. [PubMed: 22573322]
- Pena-Diaz J, Jiricny J. Mammalian mismatch repair: error-free or error-prone? *Trends Biochem Sci.* 2012; 37:206–214. [PubMed: 22475811]
- Poulogiannis G, Frayling IM, Arends MJ. DNA mismatch repair deficiency in sporadic colorectal cancer and Lynch syndrome. *Histopathology.* 2010; 56:167–179. [PubMed: 20102395]
- Rodriguez H, Loechler EL. Are base substitution and frameshift mutagenesis pathways interrelated? An analysis based upon studies of the frequencies and specificities of mutations induced by the (+)-anti diol epoxide of benzo[a]pyrene. *Mutat Res.* 1995; 326:29–37. [PubMed: 7528883]
- Shah SN, Hile SE, Eckert KA. Defective mismatch repair, micro-satellite mutation bias, and variability in clinical cancer phenotypes. *Cancer Res.* 2010; 70:431–435. [PubMed: 20068152]
- Singh US, Moe JG, Reddy GR, Weisenseel JP, Marnett LJ, Stone MP. 1H NMR of an oligodeoxynucleotide containing a propanodeoxyguanosine adduct positioned in a (CG)₃ frameshift hotspot of *Salmonella typhimurium* hisD3052: Hoogsteen base-pairing at pH 5.8. *Chem Res Toxicol.* 1993; 6:825–836. [PubMed: 8117922]
- Stachelek GC, Dalal S, Donigan KA, Campisi Hegan D, Sweasy JB, Glazer PM. Potentiation of temozolomide cytotoxicity by inhibition of DNA polymerase beta is accentuated by BRCA2 mutation. *Cancer Res.* 2010; 70:409–417. [PubMed: 20028873]
- Stojic L, Brun R, Jiricny J. Mismatch repair and DNA damage signalling. *DNA Repair (Amst).* 2004; 3:1091–1101. [PubMed: 15279797]
- Thun MJ, Carter BD, Feskanich D, Freedman ND, Prentice R, Lopez AD, Hartge P, Gapstur SM. 50-year trends in smoking-related mortality in the United States. *N Engl J Med.* 2013; 368:351–364. [PubMed: 23343064]
- Zegar IS, Chary P, Jabil RJ, Tamura PJ, Johansen TN, Lloyd RS, Harris CM, Harris TM, Stone MP. Multiple conformations of an intercalated (–)-(7S,8R,9S 10R)-N6-[10-(7,8,9,10-tetrahydrobenzo[a]pyrenyl)]-2'-deoxyadenosyl adduct in the N-ras codon 61 sequence. *Biochemistry.* 1998; 37:16516–16528. [PubMed: 9843418]
- Zegar IS, Kim SJ, Johansen TN, Horton PJ, Harris CM, Harris TM, Stone MP. Adduction of the human N-ras codon 61 sequence with (–)-(7S,8R,9R,10S)-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a] pyrene: structural refinement of the intercalated SRSR(61,2) (–)-(7S,8R,9S, 10R)-N6-[10-(7,8,9,10-tetrahydrobenzo[a]pyrenyl)]-2'-deoxyadenosyl adduct from 1H NMR. *Biochemistry.* 1996; 35:6212–6224. [PubMed: 8639561]
- Zienolddiny S, Ryberg D, Svendsrud DH, Eilertsen E, Skaug V, Hewer A, Phillips DH, te Riele H, Haugen A. Msh2 deficiency increases susceptibility to benzo[a]pyrene-induced lymphomagenesis. *Int J Cancer.* 2006; 118:2899–2902. [PubMed: 16381012]

Fig. 1.

Structure of stereoisomeric *N*⁶-BPDE adducts. The structures of the (+)-(7R, 8S, 9R, 10S)-anti-*trans*-BPDE (C₁₀S-BPDE) dA or (-)(7S, 8R, 9S, 10R)-anti-*trans*-BPDE (C₁₀R-BPDE) dA lesions.

Fig. 2.

In vitro replication assays utilizing A:C mismatched primers. KF exo⁻ and Sequenase version 2.0 were used in primer (³²P-radiolabeled 20-mers) extension reactions in which a 3' mismatched dC was positioned opposite a control, unadducted dA (C) in a 33-mer template or a template containing the C₁₀R-BPDE adduct at either position 61² or 61³. Lanes 1–3 and 4–6 represent reactions for KF exo⁻ and Sequenase 2.0, respectively.

TABLE I

In Vivo Mutagenesis With C₁₀S⁶ and C₁₀R BPDE-Adducted DNA at N-*ras* 61³

<i>N-ras</i> 61 template	Total No. plaques screened	Percentage of plaques ^a	A to G (%)	A to C (%)	A to T (%)
Unadducted 61 ³	21,096	100	0	0	0
C ₁₀ S-BPDE 61 ³	2,120	10.1	0.5	0.6	0
C ₁₀ R-BPDE 61 ³	1,599	7.6	0.2	0	0.3
Unadducted 61 ²	25,104	100	0	0	0
C ₁₀ S-BPDE 61 ²	1,898	7.6	0.26	0	0
C ₁₀ R-BPDE 61 ²	1,456	5.8	0.48	0	0
Linearized M13mp7/L2	56	0.2	NA	NA	NA

Note: The data using the adducted C₁₀S-BPDE and C₁₀R-BPDE at Ade 61² have been inserted from Chary *et al.* (1995) for comparison with the *in vivo* results for the corresponding C₁₀S- and C₁₀R-BPDE adducts at position 61³.

^aRelative to unadducted template.

NA, not applicable.

TABLE II
Catalytic Efficiencies of Replication Bypass of Unadducted, C₁₀S- and C₁₀R-BPDE Adducts at 61³ Adenine (CAA)

Enzyme	Template	Catalytic efficiency k_{cat}/K_m (M ⁻¹ min ⁻¹)			
		dTTP	dATP	dCTP	dGTP
Pol II <i>exo</i> ⁻	A	1.3 × 10 ⁶	2.7 × 10 ²	60.0	18.0
	C ₁₀ S	9.8	6.1	0.4	1.5
	C ₁₀ R	1.3 × 10 ²	4.4	5.5	5.4
KF <i>exo</i> ⁻	A	4.6 × 10 ⁷	3.5 × 10 ⁴	7.5 × 10 ⁴	1.3 × 10 ³
	C ₁₀ S	16.0	3.5 × 10 ⁴	61.0	1.5 × 10 ³
	C ₁₀ R	2.2 × 10 ⁵	6.8 × 10 ³	38.0	40.0
Seq	A	4.7 × 10 ⁷	2.9 × 10 ³	3.3 × 10 ³	5.6 × 10 ²
	C ₁₀ S	1.3 × 10 ²	2.7 × 10 ³	39.0	30.0
	C ₁₀ R	1.7 × 10 ²	4.0 × 10 ²	57.0	47.0