



## Error-Prone and Error-Free Translesion DNA Synthesis over Site-Specifically Created DNA Adducts of Aryl Hydrocarbons (3-Nitrobenzanthrone and 4-Aminobiphenyl)

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Aryl hydrocarbons such as 3-nitrobenzanthrone (NBA), 4-aminobiphenyl (ABP), acetylaminofluorene (AAF), benzo(a)pyrene (BaP), and 1-nitropyrene (NP) form bulky DNA adducts when absorbed by mammalian cells. These chemicals are metabolically activated to reactive forms in mammalian cells and preferentially get attached covalently to the  $N^2$  or C8 positions of guanine or the  $N^6$  position of adenine. The proportion of  $N^2$  and C8 guanine adducts in DNA differs among chemicals. Although these adducts block DNA replication, cells have a mechanism allowing to continue replication by bypassing these adducts: translesion DNA synthesis (TLS). TLS is performed by translesion DNA polymerases—Pol  $\eta$ ,  $\kappa$ ,  $\iota$ , and  $\zeta$  and Rev1—in an error-free or error-prone manner. Regarding the NBA adducts, namely, 2-(2'-deoxyguanosin- $N^2$ -yl)-3-aminobenzanthrone (dG- $N^2$ -ABA) and  $N$ -(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (dG-C8-ABA), dG- $N^2$ -ABA is produced more often than dG-C8-ABA, whereas dG-C8-ABA blocks DNA replication more strongly than dG- $N^2$ -ABA. dG- $N^2$ -ABA allows for a less error-prone bypass than dG-C8-ABA does. Pol  $\eta$  and  $\kappa$  are stronger contributors to TLS over dG-C8-ABA, and Pol  $\kappa$  bypasses dG-C8-ABA in an error-prone manner. TLS efficiency and error-proneness are affected by the sequences surrounding the adduct, as demonstrated in our previous study on an ABP adduct,  $N$ -(2'-deoxyguanosine-8-yl)-4-aminobiphenyl (dG-C8-ABP). Elucidation of the general mechanisms determining efficiency, error-proneness, and the polymerases involved in TLS over various adducts is the next step in the research on TLS. These TLS studies will clarify the mechanisms underlying aryl hydrocarbon mutagenesis and carcinogenesis in more detail.

**Key words:** Aryl hydrocarbon, DNA adduct, Translesion DNA synthesis, 3-Nitrobenzanthrone, Mutation

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Abbreviations: AAF, acetylaminofluorene; ABP, 4-aminobiphenyl; AP, apurinic/aprimidinic; BaP, benzo(a)pyrene; BER, base excision repair; BPDE, ( $\pm$ )-anti-benzo[a]pyrene-7,8-diol-9,10-epoxide; IPTG, isopropyl- $\beta$ -D-thiogalactopyranoside; IQ, 2-amino-3-methylimidazo-[4,5-f]quinoline;  $N$ -Aco-ABA,  $N$ -acetoxy-3-aminobenzanthrone; dG-C8-ABP,  $N$ -(2'-deoxyguanosine-8-yl)-4-aminobiphenyl; dG- $N^2$ -ABA, 2-(2'-deoxyguanosin- $N^2$ -yl)-3-aminobenzanthrone; dG-C8-ABA,  $N$ -(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone; NBA, 3-nitrobenzanthrone; NER, nucleotide excision repair; NP, 1-nitropyrene; TLS, translesion DNA synthesis; X-gal, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside; XPA, xeroderma pigmentosum complementation group A.

## INTRODUCTION

Some mutagens have reactive groups such as aldehyde and methyl in their structures, covalently bind to naked or cellular DNA via a nucleophilic reaction, and are called direct mutagens. Other mutagens are metabolically transformed to reactive intermediates in the detoxification process after mutagens are absorbed by cells, get covalently attached to cellular DNA, and are called indirect mutagens. In DNA, mutagens covalently bind to one of several positions mostly in guanine and adenine. Alkylating agents, such as methyl methanesulfonate, *N*-methyl-*N'*-nitrosoguanidine, and *N*-methyl nitrosourea, preferentially donate methyl groups to the N7 or N3 position of guanine or adenine or the *O*<sup>6</sup> position of guanine. Aryl hydrocarbons such as benzo(a)pyrene (BaP) and 1,8-dinitropyrene are metabolically activated, and their metabolites preferentially get attached covalently to the N<sup>2</sup> or C8 position of guanine. There are numerous kinds of mutagens in the environment; however, their molecular pathways to DNA adduct formation have not yet been clarified in detail.

In this review, the mechanisms underlying the mutations caused by environmental aryl hydrocarbons, particularly 3-nitrobenzanthrone (NBA) and 4-aminobiphenyl (ABP), are described. These compounds are biotransformed into reactive forms by phase I and II cellular metabolic enzymes. The formation and repair of their DNA adducts in cells are also described. Although these DNA adducts block DNA replication, cells have a mechanism allowing to continue replication by bypassing these adducts by means of specific DNA polymerases, and this process is called translesion DNA synthesis (TLS). TLS over the adducts derived from NBA and ABP is discussed using data obtained by our original TLS assay.

## FORMATION AND REPAIR OF BULKY DNA ADDUCTS

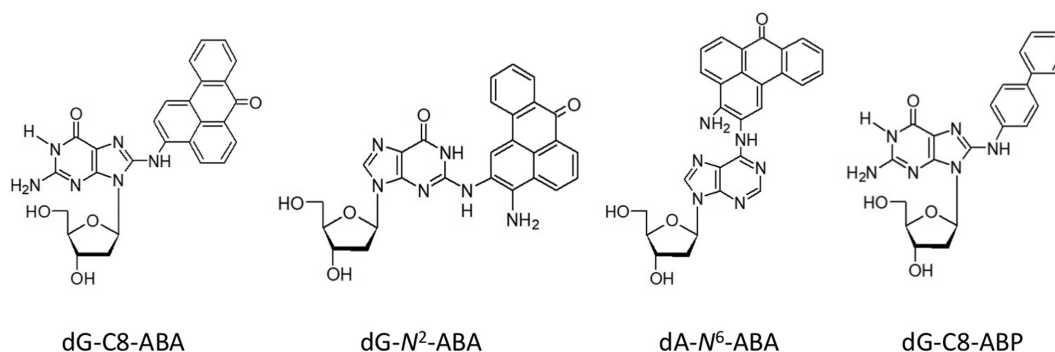
Most aryl hydrocarbons known as environmental pollutants are indirect mutagens, which are transformed to reactive forms by detoxification enzymes in some cells. The

process of biotransformation of BaP, which is abundant in the atmosphere and released during oil combustion, has been elucidated in detail (1). BaP binds to aryl hydrocarbon receptors, and functions as a transcription factor to induce *CYP1* family genes in cells. Induced CYP1A1 and CYP1B1 enzymes hydroxylate a benzene ring and eventually transform B(a)P to (±)-anti-benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE). BPDE mainly binds covalently to the N<sup>2</sup> position of guanine (2-4).

NBA is an air pollutant that originates from the diesel exhaust of cars and factories and a strong mutagen like 1,8-dinitropyrene in the Ames bacterial mutation assay (5). NBA is primarily processed by cytosolic nitroreductases, followed by activation by *N*-acetyltransferase and sulfotransferase, leading to the formation of a reactive intermediate, *N*-acetoxy-3-aminobenzanthrone (*N*-Aco-ABA), which forms covalent bonds with guanine and adenine (6,7). These adducts are 2-(2'-deoxyguanosin-N<sup>2</sup>-yl)-3-aminobenzanthrone (dG-N<sup>2</sup>-ABA), *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (dG-C8-ABA), and 2-(2'-deoxyadenosin-N<sup>6</sup>-yl)-3-aminobenzanthrone (dA-N<sup>6</sup>-ABA), listed in the order of their amounts (8,9) (Fig. 1). NBA causes lung cancer in experimental animals (10).

ABP is mainly generated by cigarette smoking and combustion of fossil fuels and is also a byproduct of the rubber, coal, textile, and printing industries (11). ABP has been identified as a major etiological agent of human bladder cancer and is a potent urinary-bladder carcinogen in experimental animals (12,13). ABP is metabolically activated by cellular *N*-acetyltransferase and forms a major DNA adduct, *N*-(2'-deoxyguanosine-8-yl)-4-aminobiphenyl (dG-C8-ABP) (Fig. 1). The mutation of the *p53* tumor suppressor gene in bladder carcinomas of patients who are occupationally exposed to ABP has been examined, and *p53* mutation hot spots coincide with the ABP-adducted sites of the *p53* gene in cells experimentally exposed to ABP (14).

In the cell, bulky DNA lesions including NBA and ABP adducts are removed by the nucleotide excision repair (NER) pathway, which involves dual incisions of adducted DNA strands, removal of the incised strand, and semicon-



**Fig. 1.** Structures of dG-C8-ABA, dG-N<sup>2</sup>-ABA, dA-N<sup>6</sup>-ABA, and dG-C8-ABP.

servative filling (DNA synthesis) of the gap (removed region). NER is performed by several proteins including various xeroderma pigmentosum (XP) family proteins, the transcription factor TFIIH complex, DNA helicase, polymerase  $\delta/\epsilon$ , and ligase with their accessory proteins (15,16). Small lesions such as alkyl and hydroxyl adducts are removed by another pathway, base excision repair (BER) (17,18). In BER, the damaged base is excised by DNA glycosylase. The excision of an abasic deoxynucleotide from the DNA strand is performed by apurinic/aprimidinic (AP) endonuclease, and a correct deoxynucleotide is inserted into the AP site by DNA polymerase  $\beta$  and some accessory proteins. Both NER and BER fix damaged DNA accurately without induction of mutations during their processes.

### TLS SYSTEM FOR BYPASSING DNA ADDUCTS

When genomic DNA containing bulky adducts proceeds to replication, the replication fork is stalled at each adduct, leading to cell death. To avoid cell death, cells have mechanisms to complete DNA replication by bypassing these adducts. There are two mechanisms for DNA replication bypass: one is damage avoidance including replication fork regression and recombination repair, which are error-free mechanisms; the other is TLS, which is either error-free or error-prone depending on the adduct structure and the DNA polymerases involved (19,20). The DNA polymerases participating in TLS are translesion DNA polymerases, which are different from the replicative DNA polymerases  $\delta$  and  $\epsilon$ . The major translesion DNA polymerases are Pol  $\eta$ ,  $\kappa$ ,  $\iota$ , and  $\zeta$  and Rev1 (21,22). When a DNA replication fork is stalled at a DNA adduct, mono-ubiquitination of proliferative cell nuclear antigen (PCNA) by Rad6/Rad18 proteins triggers the replacement of Pol  $\delta$  or  $\epsilon$  with Pol  $\eta$ ,  $\kappa$ ,  $\iota$ , or  $\zeta$  or Rev1, which continues DNA synthesis over the adduct (19,20). Given that translesion DNA polymerases are deficient in 3' to 5' exonuclease activity (proofreading activity), these polymerases readily insert an incorrect nucleotide opposite the adducted nucleotide and cause a base-change mutation.

### CELL-FREE TLS ANALYSIS (IN VITRO TLS ASSAY)

To measure the activity of each polymerase in terms of TLS over various bulky adducts, *in vitro* cell-free assays have been performed in many studies (23-28). Such an assay is composed of an oligonucleotide containing a bulky adduct at a specific site, a 5' [ $^{32}\text{P}$ ]labeled primer, four types of deoxynucleotides, and one purified DNA polymerase. Semiconservative DNA extension from the [ $^{32}\text{P}$ ]labeled primer across the adduct, extension arrest at the adduct, and incorporated nucleotides opposite the adduct are visualized by polyacrylamide gel electrophoresis and autoradiography.

*In vitro* TLS assays have revealed that Pol  $\eta$  and  $\iota$  enable a slow bypass over an  $N^2$  adduct of BPDE or 2-amino-3-

methylimidazo-[4,5-*f*]quinoline (IQ) with guanine; these adducts result in high frequencies of nucleotide misincorporation (28-30). IQ is a heterocyclic aromatic amine formed by cooking of meat or fish. Pol  $\kappa$  performs accurate and reasonably efficient bypass over the dG- $N^2$ -BPDE and dG- $N^2$ -IQ adducts. On the other hand, Pol  $\eta$  allows for an efficient error-free bypass over dG-C8-IQ, dG-C8-ABA, or *N*-[deoxyguanosine-8-yl]-1-aminopyrene (dG-C8-AP). dG-C8-AP is an aminopyrene adduct formed by the diesel exhaust-derived air pollutant 1-nitropyrene (31-35). An inefficient and error-prone bypass over dG-C8-IQ or dG-C8-AP is performed by Pol  $\kappa$  and  $\zeta$  (31) and by Pol  $\iota$  and  $\kappa$  (34,35), respectively. Pol  $\kappa$  enables an efficient and error-free bypass, whereas Pol  $\iota$  allows for an inefficient and error-prone bypass over dG-C8-ABA (32,33). Unlike the dG- $N^2$  adducts, the error-free and error-prone patterns of TLS over dG-C8 adducts are complicated.

### DEVELOPMENT OF A TLS ANALYSIS IN MAMMALIAN CELLS (IN VIVO TLS ASSAY)

*In vitro* TLS assays may not accurately reflect cellular responses to these adducts because these assays involve limited components as described above. PCNA and replication protein A (RP-A) are essential for DNA replication in the cell but are not included in *in vitro* assays. PCNA and RP-A assist DNA polymerases to efficiently bypass adducts in the assays (36). Therefore, cell-based TLS assays (*in vivo* TLS assays) using shuttle vector plasmids with these adducts have been established by several research groups.

Moriya and colleagues constructed a single-stranded pM2-based plasmid that carries a ~15-mer oligonucleotide with a site-specifically created single bulky adduct (37-39). The plasmid has a replication origin of simian virus 40 (SV40) and replicates in simian COS-7 cells. They also observed error-prone and error-free TLS over the adducts with various endogenous reactive oxygen species (38,40). Yasui *et al.* (41) investigated TLS over the acetylaminofluorene (AAF) adducts, dG- $N^2$ -AAF and dG-C8-AAF, by a similar method and found that a single dG- $N^2$ -AAF adduct reduces replication efficiency more than dG-C8-AAF does. Both adducts promote G to T transversion, with dG- $N^2$ -AAF being less mutagenic than dG-C8-AAF (41).

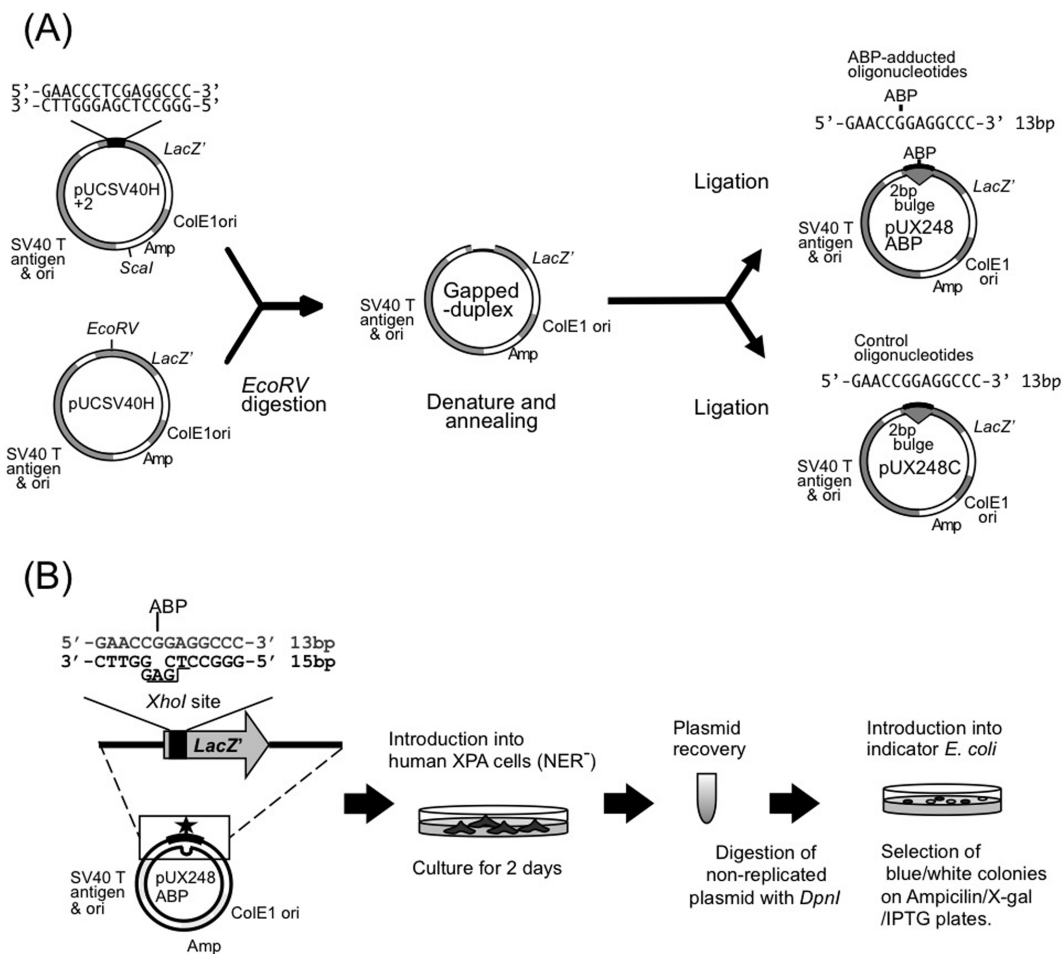
Livneh and colleagues (42,43) and Basu and colleagues (31,44-46) used another *in vivo* TLS assay. They constructed hetero-duplex gap-lesion plasmids using two types of plasmids of different lengths. A site-specifically created single bulky adduct is located in a single-strand gap region (~16 nucleotides). The gap region is filled by TLS after the plasmids are introduced into human cells. They studied TLS over various kinds of bulky adducts in human cells. Basu and colleagues found that Pol  $\eta$ ,  $\kappa$ ,  $\iota$ , and  $\zeta$  and Rev1 each plays a role in TLS over dG-C8-ABA in human cells in which TLS polymerases were knocked down by siRNA

(46). Pol  $\eta$  and  $\kappa$  are considered the major contributors to error-prone TLS over dG-C8-ABA. Rev1 is also important for mutagenesis but may perform a noncatalytic function by physically interacting with the other two TLS polymerases. In contrast, pol  $\zeta$  appears to be involved in the error-free bypass of the adduct.

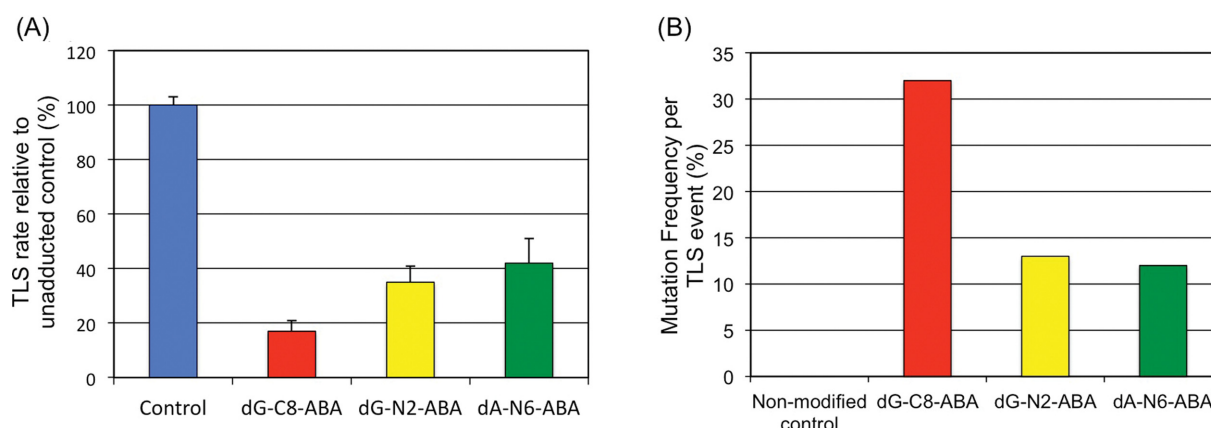
### IN VIVO TLS REPORTER ASSAY SYSTEM

Our method for the *in vivo* TLS analysis is based on that originally developed for studies on TLS in *Escherichia coli* (47,48). The constructed shuttle vector plasmid is composed of a *lacZ* mutation marker gene, ColE1 replication origin, ampicillin resistance gene (marker), an SV40 replication origin, and the T antigen gene, and the plasmid can replicate in human and *E. coli* cells (48). In the plasmid *lacZ* gene, a bulky adduct is created site-specifically on one DNA strand. The other DNA strand without the bulky adduct has a two-base bulge, and thus is two bases longer

than the strand with the adduct (Fig. 2). When the DNA strand with the adduct replicates in a human cell, the progenitor plasmids extracted from the cells form blue *E. coli* colonies on agar plates containing ampicillin, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal), and isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). When the other DNA strand without the adduct replicates in the cell, the progenitor plasmids yield white *E. coli* colonies on the plates. The proportion of blue colonies among all colonies from the plasmids with adducts is divided by the proportion of those resulting from the plasmids without the adducts; the resulting number represents the rate of TLS over the adducts. Via sequencing of the *lacZ* gene in the plasmids extracted from the blue colonies, the frequency and types of mutations caused by the adducts can be elucidated. In these assays, SV40-transformed cells derived from a patient with xeroderma pigmentosum complementation group A (XPA) are used because XPA cells lack the nucleotide excision repair system and cannot remove bulky adducts from plasmids.



**Fig. 2.** Methods for the construction of site-specifically dG-C8-ABP-adducted plasmids (A) and the *in vivo* TLS reporter assay (B). To construct NBA-adducted plasmids, CCTTCCG\*TCTCCC is used for dG-N<sup>2</sup>-ABA and dG-C8-ABA, and CCTTCCA\*TCTCCC is applied for dA-N<sup>6</sup>-ABA (G\* and A\* are ABA-adducted bases).



**Fig. 3.** Rates of TLS over different NBA adducts (A), and frequencies of mutations of the adducted bases (B). The NBA adducts, dG-N<sup>2</sup>-ABA and dG-C8-ABA are formed in CCTTCCG\*TCTCCC, while dA-N<sup>6</sup>-ABA is formed in CCTTCCA\*TCTCCC (G\* and A\* are adducted bases).

### MUTATIONS ARISING FROM DIFFERENT ADDUCTS INDUCED BY NITROBENZANTHRONE IN HUMAN CELLS

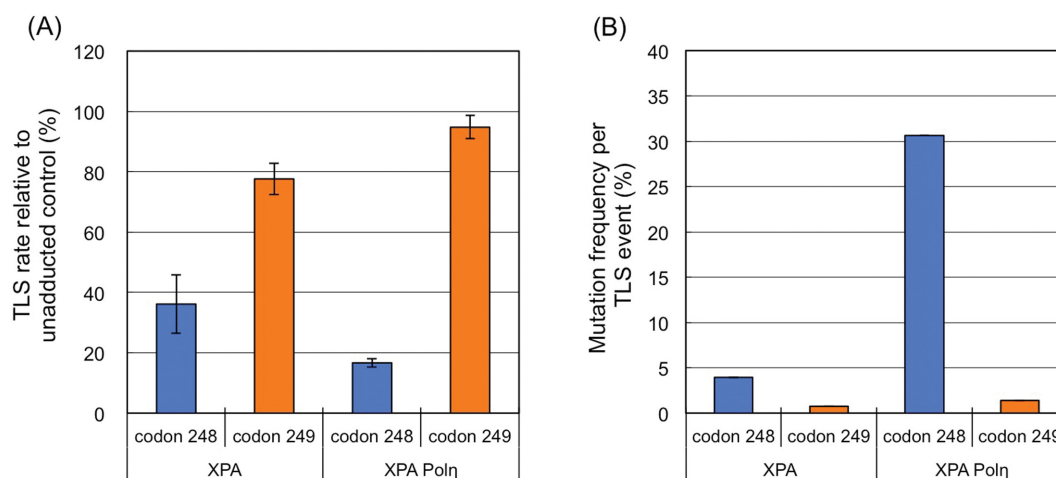
TLS over three major NBA adducts, dG-N<sup>2</sup>-ABA, dG-C8-ABA, and dA-N<sup>6</sup>-ABA, in XPA cells was analyzed with our *in vivo* TLS reporter assay system (9). The assay showed that dG-C8-ABA strongly blocks plasmid replication, while dG-N<sup>2</sup>-ABA and dA-N<sup>6</sup>-ABA block replication moderately (Fig. 3). Mutations occur more frequently during TLS over dG-C8-ABA than during TLS over dG-N<sup>2</sup>-ABA or dA-N<sup>6</sup>-ABA. The types of induced mutations are G to T transversion at dG-N<sup>2</sup>-ABA, G to A and T base substitutions at dG-C8-ABA, and A to G transition at dA-N<sup>6</sup>-ABA. NBA yields dG-C8-ABA more abundantly in cells, and dG-C8-ABA blocks DNA replication and causes

mutations more effectively than the other adducts do; therefore, dG-C8-ABA may be the DNA damage that causes NBA mutagenesis and/or carcinogenesis most frequently (9).

In contrast to human cells, the rate of TLS over dG-N<sup>2</sup>-ABA is markedly reduced when the adducted plasmid is propagated in *E. coli*, even under SOS-induced conditions, and dG-N<sup>2</sup>-ABA induces G to T mutations. Other ABA adducts are bypassed in a less mutagenic manner (49). These findings reveal host species differences in the ability to bypass these adducts.

### MUTATIONS ARISING FROM AMINOBIPHENYL-GUANINE ADDUCTS IN DIFFERENT DNA SEQUENCES

TLS over dG-C8-ABP in different DNA sequences has



**Fig. 4.** Rates of TLS over ABP adducts formed on guanine in different sequences (A), and frequencies of mutations of the adducted bases (B) in XPA cells and those overexpressing Pol η. dG-C8-ABP is formed in CCG\*GAGGC and CCGGAG\*GCC (G\* is the adducted base), which are codon 248 and 249 sequences, respectively, of the human *p53* tumor suppressor gene.

been examined in our *in vivo* TLS reporter assay system. ABP is attached to the C8-guanine of CCG\*GAGGC and CCGGAG\*GCC (G\* is the adducted base), which represent codon 248 and 249 sequences of the human *p53* tumor suppressor gene, respectively. Codons 248 and 249 are a mutation hot spot and cold spot of the *p53* gene, respectively, in bladder cancer, whereas codon 248 is a hot spot of ABP adduct formation (14).

The dG-C8-ABP of codon 248 inserted into the *lacZ* gene strongly blocks plasmid replication, whereas that of codon 249 blocks replication moderately in human XPA cells (Fig. 4). Mutations are more frequently induced in codon 248 than in codon 249. The major induced mutation is G to T transversion (50). These findings indicate that the efficiency of TLS over dG-C8-ABP is affected by the surrounding sequences. The mutation distribution in the *p53* gene in ABP-induced bladder cancer may be affected by TLS efficiency over the adducts as well as the efficiency of adduct formation in the *p53* sequence.

In our *in vivo* TLS reporter assay, Pol  $\eta$  overexpression forced by the transfection of Pol  $\eta$  expression plasmids into XPA cells potentiates DNA replication block and mutation induction at dG-C8-ABP in codon 248 of the *p53* gene (Fig. 4), and induces G to A transition as well as G to T transversion at the adduct (50). This finding suggests that Pol  $\eta$  is involved in error-prone TLS over dG-C8-ABP in codon 248 of the *p53* gene in human cells.

## CONCLUSIONS

Aryl hydrocarbons such as NBA, ABP, AAF, BaP, and NP are metabolically activated to reactive forms in certain mammalian cells and preferentially bind covalently to the  $N^2$  or C8 position of guanine, thus forming a bulky DNA adduct. Such adducts are subjected to TLS to avoid cell death. TLS is performed by Pol  $\eta$ ,  $\kappa$ ,  $\iota$ , and  $\zeta$  and Rev1 in an error-free or error-prone manner in the cell. Regarding the NBA adducts (dG- $N^2$ -ABA and dG-C8-ABA), the *in vivo* gap-lesion TLS assay suggests that Pol  $\eta$  and  $\kappa$  are the major contributors to error-prone TLS over dG-C8-ABA, whereas Pol  $\zeta$  seems to participate in error-free bypass over the adduct (46). Our *in vivo* TLS reporter assay indicates that dG- $N^2$ -ABA blocks DNA replication less than dG-C8-ABA does and is less error-prone than dG-C8-ABA in human XPA cells. dG-C8-ABA mainly causes G to T transversion (9). The research on TLS polymerases involved in error-free or error-prone bypass over dG- $N^2$ -ABA and dG-C8-ABA is in progress in TLS polymerase-deficient cells created by a genome-editing method. Our preliminary findings suggest that dG- $N^2$ -ABA is mainly bypassed by Pol  $\kappa$  in an error-free manner, whereas dG-C8-ABA is bypassed by Pol  $\eta$  in an error-free manner, but by Pol  $\kappa$  in an error-prone manner.

The stronger contribution of the C8 guanine adduct to the

replication block and error-prone TLS than that of the  $N^2$  guanine adduct may not be a universal phenomenon. dG- $N^2$ -IQ blocks DNA replication more strongly than dG-C8-IQ does. Pol  $\eta$  is mainly involved in the bypass over dG- $N^2$ -IQ and dG-C8-IQ in an error-prone and error-free manner, respectively (30,31). dG-C8-AAF blocks DNA replication slightly more strongly than dG- $N^2$ -AAF does, and the bypass of dG-C8-AAF is more error-prone than that of dG- $N^2$ -AAF (41). dG-C8-AAF allows for a higher frequency of nucleotide misincorporation than dG- $N^2$ -AAF does when Pol  $\eta$  and  $\kappa$  are employed in *in vitro* TLS assays (41). The inconsistency of the TLS efficiency over C8 guanine adducts may be due to differences in the adducted molecules, assay systems, and host mammalian cells used in three laboratories.

According to our *in vivo* reporter assay of TLS over dG-C8-ABP, it is important to note that TLS efficiency and error-proneness are affected by the surrounding DNA sequences of the adduct (50). Elucidation of the general mechanisms determining the efficiency, error-proneness, and participating polymerases of TLS over these adducts represents the next step in the research into TLS. These studies will clarify the mechanisms underlying aryl hydrocarbon mutagenesis and carcinogenesis.

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## CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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