patients. That hypermethylation of 5′CpG island in EphA7 gene is not caused by aging was confirmed by MSP ($P=0.8067$). When analysis of relation between pathological classification and MSP, the P-volume was 0.0867 if including all three types of pathological classification (well differentiated, moderately differentiated, and mucinous adenocarcinoma). Considering that mucinous type is a rare type (only two in 75 cases of colorectal cancers), we analysed the relation between MSP and pathological classification that only including well-differentiated and moderately differentiated adenocarcinomas, and detected the significant difference ($P=0.0361$). Hypermethylation of EphA7 was more frequently detected in rectal tumors than in colon cancers ($P=0.0816$). We also analysed other clinicopathological parameters including lymphatic metastases, depth of parietal invasion, macroscopic type (Bormann classification), presence of lymph (ly) and blood vessel invasions (v), according to the general rules for clinical and pathological studies on caner of the colon, rectum, and anus (Japanese society for caner of the colon and rectum, 1998), and Dukes classification, but no correlation was detected.

Immunohistochemical characterization and EphA7 level in tumors

A tissue microarray consisting of paired tumor and normal mucosa specimens from all 59 cases was made, and several immunohistological characterizations were performed (Table 4). EphA7 expression was significant different between chromogranin A positive and negative tumors ($P=0.003$).

Table 4 Correlation between EphA7 expression and immunohistochemical analysis in colorectal cancers

	Case number	*Average of log (T/N)*	*Standard deviation of log (T/N)*	*P-value*
P-53				
Positive	26	−0.300	0.573	0.899
Negative	33	−0.277	0.810	
β-Catenin				
Positive	38	−0.196	0.711	0.183
Negative	21	−0.454	0.693	
E-cadherin				
Positive	5	−0.094	0.531	0.530
Negative	54	−0.305	0.725	
Chromogranin A				
Positive	12	0.242	0.633	0.003
Negative	47	−0.424	0.669	
CD34				
Positive	37	−0.280	0.675	0.914
Negative	22	−0.301	0.781	

The tumor was considered positive when the number of stained cell was higher than 10% in the section. SAS Release 9.1 was used

Discussion

Documentation on the expression of EPH family receptor tyrosine kinases and ephrin ligands in various physiological and pathological settings is accumulating. These reports indicate that Eph receptors and their ligands may be associated with various characteristics of human tumors. The upregulation of the expression of several members of the Eph family of receptor tyrosine kinases has been reported (Easty *et al.*, 1999; Liu *et al.*, 2002; Miyazaki *et al.*, 2003; Fox and Kandpal, 2004). We previously reported that EPHB2 is overexpressed in gastric cancer (Kiyokawa *et al.*, 1994; Kataoka *et al.*, 2002) and that EphA2 is overexpressed in colorectal cancer (Kataoka *et al.*, 2004).

In the present study, we found that EphA7 was downregulated in colorectal cancers. This is the first study to report the downregulation of an Eph family gene in colorectal cancer. In the development of colorectal cancer, a series of tumor suppressor genes are inactivated by mutations, methylation, and chromosomal deletions. Velculescu and co-workers published the results of their kinome projects, which included the kinase domains in Eph, and reported that 5% of all colon cancer cell lines have a mutation in their EphA3 kinase domain (Bardelli *et al.*, 2003). We screened all the exons of EphA7 in the colon cancer cell lines used in this study but found only a passenger mutation (data not shown) at a very low frequency. The hypermethylation of 5′CpG islands has been shown to inactivate a number of cancer-associated genes, such as hMLH1 (Cunningham *et al.*, 1998), PTEN (Goel *et al.*, 2004), p14 (Shen *et al.*, 2003), and p16INK4a (Hsieh *et al.*, 1998; Wiencke *et al.*, 1999), in colorectal cancers. Since we observed a specific downregulation in the colorectal cancers, we ascribed an epigenetic origin to the cancerous changes in the colonic mucosas. Hypermethylation of the 5′CpG islands in EphA7 in five colon cancer cell lines was detected using metylation-sensitive restriction enzymes, MSP, and bisulfite sequencing. In the area of the EphA7 genomic sequence from −605 to 515 from translation start site, 80 CG sites were identified and were divided into an upstream group and a downstream group. Our results clearly showed that the methylation of the upstream CpG islands was strongly correlated with the expression of EphA7. In the DLD1, HCT116, HT29, and SW620 colon cancer cell lines, in which EphA7 expression was absent, most of the upstream CpG sites were methylated. While in 293T, which highly expressed EphA7, most of the upstream CpG sites were unmethylated; in SW480 and NCI-H1299 in which EphA7 modestly expressed, some of the upstream CpG sites were unmethylated. Based on this methylation profile, we believe that the methylation of 5′CG islands before the translation start site is responsible for EphA7 downregulation. This correlation was also confirmed by the methylation profiles in other cell lines of different tissue origins (data not shown here). Next, three colon cancer cell lines that lose their expression of EphA7 mRNA were treated with the methyltransferase inhibitor 5-aza-2′-deoxycytidine. The EphA7 expression was

restored in all of these cell lines after treatment. These data indicate that aberrant methylation of the 5′CpG islands may be one mechanism that leads to the downregulation of EphA7 in colorectal cancer.

Importantly, this phenomenon occurs *in vivo*. We checked the methylation status in 75 primary colorectal cancers and paired normal mucosas to determine whether methylation occurs *in vivo* using MSP. Methylated DNA was detected in 37(49%) primary colorectal cancer samples and not detected in matched nontumor mocosas (bisulfite sequencing results also confirmed that 30 upstream CpG sites in one of these normal mucosas were unmethylated) (Supplementary Figure 2). Methylated DNA was not detected both in tumors and matched mucosas of 32 cases (43%). Of six cases (8%), methylated DNA was detected in both of tumors and matched mucosas. Unmethylated DNA bands were detected in all normal mucosa and weakly detected in most of the primary tumors. The presence of unmethylated DNA in the primary cancers can be explained by the fact that the tumor tissue may have contained some normal epithelial cells. The faint detection of methylated DNA in normal mucosa can be explained by either tumor cell infiltration, a tumor predisposing event in the colorectal mucosa near the tumor, or some other inflammatory or reparative situation in the mucosa. The results of MSP in primary colorectal cancers and bisulfite sequencing of normal colon musosas showed that hypermethylation of EphA7 is not a colonic specific event. The hypermethylation of CpG islands is one of the most common epigenetic alterations in human cancers. Recently, researchers have begun to address the clinical significance of hypermethylation. Brock *et al.* (2003) reported that the methylation of multiple genes in esophageal adenocarcinoma was an independent and strong predictor for disease-specific recurrence and patient survival. First, we did not find any obvious strong relationship between the downregulation of EphA7 and particular clinicopathological parameters in the colorectal cancers that were examined for expressions. This finding may be due to the small number of cases (29 cases) in which EphA7 downregulated. Alternately it was because of contamination of blood cells and endothelial cells in the cancer specimens. Then we expanded colorectal cancers cases to 75 for MSP and found intriguing correlation between methylation of 5′CpG island and some of the clinicopathological parameters such as histological grade of differentiation. Eph gene family is essential for embryonic development and for differentiation of the nervous and vascular systems (Friedman and O'Leary, 1996; Adams, 2002; Aoki *et al.*, 2004). Our results showed that EphA7 may play a role in the differentiation of colorectal caner. Hypermethylation of 5′CpG island in tumor was more frequently occurred in moderately differentiated than in well-differentiated tumors ($P = 0.0361$). This may imply that EphA7 protein may take part in the regulation of cell differentiation in colorectal cancer. Furthermore, hypermethylation of EphA7 gene in tumors was significantly different between in male and female ($P = 0.0078$). From these data, we postulated that hypermethylaton of EphA7 gene may be correlated to some environmental factors such as diet, tobacco smoking, and alcohol drinking. Although the difference is not statistically significant, our MSP data showed that hypermethylation of EphA7 is more prevalent in rectum than in colon ($P = 0.0816$).

Interestingly, the colon cancer cell lines SW480 and SW620 had different EphA7 expression profiles and different CpG methylation profiles, in spite of having originated from the same cancer patient. SW480 was established from a primary adenocarcinoma of the colon (according to ATCC cell line data), and SW620 was initiated by Leibovitz *et al.* from a lymph node obtained from the same patient 1 year later, when a recurrence of the malignancy appeared. In our data, EphA7 expression was detected in SW480 and was lost in SW620. SW480 and SW620 also had different CpG methylation status in their upstream regions according to the results of bisulfite sequencing. Methylated DNA was detected in SW620, but only partially detected in SW480, and unmethylated DNA was only detected in SW480, and not in SW620 using the MSP method. This would indicate that the methylation of CpG islands in EphA7 may occur during the progression of colorectal cancer, as in this patient. The potential application of the methylation of CpG islands in the EphA7 gene as a molecular marker for predicting the occurrence and recurrence of colorectal cancer must be profoundly studied in the next round of experiments. We have already detected hypermethylation in three out of five colon adenomas (data not shown). This suggests that the downregulation of EphA7 is an early event during the initiation of colorectal carcinogenesis.

Furthermore, as shown in Figure 1, a certain subset of colon cancers exhibits EphA7 overexpression. We examined the immunohistopathological features of these cases (Table 4). Using tissue microarray containing 59 cancer tissues, which we examined EphA7 RNA expression, we immunostained p53, β-catenin, E-cadherin, chromogranin A and CD34. The prevalence of the positive of these markers was 44, 64, 6.7, 20, and 62%, respectively. Interestingly, EphA7 expression was significant greater in chromogranin A positive tumor than in the negative ones ($P = 0.003$), suggesting EphA7 expression may be paralleled to neuroendocrine cell differentiation. *In vitro* characterization of EphA7 overexpressing tumor cells is undergoing.

In conclusion, downregulation of EphA7 due to methylation in human colorectal cancer confers certain biological and histopathological tint to its carcinogenesis and differentiation.

Materials and methods

Specimens and cell lines

All human colorectal carcinoma specimens and corresponding normal tissues were obtained from surgical resections performed at Hamamatsu University School of Medicine in Japan between 1999 and 2004; the study protocol has been approved by the Institutional Review Board of Hamamatsu Univertsity

School of Medicine (12–11). Cancer tissue samples subjected to semiquantitative RT–PCR were obtained from 59 colorectal cancer patients. The distribution of the tumors by sites of origin was as follows: the cecum and ascending colon, 17 tumors; the transverse colon, two tumors; the descending colon, three tumors; the sigmoid colon, 15 tumors; and the rectum, 22 tumors. In every case, formalin-fixed and paraffin-embedded tissue sections sampled from adjacent portions of the primary tumor were stained with H&E and examined histologically. Histologically, all the tumors were classified as adenocarcinomas: well differentiated (W/D) in 44 cases, moderately differentiated (M/D) in 10, poorly differentiated (P/D) in one and mucinous adenocarcinoma in four. The clinicopathological characteristics of the 59 colorectal cancer patients are shown in Table 1. Among these 59 colorectal cancer cases, 17 cases DNAs were extracted and subjected to MSP (Table 2). Other cases were not carried out MSP, because of consumption of all samples after RNA extraction. We extracted DNAs form 58 new cases of colorectal caners for MSP. All of the tissue samples were immediately frozen in liquid nitrogen and stored at −80°C.

Five colon cancer cell lines (DLD1, HCT116, HT29, SW480, and SW620) were used in the study. The cells were routinely maintained in Dulbecco's modified Eagle's medium (NISSUI Pharmaceutical Co., Tokyo, Japan) supplemented with 1 mM L-glutamine, 10% FBS (Life Technologies, Inc.), 100 U/ml of penicillin G and 100 mg/ml of streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Semi-quantitative RT–PCR

The exponential phase of the PCR amplification of EphA7 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for the quantitative analysis. PCR amplification was performed in a DNA thermal cycler (PC-700; ASTEC, Fukuoka, Japan). In total, 30 cycles of amplification for the GAPDH internal control and 35 cycles for EphA7 were performed as follows: denaturation at 94°C for 1 min followed by primer annealing at 58°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The RT–PCR primer sets for EphA7 were designed between two exons (exons 3 and 4) to avoid genomic DNA contamination. Several other sets of primers covering all the exons of EphA7 were also tested to monitor the efficiency of amplification. The nucleotide sequences were obtained from GenBank (Accession Number: XM_004485). The sense primer was 5′-TCTTTA GTCGAGGTTCGAGG-3′ and the antisense primer was 5′-TGTGCATGCAACGTATGGTG-3′. The PCR products were 312 bp long. The sense primer for GAPDH (GenBank Accession Number: BT007877) was 5′-GAAGGTGAAGG TCGGAGTCA-3′. The antisense primer for GAPDH was 5′-TTGAGGTCAATGAAGGGGTC-3′. The PCR products were 108 bp long. The PCR products were separated by electrophoresis on 8% polyacrylamide gels at room temperature under a voltage of 150 for 1 h. The gel was then dried and subjected to autoradiography and image analysis. The expression level (radiation dose unit PSL) was measured using a Fuji BAS1000 (MacBAS; Fuji Film, Tokyo Japan). All radioisotope (RI) count values, including those for GAPDH, were transformed using a logarithm for further statistical analysis. The count of each RNA message proportional to that of GAPDH was then determined.

Quantitative Real time RT–PCR was carried out using QuantiTect SYBR Green PCR kit (QIAGEN) and Roche Molecular Biochemicals LightCycler. The transcript of housekeeping gene porphobilinogen deaminase (PBGD) was used as an internal control. PBGD-pGEM-T Easy vector and EphA7-pcDNA3.1(+) plasmid DNA were diluted to make the standard curve. The primer sets and reaction conditions for amplifying PBGD and EphA7 are available when requiring. cDNAs from 12 cases of colorectal cancers that semiquantitative RT–PCR carried out were subjected to quantitative real time RT–PCR. The volume of tumor/normal mucosa of EphA7/PBGD was compared with that of EphA7/GAPDH using isotope.

Statistical analysis

The ratio of EphA7 expression in the tumor to that in normal tissue in each case was transformed using a common logarithm. For statistical comparisons of these log-transformed data among two or more groups, an ANOVA was used. When comparing two groups, a t-test was used. All statistical analyses were performed using SPSS software, version 11.5 (SPSS Japan Inc., Tokyo, Japan). For all of the statistical tests, a two-sided P-value of less than 0.05 was considered statistically significant. The SAS 9.1 software was used when analysis of correlation between EphA7 expression, MSP results, immunohistochemical staining results, and clinicopathological parameters.

Methylation-sensitive restriction enzyme and PCR

The methylation status of the 5′CpG island upstream of translation start site was determined by digestion with methylation-sensitive restriction enzymes followed by PCR. In total, 1 μg of genomic DNA was digested using *Msp*I and *Hpa*II (TOYOBO, Japan) restriction enzymes. The digested DNAs were precipitated with ethanol and dissolved in 30 μl of dH_2O. Then, 1 μl aliquots were analysed by PCR in 30 μl reactions, followed by a 1.5% agarose gel check. The primer sequences for the methylation sensitive-restriction PCR were 5′-GCTATAGAAAAGAGTGTAAG-3′ (forward) and 5′-CTCCACACTCCAATAATATC-3′ (reverse). The PCR products were 378 bp long and included three enzyme restriction sites (5′CCGG). The basic principle of this method was described by Endo *et al.* (1995).

Methylation-specific PCR (MSP)

Genomic DNA was modified by sodium bisulfite, as described by Clark *et al.* (1994) and Herman *et al.* (1996). Primers were designed to discriminate between methylated and unmethylated alleles following bisulfite treatment. Primer sequences were chosen for the regions containing frequent CpG and CpG pairs near the 3′ end of the primers to provide maximal discrimination between methylated and unmethylated DNA. In total, 2 μl aliquots were amplified in a 20 μl reaction mixture consisting of 1× buffer (10 mM Tris-HCl, 2.0 mM $MgCl_2$, 50 mM KCl, pH 8.3), 0.5 U HotStarTaq (Qiagen), 260 μM dNTPs, and 0.3 μM of the primer sets. The PCR conditions were as follows: 95°C for 15 min, then 35 cycles of 94°C for 30 s, 58°C (for detection of methylated DNA) or 53°C (for detection of unmethylated DNA) for 30 s, 72°C for 1 min and finally 10 min at 72°C. The methylation specific primer set was 5′-ATTTGATTTCGTTCGGTATC-3′ (forward) and 5′-CTC CGACTACAAACCGACCG-3′ (reverse). The unmethylation specific primer set was 5′-ATTTGATTTTGTTTGGTATT-3′ (forward) and 5′-CTCCAACTACAAACCAACCA-3′ (reverse). Primer sets for detection of methylated and unmethylated DNA were located at the same sites of genomic sequence (forward primer was located at −432 to −413 from translation start site; reverse primer was located at −223 to −204) (Figure 6a). The PCR products were 229 bp long. The PCR

products were run on an 8% nondenaturing polyacrylamide gel, followed by ethidium bromide staining.

Bisulfite genomic sequencing

In total, 2 μl of bisulfite-treated genomic DNA was PCR amplified by three primer sets in a 30 μl reaction mixture consisting of 1× buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 260 μM of each dNTP, 400 μM of primer, and 1.2 U of *Taq* polymerase. The PCR conditions were as follows: 95°C for 3 min, then 35 cycles of 94°C for 45 s, 50°C or 58°C or 60°C for 1 min, 72°C for 1 min and finally 10 min at 72°C. The three primer sets used for bisulfite sequencing were 5′-TATAGAAAAGAGTGTAAGTTG-3′ (forward) and 5′-CTCCACACTCCAATAATATC-3′ (reverse), PCR product = 375 bp; 5′-GATATTATTGGAGTGTGGAG-3′ (forward) and 5′-CCATCACCTTACCTTCCTTC-3′ (reverse), PCR products = 356 bp; and 5′-AAGGAAGGTAAGGTGATGGG-3′ (forward) and 5′-CCAGTCTCCAACCCAACTCT-3′ (reverse), PCR product = 424 bp. We could not find the ideal primer sequence without C for reverse primer flank to downstream CpG island, then designed this primer according to the protocol available from http://www.mdanderson.org/leukemia/methylation/bpcr.html. The PCR products were purified using a QIAquick PCR purification kit (QIAGEN). The PCR products were subjected to direct sequencing or cloned into pGEM-T Easy vector (Promega); 12 colonies of each sample were picked up and subjected to PCR using primer set of T7 and SP6, and sequenced using the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Japan) on a 3100 Genetic Analyser (Applied Biosystems, HITACHI Japan), according to the manufacturer's protocol.

5-aza-2′-deoxycytidine treatment of colon cancer cell lines

Cells were plated at a density of 3×10^5 cells/100 mm on day 0 and treated with freshly prepared 5-aza-2′-deoxycytidine (Sigma) after 24 h. In total, 10 μl of 2 mM 5-aza-2′-deoxycytidine were added to a total medium volume of 10 ml (final concentration, 2 μM). The medium was changed 24 h after treatment. RNA was obtained at 2 and 5 days after the start of treatment. Total RNA was extracted, and reverse transcription was performed. The expression of EphA7 mRNA in colon cancer cell lines was detected by semiquantitative RT–PCR.

Genomic mutation screening in cell lines by SSCP

Five colon cancer cell lines (DLD1, HT29, HCT116, SW480, and SW620), six gastric cancer cell lines (TMK1, MKN1, MKN28, MKN74, KATOIII, and AGS), and three lung cancer cell lines (NCI-H460, NCI-H1299, and A549) were screened for genomic mutations in EphA7 using SSCP in all 17 exons. SSCP was performed under three conditions: at room temperature with or without 5% glycerol and at 4°C without glycerol. The sequences of the primer sets are available on request.

Abbreviations

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MSP, methylation specific PCR; PCR, polymerase chain reaction.

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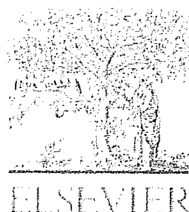

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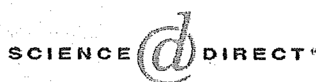

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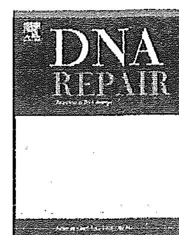

Specificity of replicative and SOS-inducible DNA polymerases in frameshift mutagenesis: Mutability of *Salmonella typhimurium* strains overexpressing SOS-inducible DNA polymerases to 30 chemical mutagens

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ABSTRACT

DNA replication is frequently hindered because of the presence of DNA lesions induced by endogenous and exogenous genotoxic agents. To circumvent the replication block, cells are endowed with multiple specialized DNA polymerases that can bypass a variety of DNA damage. To better understand the specificity of specialized DNA polymerases to bypass lesions, we have constructed a set of derivatives of *Salmonella typhimurium* TA1538 harboring plasmids carrying the *polB*, *dinB* or *mucAB* genes encoding *Escherichia coli* DNA polymerase II, DNA polymerase IV or DNA polymerase RI, respectively, and examined the mutability to 30 chemicals. The parent strain TA1538 possesses CGCGCGCG hotspot sequence for −2 frameshift. Interestingly, the chemicals could be classified into four groups based on the mutagenicity to the derivatives: group I whose mutagenicity was highest in strain YG5161 harboring plasmid carrying *dinB*; group II whose mutagenicity was almost equally high in strain YG5161 and strain TA98 harboring plasmid carrying *mucAB*; group III whose mutagenicity was highest in strain TA98; group IV whose mutagenicity was not affected by the introduction of any of the plasmids. Introduction of plasmid carrying *polB* did not enhance the mutagenicity except for benz[a]anthracene. We also introduced a plasmid carrying *polA* encoding *E. coli* DNA polymerase I to strain TA1538. Strikingly, the introduction of the plasmid reduced the mutagenicity of chemicals belonging to groups I, II and III, but not the chemicals of group IV, to the levels observed in the derivative whose SOS-inducible DNA polymerases were all deleted. These results suggest that (i) DNA polymerase IV and DNA polymerase RI possess distinct but partly overlapping specificity to bypass lesions leading to −2 frameshift, (ii) the replicative DNA polymerase, i.e., DNA polymerase III, participates in the mutagenesis and (iii) the enhanced expression of *E. coli polA* may suppress the access of Y-family DNA polymerases to the replication complex.

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1. Introduction

Cellular DNA is continuously exposed to a variety of endogenous and exogenous genotoxic agents. Although DNA repair mechanisms are in operational to remove DNA lesions, DNA polymerases have to often encounter the lesions that are escaped from the repair mechanisms. DNA damages, such as ultraviolet light (UV) photoproducts or carcinogen adducts, strongly block the progress of DNA replication, and thus daughter-strand gaps are generated downstream of the lesions [1]. In *Escherichia coli*, the single-stranded regions are recognized by RecA protein, which mediates recombination to fill in the gaps with a homologous DNA sequence that is derived from the replicated sister chromatids [2]. In addition, the binding of RecA to the single-stranded regions activates RecA protein itself to mediate the cleavage of LexA repressor, which triggers the expression of more than 30 genes in the chromosome. This damage-inducible gene expression is referred to as an SOS response [3]. Interestingly, some of DNA polymerases in *E. coli* (see below) are regulated as part of the SOS response, and the SOS-inducible DNA polymerases appear to be involved in translesion DNA synthesis (TLS), which directly bypasses the lesions to fill in the gaps [4–6]. Some TLS reactions they catalyze are error-prone, i.e., incorporating incorrect bases in the nascent strand, while others are error free [7]. Thus, filling in the gaps by TLS appears to contribute to mutagenesis as well as to DNA damage tolerance, while filling-in reactions by homologous recombination are supposed to be non-mutagenic.

In *E. coli*, there are five DNA polymerases, i.e., DNA polymerases I–V [8]. DNA polymerase I encoded by *polA* is the first DNA polymerase to be described and is involved in lagging strand DNA synthesis, i.e., processing of Okazaki fragment, DNA repair and initiation of ColE1 plasmids such as pBR322 [9,10]. DNA polymerase II encoded by *polB* is a damage (SOS)-inducible DNA polymerase [11,12] and is involved in the process to restart the synthesis of damaged DNA [13,14]. This polymerase is reported to bypass 2-acetylaminofluorene adducts, which results in −2 frameshift [15,16]. Unlike DNA polymerase I, which belongs to A-family DNA polymerase, DNA polymerase II is a member of B family, in which mammalian replicative DNA polymerases such as DNA polymerase delta are included [17]. DNA polymerase III holoenzyme, which is composed of multiple subunits, is responsible for the chromosome replication of *E. coli* and the catalytic subunit is encoded by *dnaE* (or *polC*) [18,19]. This enzyme is classified into C family, in which prokaryotic replicative DNA polymerases are categorized [8]. DNA polymerase IV and DNA polymerase V belong to Y family, whose members are mostly involved in TLS, and the expression of *dinB* and *umuDC* encoding DNA polymerase IV and DNA polymerase V, respectively, is regulated as part of the SOS response [20–24]. DNA polymerase IV is shown to be involved in −1 frameshift mutagenesis induced by 4-nitroquinoline N-oxide and benzo[a]pyrene [25,26], and DNA polymerase V is known to play important roles in mutagenesis induced by UV and a variety of genotoxic compounds [27,28]. However, current knowledge about the roles of replicative, i.e., DNA polymerase I and DNA polymerase III, and SOS-inducible specialized DNA polymerases, i.e., DNA polymerase II, DNA polymerase IV and DNA polymerase V, in mutagenesis is still limited because synthetic oligonucleotides bearing specific DNA lesions are required for in vitro and in vivo analyses to address the question.

Salmonella typhimurium is a Gram negative bacterium, whose genome sequence is 70–90% homologous to *E. coli* [29]. Some of *S. typhimurium* strains have been widely used to detect a variety of environmental mutagens and carcinogens as tester strains of Ames test [30]. One of such strain TA1538 possesses CGCGCGCG sequence in the *hisD* gene, which is a mutational hot spot for −2 (–CG) frameshift [31,32]. The strain bears a deep-rough *rfa* mutation, which increases the permeability to hydrophobic compounds such as polycyclic aromatic hydrocarbons (PAH) [33]. In addition, the strain is deficient in the capacity to excise bulky DNA adducts by the *uvrB* mutation, so that the DNA adducts are more likely to be bypassed rather than removed by repair enzymes [33].

In a previous study, we have systematically disrupted the genes of *S. typhimurium* TA1538 encoding SOS-inducible DNA polymerases, i.e., $polB_{ST}$, $dinB_{ST}$, $umuDC_{ST}$ and *samAB*, and concluded that different sets of DNA polymerases are engaged in lesion bypass in the CGCGCGCG sequence depending upon the environmental threats by chemicals [34]. We also proposed that not only SOS-inducible DNA polymerases but also the main replicative DNA polymerase, i.e., DNA polymerase III, plays important roles in −2 frameshift [34].

In this study, we generated a set of isogenic derivatives of *S. typhimurium* TA1538 by introducing plasmids carrying *polB*, *dinB* or *mucAB* encoding *E. coli* DNA polymerase II, DNA polymerase IV or DNA polymerase RI, respectively, and examined the mutability to 30 chemicals. We introduced the plasmid carrying *mucAB*, i.e., pKM101, instead of a plasmid carrying *E. coli umuDC*, because DNA polymerase RI is a homologue of *E. coli* DNA polymerase V [35], and the derivative of TA1538 harboring plasmid pKM101, i.e., strain TA98, has been widely used as a standard tester strain of Ames test [30]. We also introduced a plasmid carrying the *polA* gene of *E. coli* to strain TA1538 and examined the mutability to investigate the possible involvement of DNA polymerase I in TLS leading to frameshift. Intriguingly, the introduction of the *polA* plasmid completely suppressed the mutations depending on the activities of $dinB_{ST}$ and $umuDC_{ST}$ of *S. typhimurium*. Collectively, the present results suggest that (1) DNA polymerase IV and DNA polymerase RI has distinct but partly overlapping specificity to bypass lesions leading to −2 frameshift, (2) the replicative DNA polymerase, i.e., DNA polymerase III, substantially contributes to −2 frameshift and (3) the enhanced expression of *E. coli* polymerase I inhibits the access of Y-family DNA polymerases to the replication complex where TLS occurs. In addition, our results raise an interesting possibility that strain YG5161 harboring plasmid pYG768 carrying *dinB* could be a superior tester strain to strain TA98 to detect the mutagenicity of environmental PAHs.

2. Materials and methods

2.1. Strains and plasmids

The strains and plasmids used in this study are listed in Table 1. Strain YG5160 and strain YG5161 were constructed

Table 1 – *S. typhimurium* strains and plasmids

Strain or plasmid	Description	Source
Strains		
TA1535	*hisG46, gal, Δ (chl, uvrB, bio), rfa*	Maron and Ames [30]
TA1537	*hisC3076, gal, Δ (chl, uvrB, bio), rfa*	Maron and Ames [30]
TA1538	*hisD3052, gal, Δ (chl, uvrB, bio), rfa*	Maron and Ames [30]
TA98	As TA1538 but harbors plasmid pKM101	Maron and Ames [30]
YG5160	As TA1538 but harbors plasmid pYG787	This study
YG5161	As TA1538 but harbors plasmid pYG768	This study
YG6215	As TA1538 but $\Delta umuDC_{ST}$::Km^r, $\Delta samAB$::Cm^r, $\Delta dinB_{ST}$::Sp^r, $\Delta polB_{ST}$::Tc^r	Kokubo et al. [34]
Plasmids		
pKM101	Plasmid carrying the *mucAB* genes	Maron and Ames [30]
pYG768	Derivative of pWSK29 with the *E. coli dinB* gene	Kim et al. [36]
pYG787	Derivative of pWKS30 with the *E. coli polB* gene	Kokubo et al. [34]
pIMA-1	Derivative of pWKS30 with the *E. coli polA* gene	Imai and Yamamoto (unpublished)

by introduction of plasmid pYG787 carrying *polB* and plasmid pYG768 carrying *dinB*, respectively, into strain TA1538 [34,36]. Plasmid pIMA-1 carrying *E. coli polA* [9] was constructed by the insertion of a 3.5-kb fragment of the *polA* gene between EcoRI and SalI sites of plasmid pWKS30 (Imai and Yamamoto, unpublished). The direction of transcription of the *polA* gene in the plasmid is opposite to that of the *lacZ* gene. The plasmid could complement the killing sensitivity of a *polA* strain of *E. coli* to ultraviolet light and methyl methanesulfonate. Transformation was conducted by electroporation [37].

2.2. *Chemicals*

The names, abbreviations, CAS registry numbers and sources of the chemicals used in this study are listed in Table 2. The chemical structures are presented in Fig. 1.

2.3. *Media*

Luria–Bertani broth and agar were used for bacterial culture [38]. Vogel–Bonner minimal agar plates and top agar were prepared as previously described, and used for the His^+ reversion assay with *S. typhimurium* [30]. Nutrient broth (Difco, MI, USA) with ampicillin (AP, 25 μg/ml) was used for pre-cultures of the strains for the reversion assay.

2.4. *Mutagenicity assay*

The mutagenicity assay was carried out with a pre-incubation procedure [30]. Briefly, 0.1 ml overnight culture was incubated with the chemicals dissolved in 0.1 ml solvent and 0.5 ml S9 mix for 20 min at 37 °C. When S9 mix is not required, 0.5 ml of 1/15 M phosphate buffer pH 7.4 was added. The mixture was then poured onto agar plates with soft agar and incubated for 2 days at 37 °C. Each chemical was assayed with four to seven doses on duplicate plates with four strains, i.e., strain TA1538, TA98, YG5160 and YG5161, in parallel. In the series of experiments, we regarded the effects of introduction of plasmids, i.e., pYG787, pYG768 or pKM101, on the mutability of strain T1538 as significant when the transformed strains displayed more than and including 50% higher or lower mutability, compared to the parent strain TA1538.

3. Results

3.1. *Specificity of SOS-inducible DNA polymerases in frameshift induced by 30 chemicals*

To assign the role of SOS-inducible DNA polymerases in bypass of DNA lesions, we introduced plasmids carrying *E. coli polB*, *dinB* or *mucAB* encoding different SOS-inducible DNA polymerases to strain TA1538 and examined their mutability to 30 chemicals. The dose–response curves are presented in Fig. 2, and the numbers of revertants per microgram per plate of each chemical and strain are summarized in Table 3. To make the comparison easier, we also calculated the relative mutability of each derivative by assigning the number of revertants per microgram in strain TA1538 as 1.0. According to the mutagenicity, we classified the 30 chemicals into four groups as follows.

Group I includes benzo[a]pyrene and other seven chemicals. The mutagenicity of these compounds was highest in strain YG5161 harboring plasmid pYG768 carrying *dinB* encoding DNA polymerase IV, followed by strain TA98 harboring plasmid pKM101 carrying *mucAB* encoding DNA polymerase RI. The mutagenicity of the chemicals to strain YG5160 harboring plasmid pYG787 carrying *polB* encoding DNA polymerase II was very similar to the parent strain TA1538 except for benzo[a]pyrene-7,8-dihydroepoxide and 1-aminoanthracene where introduction of plasmid pYG787 appeared to alleviate the mutagenicity by 50% and 40%, respectively. For benzo[a]pyrene, the ratio of the mutability of strain YG5161, TA98, YG5160 and TA1538 was 7:2:1:1. The compounds in this group are derivatives of benzo[a]pyrene except for 3-methylcholanthrene, 1-aminoanthracene and 2-aminoanthracene.

Group II includes ENNG and other four chemicals. The mutagenicity of these compounds was almost equally high in strain YG5161 and strain TA98. The introduction of plasmid pYG787 carrying *polB* did not enhance the mutagenicity. Rather, plasmid pYG787 seemed to reduce the mutagenicity of 6-aminochrysene by 60%. The ratio of the mutability of strain YG5161, TA98, YG5160 and TA1538 was 20:19:1:1 for ENNG. The compounds in this group are PAHs and the derivative except for ENNG.

Table 2 – Names, abbreviations, CAS registry numbers and sources of the chemicals

Chem. no.	Chemical	CAS registry numbers	Sources[a]
1	Benzo[a]pyrene-7,8-dihydroepoxide	36504-65-1	Mi
2	Benzo[a]pyrene diol epoxide	58917-67-2	Mi
3	10-Azabenzo[a]pyrene	189-92-4	1
4	Benzo[a]pyrene	50-32-8	W
5	3-Nitro-benzo[a]pyrene	70021-98-6	4
6	3-Methylcholanthrene	56-49-5	S
7	1-Aminoanthracene	610-49-1	S
8	2-Aminoanthracene	613-13-8	W
9	7,12-Dimethylbenz[a]anthracene (DMBA)	57-97-6	W
10	6-Aminochrysene	2642-98-0	S
11	1-Nitro-benzo[a]pyrene	70021-99-7	4
12	Benzo[a]pyrene-4,5-dihydroepoxide	64437-52-1	Mi
13	N-Ethyl-N′-nitro-N-nitrosoguanidine (ENNG)	4245-77-6	5
14	1-Nitropyrene	5522-43-0	T
15	1,8-Dinitropyrene	42397-65-9	T
16	6-Nitro-benzo[a]pyrene	63041-90-7	4
17	1-Nitro-6-azabenzo[a]pyrene	138835-35-5	4
18	3-Nitro-6-azabenzo[a]pyrene	138835-36-6	4
19	Furylfuramide	3688-53-7	W
20	Aflatoxin B1	1162-65-8	S
21	Benzo[a]pyrene-7,8-tetrahydroepoxide	36504-67-3	Mi
22	Acridine orange	65-61-2	Me
23	Benz[a]anthracene	56-55-3	S
24	2-Nitrofluorene	607-57-8	T
25	2-[2-(Acetylamino)-4-[bis-(2-methoxy-ethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2H-benzotriazole (PBTA-1)	194590-84-6	3
26	2-Amino-6-methyldipyrido[1,2-a:3′,2′-d]imidazole (Glu-P-1)	67730-11-4	W
27	Aminophenylnorharman	219959-86-1	3
28	N-Hydroxyacetylaminofluorene (N-OH-AAF)	53-95-2	Mi
29	4-Nitroquinoline-1-oxide (4-NQO)	56-57-5	T
30	2-Acetylaminofluorene	53-96-3	T

[a] The chemicals were purchased from the following sources at the highest grade of purity: Wako Pure Chemical (W); Tokyo Kasei Kogyo (T); Sigma–Aldrich (S); Merck (Me); Midwest Research Institute (Mi); Nacalai Tesque (N). Commercially unavailable chemicals were provided by the follwing persons: Dr. Ken-ichi Saeki, Nagoya City University, Japan (1); Dr. Takeji Takamura-Enya, National Cancer Center Research Institute, Tokyo, Japan (2); Dr. Yukari Totsuka, National Cancer Center Research Institute, Tokyo, Japan (3); Dr. Kiyoshi Fukuhara, National Institute of Health Sciences, Tokyo, Japan (4); laboratory stock (5).

Group III includes 1-nitropyrene and other 10 chemicals. The mutagenicity of these compounds was highest in strain TA98. Introduction of plasmid pYG768 carrying *dinB* displayed moderate (less than three-fold) enhancing effects on the mutagenicity of this group of chemicals. The introduction of plasmid pYG787 carrying *polB* enhanced the mutagenicity of benz[a]anthracene three-fold reproducibly, although it had no enhancing effects on other chemicals. For the mutagenicity of 1,8-dinitropyrene, 1-nitro-6-azabenzo[a]pyrene and furylfuramide, plasmid pYG787 reduced the mutagenicity by half. The ratio of the mutability of strain TA98, YG5161, YG5160 and TA1538 was 16:1:1:1 for 1-nitropyrene. The compounds in this group include structurally unrelated compounds such as furylfuramide, aflatoxin B1 and acridine orange.

Group IV includes 2-acetylaminofluorene and other five compounds. The characteristic of this group was that the mutagenicity was not enhanced by the introduction of any of the plasmids encoding SOS-inducible DNA polymerases. The ratio of the mutability of strain YG5161, TA98, YG5160 and TA1538 was 1:1:1:1 for 2-acetylaminofluorene. The compounds in this group are aromatic amines except for 4-NQO.

3.2. −1 Frameshit and base substitutions by benzo[a]pyrene and ENNG promoted by DNA polymerase IV and DNA polymerase RI

Since DNA polymerase IV encoded by *dinB* appeared to promote −2 frameshift induced by benzo[a]pyrene (group I chemical) and ENNG (group II chemical), we examined the possibility whether the polymerase also promotes other types of mutations, i.e., −1 frameshift and base substitutions, by the chemicals. To this end, we took advantage of other *S. typhimurium* strains, i.e., TA1537 and TA1535, which detects mutagens that cause −1 frameshift in CCC sequence in the *hisC* gene and base substitutions in GGG sequence in the *hisG* gene, respectively [30]. For benzo[a]pyrene-induced mutagenesis, the introduction of plasmid pYG768 carrying *dinB* into strain TA1537 slightly enhanced the mutagenicity, but the effect of enhancing mutagenesis was much lower compared to the effect of plasmid pKM101 carrying *mucAB* encoding DNA polymerase RI (Fig. 3A). For base substitutions, DNA polymerase IV seemed inactive and virtually no enhancement was observed in strain TA1535 with plasmid pYG768. In contrast, DNA polymerase RI actively promoted the base substitution mutations. As has been observed

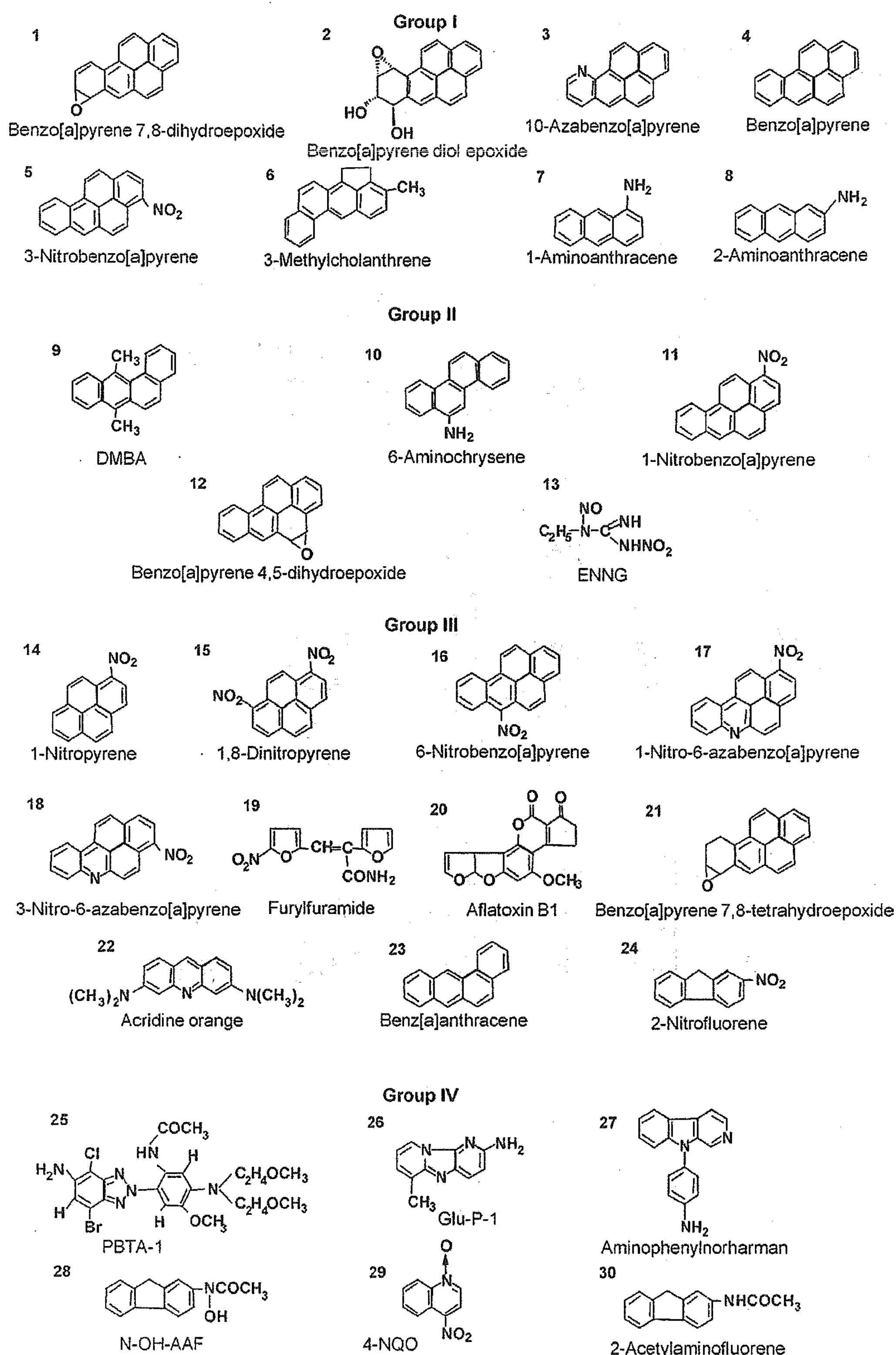

Fig. 1 – Structures of the chemical mutagens used in this study.

in −2 frameshift, introduction of plasmid pYG787 carrying *polB* had almost no effects on any types of mutations induced by benzo[a]pyrene. These results suggest that the efficiency of error-prone bypass across lesions by DNA polymerase IV strongly depends on the types of mutations and the sequence context surrounding the lesions. For ENNG-induced mutagenesis, both DNA polymerase IV and DNA polymerase RI appeared to promote −1 frameshift and base

substitutions substantially (Fig. 3B). Introduction of plasmid pYG787 carrying *polB* had almost no effects on the mutability. Unlike ENNG-induced mutagenesis, −2 frameshift, −1 frameshift and base substitutions induced by N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) were not enhanced by the introduction of either plasmid pYG768 or pKM101 (data not shown). These results suggest that both DNA polymerase IV and DNA polymerase RI bypass ethyl, but not methyl, adducts in DNA leading to −2 and −1 frameshifts and base substitutions.

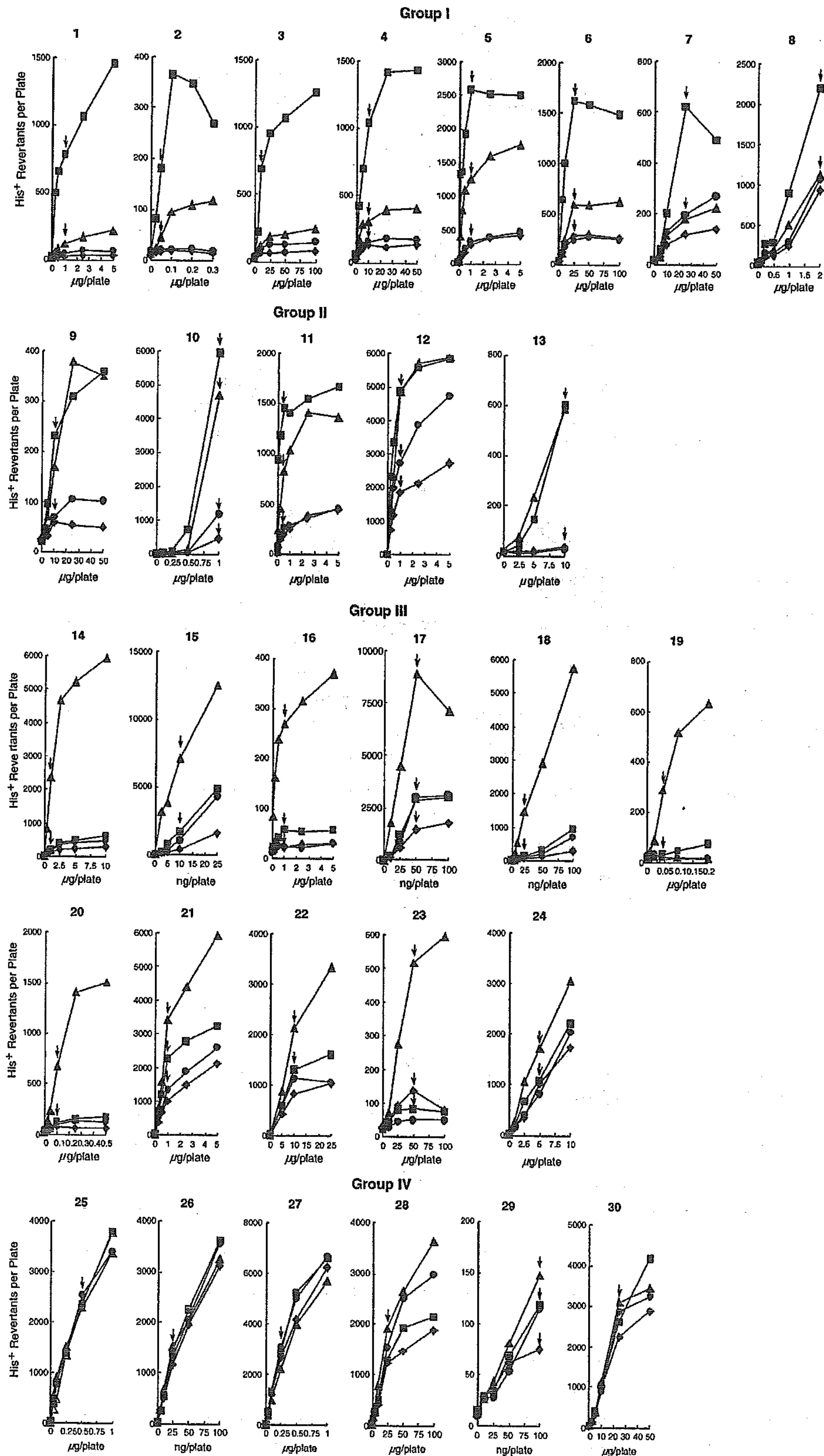

Table 3 – Mutagenicity of 30 chemicals in *S. typhimurium* strains harboring plasmids carrying genes encoding *E. coli* SOS DNA polymerases

Number	Chemical	Group	S9	TA1538 no plasmid	TA98 pKM101 (DNA pol RI)	YG5160 pYG787 (DNA pol II)	YG5161 pYG768 (DNA pol IV)
1	Benzo[a]pyrene-7,8-dihydroepoxide	I	–	44 (1.0)	113 (2.6)	22 (0.5)	776 (17.6)
2	Benzo[a]pyrene diol epoxide	I	–	420 (1.0)	920 (2.2)	320 (0.8)	3620 (8.6)
3	10-Azabenz[a]pyrene	I	+	9 (1.0)	12 (1.3)	6 (0.7)	68 (7.6)
4	Benzo[a]pyrene	I	+	15 (1.0)	30 (2.0)	13 (0.9)	104 (6.9)
5	3-Nitro-benzo[a]pyrene	I	–	316 (1.0)	1244 (3.9)	277 (0.9)	2580 (8.2)
6	3-Methylcholanthrene	I	+	10 (1.0)	23 (2.3)	11 (1.1)	65 (6.5)
7	1-Aminoanthracene	I	+	8 (1.0)	7 (0.9)	5 (0.6)	25 (3.1)
8	2-Aminoanthracene	I	+	540 (1.0)	564 (1.0)	468 (0.9)	1102 (2.0)
9	DMBA	II	+	7 (1.0)	17 (2.4)	6 (0.9)	23 (3.3)
10	6-Aminochrysene	II	+	1200 (1.0)	4693 (3.9)	461 (0.4)	5955 (5.0)
11	1-Nitro-benzo[a]pyrene	II	–	524 (1.0)	1640 (3.1)	400 (0.8)	2896 (5.5)
12	Benzo[a]pyrene-4,5-dihydroepoxide	II	–	2724 (1.0)	4836 (1.8)	1856 (0.7)	4900 (1.8)
13	ENNG	II	–	3 (1.0)	58 (19.3)	4 (1.3)	60 (20)
14	1-Nitropyrene	III	–	154 (1.0)	2354 (15.3)	112 (0.7)	194 (1.3)
15	1,8-Dinitropyrene	III	–	110100 (1.0)	708300 (6.4)	39300 (0.4)	171600 (1.6)
16	6-Nitro-benzo[a]pyrene	III	–	24 (1.0)	268 (11.2)	21 (0.9)	58 (2.4)
17	1-Nitro-6-azabenzo[a]pyrene	III	–	60500 (1.0)	178180 (2.9)	29700 (0.5)	58020 (1.0)
18	3-Nitro-6-azabenzo[a]pyrene	III	–	3560 (1.0)	57640 (16.2)	2480 (0.7)	5240 (1.5)
19	Fulylfuramide	III	–	480 (1.0)	5760 (12.0)	220 (0.5)	620 (1.3)
20	Aflatoxin B1	III	+	990 (1.0)	6680 (6.7)	750 (0.8)	1160 (1.2)
21	Benzo[a]pyrene-7,8-tetrahydroepoxide	III	–	1332 (1.0)	3404 (2.6)	1000 (0.8)	2252 (1.7)
22	Acridine orange	III	+	113 (1.0)	234 (2.1)	83 (0.7)	131 (1.2)
23	Benz[a]anthracene	III	+	1 (1.0)	10 (10.0)	3 (3.0)	2 (2.0)
24	2-Nitrofluorene	III	–	162 (1.0)	341 (2.1)	203 (1.3)	215 (1.3)
25	PBTA-1	IV	+	5074 (1.0)	4568 (0.9)	4768 (0.9)	4720 (0.9)
26	Glu-P-1	IV	+	52800 (1.0)	61120 (1.2)	46480 (0.9)	56640 (1.1)
27	Aminophenylnorharman	IV	+	12352 (1.0)	8880 (0.7)	10688 (0.9)	11456 (0.9)
28	N-OH-AAF	IV	–	62 (1.0)	76 (1.2)	49 (0.8)	52 (0.8)
29	4-NQO	IV	–	1150 (1.0)	1470 (1.3)	750 (0.7)	1180 (1.0)
30	2-Acetylaminofluorene	IV	+	114 (1.0)	124 (1.1)	89 (0.8)	105 (0.9)

Each chemical was assayed with four to seven doses on duplicate plates with four strains in parallel. The assays with chemical nos. 2, 4, 7, 8, 10, 12, 15, 17, 23 and 29 were repeated to confirm the initial results. The numbers of His+ revertants per plate per microgram of each strain are calculated at the doses indicated with arrows in Fig. 2. The numbers in parentheses represent the values relative to the numbers of His+ revertants per microgram in TA1538 (no plasmid). Difference of the relative mutability two-fold or more was regarded as significant effects of the introduction of plasmids on the mutability.

Group I: the chemicals whose mutagenicity was highest in strain YG5161 harboring plasmid pYG768 carrying *dinB* (DNA pol IV).

Group II: the chemicals whose mutagenicity was equally high in both strain YG5161and strain TA98 harboring plasmid pKM101 carrying *mucAB* (DNA pol RI).

Group III: the chemicals whose mutagenicity was highest in strain TA98.

Group IV: the chemicals whose mutagenicity was not substantially modulated by the introduction of any of the plasmids.

Fig. 2 – Responses of S. *typhimurium* tester strains to 30 chemical mutagens. The chemicals are: benzo[a]pyrene-7,8-dihydroepoxide (1); benzo[a]pyrene diol epoxide (2); 10-azabenzo[a]pyrene (3); benzo[a]pyrene (4); 3-nitro-benzo[a]pyrene (5); 3-methylcholanthrene (6); 1-aminoanthracene (7); 2-aminoanthracene (8); DMBA (9); 6-aminochrysene (10); 1-nitro-benzo[a]pyrene (11); benzo[a]pyrene-4,5-dihydroepoxide (12); ENNG (13); 1-nitropyrene (14); 1,8-dinitropyrene (15); 6-nitro-benzo[a]pyrene (16); 1-nitro-6-azabenzo[a]pyrene (17); 3-nitro-6-azabenzo[a]pyrene (18); furylfuramide (19); aflatoxin B1 (20); benzo[a]pyrene-7,8-tetrahydroepoxide (21); acridine orange (22); benz[a]anthracene (23); 2-nitrofluorene (24); PBTA-1 (25); Glu-P-1 (26); aminophenylnorharman (27); N-OH-AAF (28); 4-NQO (29); 2-acetylaminofluorene (30). The strains used are: TA1538 (circles ●); YG5160 (diamonds ◆); YG5161 (squares ■); TA98 (triangles ▲). The arrow indicates the dose that was used for the calculation of His+ revertants per microgram per plate in Table 3.

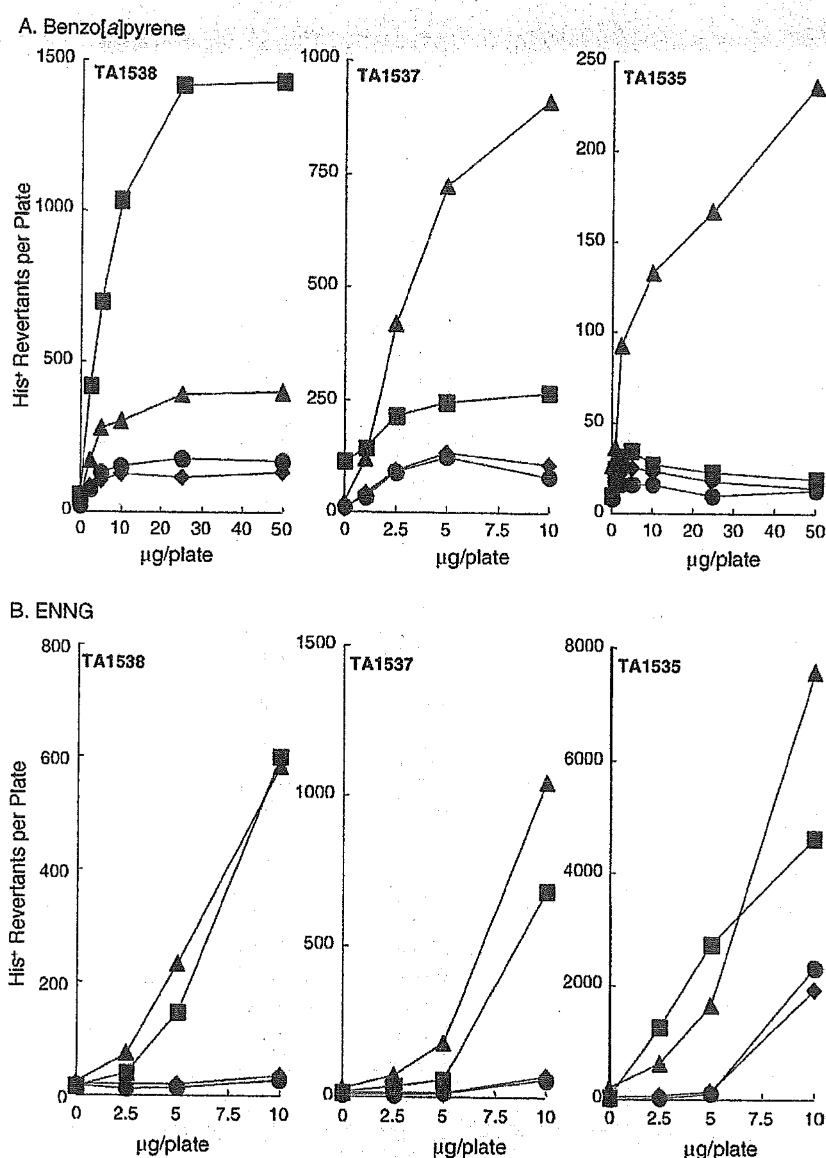

Fig. 3 – Responses of *S. typhimurium* tester strains to benzo[a]pyrene and ENNG. Benzo[a]pyrene plus S9 mix (A) or ENNG (B) was assayed with four to six doses on duplicate plates with four strains, i.e., the parent strain without plasmids (circles ●), the derivative harboring plasmid pYG787 carrying *polB* (diamonds ♦), the derivative harboring plasmid pYG768 carrying *dinB* (squares ■) and the derivative harboring plasmid pKM101 carrying *mucAB* (triangles ▲) in parallel. The parent strains were *S. typhimurium* TA1538, TA1537 and TA1535 for the detection of −2 frameshift, −1 frameshift and base substitutions, respectively, and they were transformed with one of three plasmids, i.e., pYG787, pYG768 and pKM101.

3.3. *Effects of the introduction of plasmid carrying polA encoding E. coli DNA polymerase I on the mutability of strain* TA1538

The mutagenicity of the compounds of group IV was not influenced by introduction of any of the plasmids carrying genes encoding SOS-inducible DNA polymerases (Fig. 2). This suggests the involvement of replicative DNA polymerases, i.e., DNA polymerase I and/or DNA polymerase III, in the mutagenesis. To examine the possible involvement of DNA polymerase I, we introduced plasmid pIMA-1 carrying *polA* encoding DNA polymerase I to strain TA1538 and its derivative YG6215, which lacks all the genes encoding SOS-inducible DNA polymerases [34], and compared the mutability to the group IV compounds, i.e., PBTA-1, Glu-P-1, aminophenylnorharman, 4-NQO and 2-acetylaminofluorene (Fig. 4). We also examined the mutagenicity of 2-aminofluorene, a derivative of 2-acetylaminofluorene. The introduction of plasmid pIMA-1 did not affect the mutability of strain TA1538 and YG6215 to the group IV compounds and 2-aminofluorene. We also examined the mutability of strain TA1538 and YG6215 harboring plasmid pIMA-1 carrying *polA* to other chemicals belong to group I, i.e., benzo[a]pyrene, 10-azabenzo[a]pyrene, 3-methylcholanthrene and 1-aminoanthracene, group II,

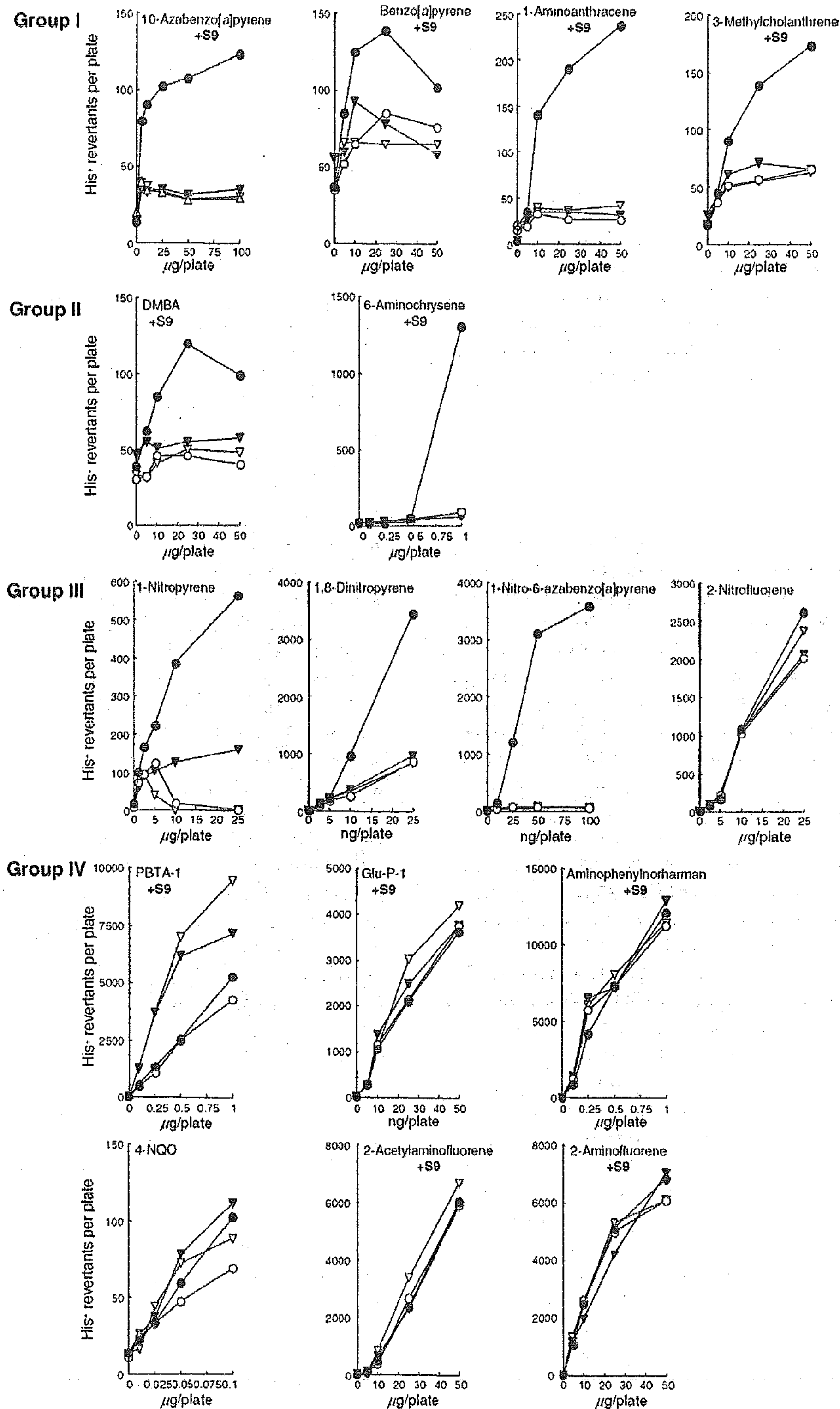

Fig. 4 – Effects of introduction of plasmid pIMA-1 carrying *polA* on the mutability of strain TA1538 and YG6215 to chemicals. The strains used were *S. typhimurium* TA1538 harboring the vector pWKS30 (closed circles ●), TA1538 harboring plasmid pIMA-1 (open circles ○), YG6215 harboring the vector pWKS30 (closed triangles ▼) and YG6215 harboring plasmid pIMA-1 (open triangles ▽). Each chemical was assayed with five to six doses on triplicate plates with four strains in parallel. The dose response curves of PBTA-1 plus S9 was unique in that strain YG6215 lacking all SOS-inducible DNA polymerases displayed higher mutability than strain TA1538 [34].

i.e., DMBA and 6-aminochrysene and group III, i.e., 1-nitropyrene, 1,8-dinitropyrene, 1-nitro-6-azabenzo[a]pyrene and 2-nitrofluorene. Surprisingly, the introduction of plasmid pIMA-1 reduced the mutability of strain TA1538 to the level of strain YG6215 harboring the vector plasmid (Fig. 4). Only exception was 2-nitrofluorene where the mutability of strain TA1538 and YG6215 was not affected by the introduction of plasmid pIMA-1 as in the case of group IV compounds. The dose–response curves of strain YA1538 harboring plasmid pIMA-1 almost completely overlapped those of strain YG6215 harboring the vector plasmid. The mutability of strain YG6215 was not affected by the introduction of plasmid pIMA-1 carrying *polA* except for 1-nitropyrene where strain YG6215 as well as strain TA1538 exhibited an enhanced killing sensitivity to the chemical when the *polA* plasmid was introduced.

4. Discussion

DNA polymerase III holoenzyme replicates the chromosome of *E. coli* with high fidelity but its progress is occasionally blocked by DNA lesions, and DNA polymerase V encoded by *umuDC* bypasses the lesions to assist the chromosome replication, which mostly results in base substitutions [10,28]. Less is known, however, about the mechanisms of translesion bypass leading to frameshift [39–41]. To assign the role of each SOS-inducible DNA polymerases of *E. coli* in TLS leading to frameshift, we have introduced plasmids carrying the genes encoding SOS-inducible DNA polymerases to strain TA1538 possessing CGCGCGCG sequence in the *hisD* gene as a −2 frameshift hot spot [31], and examined the mutability to 30 chemical mutagens. The *mucB* gene is expressed 13,000 molecules per cell from plasmid pKM101 when LexA repressor is inactivated [42], and the expression level is much higher than the chromosomal expression level of the *umuC* gene, i.e., about 200 molecules per cell upon SOS induction [43]. Similarly, the *dinB* gene is expressed 25,000–50,000 molecules per cell from plasmid pYG768 in the presence of DNA damage, which is 10–20 times higher than the expression level of *dinB* from the chromosome upon SOS induction [25]. The exact expression levels of DNA polymerase I and DNA polymerase II from plasmid pIMA-1 and pYG787, respectively, are unclear, but they could be at least two to three times higher than those from the chromosome because both plasmids are derivatives of low-copy-number plasmid pWKS30 [44]. DNA polymerase I and DNA polymerase II encoded by *polA* and *polB*, respectively, are expressed 400 and 30–50 molecules per cell from the chromosome and the expression of *polB* is enhanced about seven-fold by DNA damage [10,45]. Thus, we assume the expression levels are about 1000 for DNA polymerase I, 500–1000 for DNA polymerase II, 10–20 for DNA polymerase III [10], 25,000–50,000 for DNA polymerase IV and 13,000 for DNA polymerase RI per cell when the chromosome DNA is damaged by chemicals. Because of the high levels of expression of these DNA polymerases from the plasmids, we could observe distinct enhancing effects on the mutability of strain TA1538 to 30 chemicals (Fig. 2, Table 3). Based on the mutagenicity, we classified the chemicals into four groups as follows.

Group I includes eight chemicals that exhibited highest mutagenicity in strain YG5161 harboring plasmid pYG768 carrying *dinB*. The introduction of plasmid pKM101 also enhanced the mutagenicity of some compounds, but the extent of the enhancement was much less compared to the effects of plasmid pYG768. In fact, the introduction of plasmid pKM101 into strain TA1538 did not enhance the mutagenicity of 1-aminoanthracene and 2-aminoanthracene while plasmid pYG768 enhanced the mutagenicity more than two times. These results suggest that DNA polymerase IV encoded by *dinB* efficiently bypasses DNA lesions induced by group I compounds in CGCGCGCG sequence leading to −2 frameshift. This is consistent with our previous results that deletion of endogenous *dinB* gene of *S. typhimurium*, i.e., $dinB_{ST}$, significantly reduced the mutability of strain TA1538 to 10-azabenzo[a]pyrene, benzo[a]pyrene, 1-aminoanthracene and 2-aminoanthracene [34]. DNA polymerase IV could have specificity to bypass across guanine bases modified with the polycyclic aromatics, leading to −2 frameshift. In contrast to −2 frameshift, the introduction of plasmid pYG768 did not enhance base substitutions induced by benzo[a]pyrene (Fig. 3A). *E. coli* DNA polymerase IV is reported to bypass N^2-guanine adducts of benzo[a]pyrene diol epoxide in vitro with higher efficiency and high fidelity compared to DNA polymerase II and DNA polymerase V [46]. Thus, we suggest that DNA polymerase IV correctly incorporate cytosine opposite the adducted guanine in the GGG sequence in the *hisG* gene, thereby leading to no base substitutions. Correct insertion of cytosine opposite the adducted guanine in the CGCGCGCG sequence in *hisD*, however, may induce a two-bp slippage leading to −2 frameshift [47].

Group II includes five chemicals that displayed equally high mutagenicity to strain YG5161 and strain TA98. They are derivatives of PAHs, i.e., DMBA and benzo[a]pyrene-4,5-dihydroepoxide, an aromatic amine, i.e., 6-aminochrysene, a nitroaromatic, i.e., 1-nitrobenzo[a]pyrene and an alkylating agent, i.e., ENNG. The results that strains YG5161 and TA98 exhibited similar mutability to the compounds suggest that lesions induced by group II compounds can be bypassed by either DNA polymerase IV or DNA polymerase RI at similar efficiency. In fact, the previous study indicates that deletion of either $dinB_{ST}$ or $umuDC_{ST}$ reduced the mutagenicity of DMBA and 6-aminochrysene [34]. These compounds appear to require the presence of at least two specialized DNA polymerases to bypass the lesions. Of group II compounds, ENNG is exceptional because it is a simple alkylating agent that induces a mutagenic guanine base, i.e., O^6-ethylguanine [48]. Since the lesion in DNA directs the incorporation of thymine as well as cytosine during DNA synthesis, it was expected that this compound enhanced base substitutions in strain TA1535 (Fig. 3 B). It was a big surprise, however, that the simple alkylating agent was also capable of inducing −1 and −2 frameshifts in the repetitive sequences in strains TA1537 and TA1538, respectively, and that the bypass reactions leading to frameshifts appeared to be mediated by DNA polymerase IV or DNA polymerase RI. We also observed that the introduction of plasmids pYG768 and pKM101 enhanced the mutability of strain TA1538 against ethylnitrosourea (ENU), which induces O^6-ethylguanine in DNA (unpublished results). Eckert and Hile reported that frameshift errors are generated during in vitro DNA synthesis of ENU-treated template single-stranded DNA by mammalian DNA alpha-primase and DNA polymerase beta

[49]. Since humans possess Y-family DNA polymerases, i.e., DNA polymerase eta, iota, kappa and REV1 [8], it seems worth examining the abilities to bypass O^6-ethylguanine in repetitive sequences leading to frameshifts.

Since the newly established strain YG5161 harboring plasmid pYG768 exhibited higher sensitivity to groups I and II compounds than the standard Ames tester strain TA98 harboring plasmid pKM101, strain YG5161 could be useful for the sensitive detection of environmental mutagens and carcinogens such as benzo[a]pyrene and its derivatives. Actually, 10-azabenzo[a]pyrene, benzo[a]pyrene and 3-nitrobenzo[a]pyrene are present in polluted air and soot of combustion of coal [50,51], and some of them are identified in cigarette smoke [52].

Group III includes 1-nitropyrene and other 10 compounds. They include structurally unrelated compounds such as furylfuramide, aflatoxin B1 and acridine orange. The previous study indicated that deletion of *umuDC*$_{ST}$ significantly reduced the mutagenicity of 1-nitropyrene, 1,8-dinitropyrene, 1-nitro-6-azabenzo[a]pyrene and 3-nitro-6-azabenzo[a]pyrene [34], which are all included in this group. DNA adducts induced by group III compounds appeared to be more efficiently bypassed by DNA polymerase RI leading to −2 frameshift, compared to DNA polymerase IV or DNA polymerase II. Of the compounds, the mutagenicity of 3-nitro-6-azabenzo[a]pyrene is reduced by more than 95% by the deletion of *umuDC*$_{ST}$ [34]. Since the endogenous DNA polymerase V encoded by *umuDC*$_{ST}$ is capable to bypass DNA adduct(s) induced by this compound, the exogenous expression of DNA polymerase RI from plasmid pKM101 merely enhanced the mutagenicity by less than three-fold. Although DNA polymerase II encoded by *polB* had virtually no enhancing effects or rather suppressing effects on the mutagenicity of the chemicals examined, the introduction of plasmid pYG787 carrying *polB* specifically and repeatedly enhanced the mutagenicity of benz[a]anthracene, which is a potent carcinogen (Table 3). This compound induces adducts in guanine N^2 and adenine N6 atoms upon metabolic activation [53,54]. Thus, guanine N^2-adducts by the active metabolites of benz[a]anthracene in the CG repetitive sequence could be bypassed by DNA polymerase II leading to −2 frameshift. Since DNA polymerase II is a member of B-family DNA polymerase, its mammalian counterpart such as DNA polymerase delta may have an ability to bypass the adducts in the repetitive sequences.

Group IV includes PBTA-1 and other five compounds. PBTA-1 is a potent aromatic amine mutagen in a polluted river [55]. The characteristic of this group of compounds is that the introduction of any of the plasmids encoding SOS-inducible DNA polymerase had no enhancing effects on the mutagenicity. In the previous study, we reported that the mutagenicity of PBTA-1, Glu-P-1, aminophenylnorharman, N-OH-AAF, 4-NQO and 2-acetylaminofluorene, which are all belong to group IV in this study, are not reduced by the deletions of any of *S. typhimurium* genes encoding SOS-inducible DNA polymerases [34]. Thus, we suggested that the replicative DNA polymerase, i.e., DNA polymerase III holoenzyme, is responsible for the translesion bypass across DNA adducts induced by the chemicals in the CG repetitive sequence leading to −2 frameshift [34]. Although we cannot strictly rule out the possibility that DNA polymerase I is involved in the translesion, we prefer the possibility that DNA polymerase III holoenzyme is responsible for the bypass reactions because the introduction of plasmid pIMA-1 carrying *polA* did not enhance the mutagenicity (Fig. 4). The group IV compounds are all aromatic amines except for 4-NQO and some of them are proved to induce guanine C8 adducts in DNA [56–58]. Thus, we suggest that DNA polymerase III holoenzyme efficiently skips over guanine C8 adducts by aromatic amines in certain sequence context such as CGCGCGCG, thereby inducing −2 frameshift. Involvement of the replicative DNA polymerase may make this repetitive sequence a hot spot for frameshift mutagenesis.

In the previous study, we systematically disrupted one or all the genes of *S. typhimurium* strain TA1538 encoding SOS-inducible DNA polymerases and examined the mutability to chemical mutagens [34]. It is in contrast with the present study where the expression of SOS-inducible DNA polymerases is enhanced. As expected, most of the chemicals exhibited contrastive responses. They displayed enhanced mutagenicity in the presence of enhanced expression of the DNA polymerase, and diminished mutagenicity in the absence of the gene encoding the polymerase. Curiously, some compounds exhibited unexpected mutagenicity in the plasmid-bearing strains and the deletion strains. For example, 3-nitrobenzo[a]pyrene, 1-nitrobenzo[a]pyrene and 2-nitrofluorene were classified into groups I, II and III, respectively (Table 3, Fig. 1). The mutagenicity was enhanced by the introduction of plasmid pYG768 carrying *dinB* encoding DNA polymerase IV and/or pKM101 carrying *mucAB* encoding DNA polymerase RI. However, the mutagenicity of these compounds was not reduced by deletion of any of the genes encoding SOS-inducible DNA polymerases (class IV compounds in the previous study [34]). These results suggest that DNA polymerase III holoenzyme is responsible for the translesion bypass across the lesions induced by the chemicals in physiological conditions, but the Y-family DNA polymerases can take over the translesion reactions when the expression levels are enhanced. In other words, DNA polymerase III holoenzyme, DNA polymerase IV and DNA polymerase RI share, at least in part, the specificity to bypass the lesions and the polymerase actually involved in the translesion depends upon the cellular expression levels or the concentrations of the DNA polymerase in the replication complex.

To examine the possible involvement of DNA polymerase I in the frameshift mutagenesis, we introduced plasmid pIMA-1 carrying *polA* to strain TA1538 and strain YG6215, in which all the genes encoding SOS-inducible DNA polymerase are deleted [34]. Strikingly, the introduction of plasmid pIMA-1 sharply reduced the mutability of strain TA1538 against groups I, II and III compounds to that of strain YG6215 (Fig. 4). The dose–response curves of strain TA1538 harboring plasmid pIMA-1 with benzo[a]pyrene, 10-azabenzo[a]pyrene, 1-aminoanthracene, 3-methylcholanthrene (group I), DMBA, 6-aminochrysene (group II), 1,8-DNP and 1-nitro-6-azabenzo[a]pyrene (group III) almost overlapped those of strain YG6215 harboring plasmid pIMA-1 or the vector plasmid pWKS30. Because the mutability of strain TA1538 harboring plasmid pIMA-1 appears to be similar to that of strain YG6215 harboring the vector, we suggest that the enhanced expression of DNA polymerase I prevents the access of the Y-family DNA polymerases, i.e., DNA polymerase IV and DNA polymerase V, to the replication complex

where translesion bypass actually occurs. It is known that all five DNA polymerases in *E. coli* interact with the beta-subunit of DNA polymerase III holoenzyme [59–61]. Thus, there should be some competition for the polymerases to interact with the beta clamp. The beta-subunit assembles in a donut-like shape as a dimer and tethers DNA polymerase to a template/primer DNA, thereby preventing a falling off of polymerase from template DNA [62]. We speculate the order of the affinity of each DNA polymerase to the beta clamp or the replication complex may be DNA polymerase III > DNA polymerase I > DNA polymerase II > DNA polymerase IV = DNA polymerase V. This assumption is based on the observation that the introduction of plasmid pIMA-1 reduced the mutagenicity of compounds of groups I, II and III, which require the presence of the Y-family DNA polymerases for the maximum mutagenesis, but not group IV, whose mutagenicity is depended upon DNA polymerase III holoenzyme (Fig. 4). In addition, Foster suggested that DNA polymerase II may be dominant over DNA polymerase IV in the replication complex because the *dinB* mutator effects are more pronounced in the stationary-phase mutagenesis when the *polB* gene is deleted [63]. At present, we do not know which of DNA polymerase I or DNA polymerase II has a higher affinity to the replication complex. We prefer the possibility, however, that DNA polymerase I is dominant over DNA polymerase II because it is involved in lagging strand DNA synthesis during the chromosome replication. An alternative explanation for the suppressive effects of plasmid pIMA-1 (Fig. 4) is that DNA polymerase I expressed from the plasmid bypasses the lesions induced by the chemicals of groups I–III in an error-free manner, thereby reducing the mutagenicity. However, we think it less likely because the suppressive effects would vary with chemicals or lesions if DNA polymerase I mediated the error-free TLS. Each DNA polymerase including DNA polymerase I should have specificity to bypass the lesions. Hence, the introduction of pIMA-1 would suppress the mutagenicity of some compounds efficiently but not others. In fact, the strong suppressive effects were observed with almost all the compounds of groups I–III we examined. This is in contrast with the suppressive effects of DNA polymerase II expressed from plasmid pYG787, which reduced the mutagenicity of some of the compounds of groups I–III with various efficiencies (see below more detail). Thus, we prefer the possibility that DNA polymerase I expressed from the plasmid inhibits the access of the Y-family DNA polymerases to the replication complex. Nevertheless, it is important to examine whether a catalytically dead mutant of DNA polymerase I exhibits the suppressive effects on the mutagenicity of groups I–III chemicals to distinguish the possibilities.

In contrast to the clear suppressive effects by plasmid pIMA-1 carrying *polA*, the suppressive effects of plasmid pYG787 carrying *polB* on strain TA1538 were moderate. The introduction of plasmid pYG787 reduced the mutagenicity of benzo[a]pyrene-7,8-dihydroepoxide and 1-aminoanthracene (group I), 6-aminochrysene (group II), 1,8-dinitropyrene, 1-nitro-6-azabenzo[a]pyrene and furylfuramide (group III) by 40–60% (Table 3). DNA polymerase II may mediate the error-free translesion across DNA adducts induced by these compounds. In the previous study, deletion of $polB_{ST}$ reduced the −2 frameshift mutations induced by benzo[a]pyrene-7,8-tetrahydroepoxide, 3-methycholanthrene, 1-nitropyrene, 1,8-nitropyrene, 1-nitro-6-azabenzo[a]pyrene and 3-nitro-6-azabenzo[a]pyrene by 30–60% [34]. Thus, it seems that the enhanced expression as well as the lack of expression diminished the mutagenicity of 1,8-dinitropyrene and 1-nitro-6-azabenzo[a]pyrene. In other words, DNA polymerase II could have an optimal cellular concentration to enhance the translesion DNA synthesis leading to −2 frameshift. This is contrast to DNA polymerase IV and DNA polymerase RI, which enhance the mutagenesis when the levels of their expression are elevated. *E. coli* DNA polymerase II is reported to be involved in the immediate recovery of DNA synthesis after UV irradiation [13,14]. It is tempting to speculate that it might be required to re-synthesize the primer strand to reach the lesion when the primer strand was degraded. This degradation might occur when DNA polymerase III holoenzyme encountered the lesion and stopped the replication. If the expression level of DNA polymerase II was enhanced, it might promote error-free bypass reactions across the lesions while the lack of DNA polymerase II might lead to poor translesion DNA synthesis by DNA polymerase IV or DNA polymerase V.

In summary, our results suggest that DNA polymerase IV and DNA polymerase RI possess distinct but partly overlapping specificity to bypass lesions leading to −2 frameshift, and also that the replicative DNA polymerase, i.e., DNA polymerase III holoenzyme, participates in the bypass reactions in the CG repetitive sequence. Although DNA polymerase III holoenzyme is responsible for the translesion, the Y-family DNA polymerase may take over the primer termini, thereby enhancing the bypass reactions, when the expression of the polymerases is enhanced. Based on the suppressive effects of plasmid pIMA-1, we speculate that the order of DNA polymerases in *E. coli* to access to the replication complex could be DNA polymerase III > DNA polymerase I > DNA polymerase II > DNA polymerase IV = DNA polymerase V. Our results also raise an interesting possibility that strain YG5161 harboring plasmid pYG768 is a sensitive tester strain to identify the mutagenicity of environmental PAHs.

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IL-10 deficiency leads to somatic mutations in a model of IBD

Running Title: Mutations of chronic colitis

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