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Escherichia coli DNA polymerase III is responsible for the high level of spontaneous mutations in *mutT* strains

Masami Yamada,[1] Masatomi Shimizu,[1,2] Atsushi Katafuchi,[1] Petr Grúz,[1] Shingo Fujii,[3] Yukio Usui,[2] Robert P. Fuchs[3] and Takehiko Nohmi[1]*
[1]Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo 158-8501, Japan.
[2]Division of Medical Nutrition, Tokyo Health Care University, Tokyo 154-8568, Japan.
[3]Unité Mixte de Recherche 7258, CRCM, Genome Instability and Carcinogenesis, Centre National de la Recherche Scientifique, Marseille F-13009, France.

Summary

Reactive oxygen species induce oxidative damage in DNA precursors, i.e. dNTPs, leading to point mutations upon incorporation. *Escherichia coli mutT* strains, deficient in the activity hydrolysing 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphate (8-oxo-dGTP), display more than a 100-fold higher spontaneous mutation frequency over the wild-type strain. 8-oxo-dGTP induces A to C transversions when misincorporated opposite template A. Here, we report that DNA pol III incorporates 8-oxo-dGTP ≈ 20 times more efficiently opposite template A compared with template C. Single, double or triple deletions of pol I, pol II, pol IV or pol V had modest effects on the *mutT* mutator phenotype. Only the deletion of all four polymerases led to a 70% reduction of the mutator phenotype. While pol III may account for nearly all 8-oxo-dGTP incorporation opposite template A, it only extends ≈ 30% of them, the remaining 70% being extended by the combined action of pol I, pol II, pol IV or pol V. The unique property of pol III, a C-family DNA polymerase present only in eubacteria, to preferentially incorporate 8-oxo-dGTP opposite template A during replication might explain the high spontaneous mutation frequency in *E. coli mutT* compared with the mammalian counterparts lacking the 8-oxo-dGTP hydrolysing activities.

Introduction

Excess oxidation is a major threat to the genomic integrity of most living organisms. Reactive oxygen species (ROS) are produced by normal cellular respiration, cellular injury or by exposure to environmental carcinogens and radiation. ROS generate a variety of altered purines and pyrimidines in DNA (Bjelland and Seeberg, 2003; Kamiya, 2003), thereby playing important roles in mutagenesis, carcinogenesis and ageing (Ames, 1983; Jackson and Loeb, 2001). It should be emphasized, however, that oxidized bases in DNA are introduced not only by direct oxidation of DNA but also by incorporation of oxidized deoxynucleoside triphosphates (dNTPs) into DNA by DNA polymerases (pols) (Michaels and Miller, 1992; Sekiguchi and Tsuzuki, 2002; Nakabeppu *et al.*, 2006; Katafuchi and Nohmi, 2010).

7,8-Dihydro-8-oxo-dGTP (8-oxo-dGTP), a major form of oxidized dGTP in the cellular nucleotide pool, is a mutagenic substrate for DNA synthesis and the incorporation results in A:T to C:G mutations (Treffers *et al.*, 1954; Yanofsky *et al.*, 1966; Akiyama *et al.*, 1989; Tajiri *et al.*, 1995). When incorporated opposite A in the template DNA, 8-oxo-G can pair with incoming dCMP in the next round of DNA replication, then causing A:T to C:G mutations (Michaels and Miller, 1992; Kasai, 2002). To counteract the mutagenic 8-oxo-dGTP, *Escherichia coli* possesses a sanitizing enzyme, i.e. MutT, to hydrolyse 8-oxo-dGTP to the monophosphate form. When the *mutT* gene is inactivated, the mutation frequency of A:T to C:G transversions increases more than a 100-fold over the wild-type level (Yanofsky *et al.*, 1966; Maki and Sekiguchi, 1992; Fowler *et al.*, 2003). The high spontaneous A:T to C:G mutations in the *mutT* strain are almost completely suppressed when the *mutT* cells are cultured in anaerobic conditions, indicating the essential role of oxygen in the mutagenesis (Fowler *et al.*, 1994; Sakai *et al.*, 2006; Setoyama *et al.*, 2011).

In mammals including humans, enzymes that possess similar activities to *E. coli* MutT are identified (Mo *et al.*, 1992). Expression of human *MTH1* (*mutT* homologue-1) cDNA in *E. coli mutT* strain significantly suppresses the frequency of spontaneous mutations (Sakumi *et al.*, 1993; Furuichi *et al.*, 1994). Suppressive effects are also observed when mouse or rat cDNA is expressed in the *mutT* cells (Cai *et al.*, 1995; Kakuma *et al.*, 1995). These observations imply that the mammalian proteins may sanitize the nucleotide pools in the organisms, thereby reducing the spontaneous mutagenesis and carcinogenesis. In

Accepted 1 October, 2012. *For correspondence. E-mail nohmi@nihs.go.jp; Tel. (+81) 3 3700 1564; Fax (+81) 3 3700 1622.

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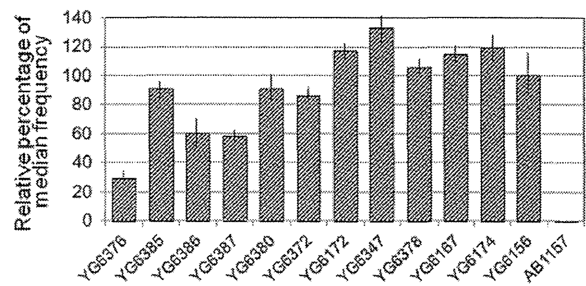
fact, deletion of the *Mth1* gene results in high frequency of tumour formation in several organs of mice (Tsuzuki *et al.*, 2001). However, deletion of the mouse *Mth1* gene enhances the spontaneous mutation frequency only twofold, which is a striking difference from the strong mutator effects of *E. coli mutT* (Egashira *et al.*, 2002). Although mammalian cells possess more than one enzyme to sanitize oxidized nucleotides (Bessman *et al.*, 1996; Cai *et al.*, 2003; Ishibashi *et al.*, 2003), there may be other reasons to account for the marked difference in spontaneous mutagenesis between *E. coli mutT* and the mammalian counterparts.

In this study, we explored the potential involvement of the different *E. coli* DNA polymerases in the *mutT* mutator phenotype. *E. coli* possesses five pols, i.e. pol I (A family), pol II (B family), pol III (C family), pol IV (Y family) and pol V (Y family) (Nohmi, 2006), and pol III holoenzyme (pol III HE) is mainly responsible for the chromosome replication (McHenry, 2011). Pol III HE is a large dimeric complex, which is composed of pol III core complex including ε proofreading subunit, sliding clamp (β subunit) and the clamp loader (McHenry, 2011). Although A-, B- and Y-family pols are present in mammals, C-family pols are present only in eubacteria (Ito and Braithwaite, 1991; Filee *et al.*, 2002). We disrupted the genes encoding pols I, II, IV and V, and characterized their mutator phenotypes. We also examined the biochemical properties of pol III*, the HE without β subunit (McHenry, 1988), incorporating 8-oxo-dGTP into DNA and effects of addition of β subunit on extension reaction upon incorporation of 8-oxo-dGTP by pol III* *in vitro*. Our results indicate that pol III HE may be responsible for the misincorporation of 8-oxo-dGTP into DNA and suggest that the erroneous nature of pol III HE uniquely present in bacteria might account for the strong mutator effects of *mutT* in *E. coli*.

Results

Deletion of the genes encoding pols in a mutT background

To examine what pols are involved in the high spontaneous mutations in a *mutT* background, we deleted the genes encoding pol I (*polA*), pol II (*polB*), pol IV (*dinB*) or pol V (*umuDC*) in a *mutT* background. Deletion of *mutT* increased median frequency of rifampicin-resistant mutations more than 100 times without any damaging treatments to DNA (Fig. 1). None of single deletions of the *pol* genes reduced the median frequency of the *mutT*-deficient strain. Since pols IV and V preferentially incorporate 8-oxo-dGTP opposite template A *in vitro* (Yamada *et al.*, 2006), we deleted both the *dinB* and *umuDC* genes and examined the spontaneous mutation frequency. However, the deletion of genes encoding two Y-family pols did not decrease



Pol I	Δ	+	Δ	Δ	Δ	Δ	+	Δ	+	+	+	+	+
Pol II	Δ	Δ	+	Δ	Δ	+	+	+	Δ	+	+	+	+
Pol IV	Δ	Δ	Δ	+	Δ	Δ	Δ	+	+	Δ	+	+	+
Pol V	Δ	Δ	Δ	Δ	+	+	Δ	+	+	+	Δ	+	+
MutT	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	Δ	+

Fig. 1. Mutation frequency of *mutT* strains with deficiency of DNA polymerase(s). Relative values (percentage) and the standard deviations of median frequency of rifampicin resistance mutations of *mutT* derivatives of *E. coli* are presented. Mutation assays were conducted at 30°C for strains with ΔKF and at 37°C for the other strains. The frequencies of strain YG6156 (Δ*mutT*) at 30°C and 37°C were set as 100%. The average mutation frequencies of YG6156 were $184 \pm 58 \times 10^{-8}$ at 30°C ($n = 12$) and $185 \pm 57 \times 10^{-8}$ at 37°C ($n = 9$). The mutation frequency of AB1157 at 37°C (the wild-type strain) was $1.3 \pm 0.3 \times 10^{-8}$ ($n = 3$). n represents number of repeated experiments. Mutation frequencies of other strains are presented in Table S1. The table under the graph indicates which polymerases are deficient (Δ) and proficient (+) in each strain.

the median frequency. We also deleted both the *polA* and *dinB* genes and examined the mutation frequency. Although pol I Klenow fragment (KF) and pol IV possess ability to extend primer DNA having 8-oxo-dGMP at the termini *in vitro* (see below and Fig. S3), deletion of the *polA* and *dinB* genes did not reduce the mutator phenotype substantially. Interestingly, when we deleted *polA*, *umuDC* and either *polB* or *dinB*, the spontaneous mutation frequency decreased by 40%. When we deleted all four pol genes, i.e. *polA*, *polB*, *dinB* and *umuDC*, the mutation frequency decreased by 70%. It should be emphasized, however, that the resulting penta mutant YG6376, i.e. Δ*mutT*, Δ*polA*, Δ*polB*, Δ*dinB* and Δ*umuDC*, still manifested more than 50 times higher spontaneous mutation frequency than the wild-type strain AB1157 ($74 \pm 20 \times 10^{-8}$, YG6376 versus $1.3 \pm 0.3 \times 10^{-8}$, AB1157). These results suggest that pol III plays an important role in high spontaneous mutations in the *mutT* background, and also that other pols might have additional and redundant roles in the mutagenesis.

*Incorporation of 8-oxo-dGTP into DNA by pol III** in vitro

Next, we examined the specificity of pol III* incorporating 8-oxo-dGTP into DNA *in vitro* (Fig. 2). Pol III* preferentially

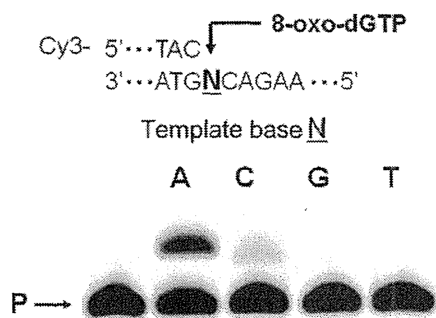


Fig. 2. Incorporation of 8-oxo-dGTP by pol III*. The Cy3-labelled 18-mer primer/36-mer template (sequences 1, 0.1 μM) was treated with pol III* (1 nM) in the presence of 100 μM 8-oxo-dGTP. The reaction mixtures were incubated at room temperature for 1 min. The samples were analysed by denaturing polyacrylamide gel electrophoresis and visualized by the Molecular Imager as described in *Experimental procedures*. The alphabets shown in the figure indicate as follows: N, template base; A, adenine; C, cytosine; G, guanine; T, thymine; P, primer.

incorporated 8-oxo-dGTP opposite template A in DNA. To examine the specificity quantitatively, we conducted kinetic analyses incorporating 8-oxo-dGTP into DNA (Fig. S1). Pol III* incorporated 8-oxo-dGTP opposite template A at concentration range of 8-oxo-dGTP from 0.05 to 10 μM (Fig. S1A). In contrast, pol III* incorporated 8-oxo-dGTP opposite template C at higher concentrations of 8-oxo-dGTP, i.e. 10 to 500 μM (Fig. S1B). The f_{inc} values for incorporation, i.e. the ratio between efficiency (V_{max}/K_m) of incorporation of 8-oxo-dGTP and that of incorporation of normal dNTP, were 5.6×10^{-2} and 2.9×10^{-3}, respectively, opposite template A and template C (Table 1). It indicates that pol III* incorporates 8-oxo-dGTP opposite template A about 20 times more efficiently than opposite template C. It is remarkable that the apparent K_m value for incorporation of 8-oxo-dGTP opposite template A was 2.5 μM, which is similar to the values for incorporation of normal dTTP and dGTP opposite templates A and C respectively (1.6 μM and 3.2 μM). The apparent K_m value for incorporation of 8-oxo-dGTP opposite template C was 212 μM.

Excision of 8-oxo-dG at the end of the primer

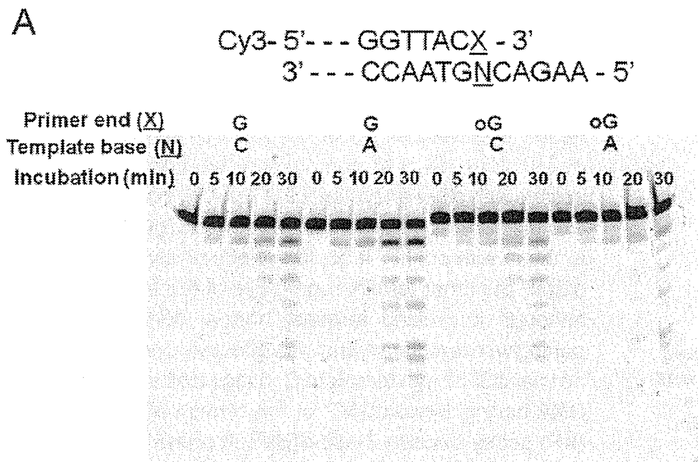
The incorporated 8-oxo-dG opposite template A forms a mismatch, which is usually excised as an improper base by proofreading activities of pols. Thus, we examined whether incorporated 8-oxo-dGMP is excised by the proofreading activity of pol III* (Fig. 3A). Strikingly, 8-oxo-dGMP was not excised from the primer DNA regardless of the pairing template base of C or A efficiently. In contrast, pol III* effectively excised terminal normal dGMP incorrectly pairing with template A, and even correctly pairing with template C. When we plotted percentage of digestion of primer DNA periodically, it became evident that the order of primer most rapidly digested was primer having G/A mismatch at the termini > primer having G/C > primer having 8-oxo-G/A = primer having 8-oxo-G/C (Fig. 3B). Single-stranded DNA having 8-oxo-dGMP at the termini was rapidly degraded by pol III* as well as that having normal dGMP (Fig. 3C). For comparison, we conducted similar assays with other enzymes, i.e. T7 pol, *E. coli* pol I (KF) and exonuclease III. T7 pol displayed similar digesting patterns to those with pol III*. It could not effectively excise 8-oxo-dGMP at primer termini regardless of the template bases although it excised terminal normal dGMP incorrectly pairing with template A and less effectively correctly pairing normal dGMP with template C. It digested single-stranded DNA having 8-oxo-dGMP at the termini effectively. Pol I (KF) poorly excised 8-oxo-dGMP at primer termini as pol III* and T7 pol. In contrast to pol III* and T7 pol, pol I (KF) did not digest single-stranded DNA having 8-oxo-dGMP at the termini. Exonuclease III digested primer DNA having terminal 8-oxo-dGMP as well as normal dGMP. It did not digest single-stranded DNA regardless of the presence or the absence of 8-oxo-dGMP at the termini.

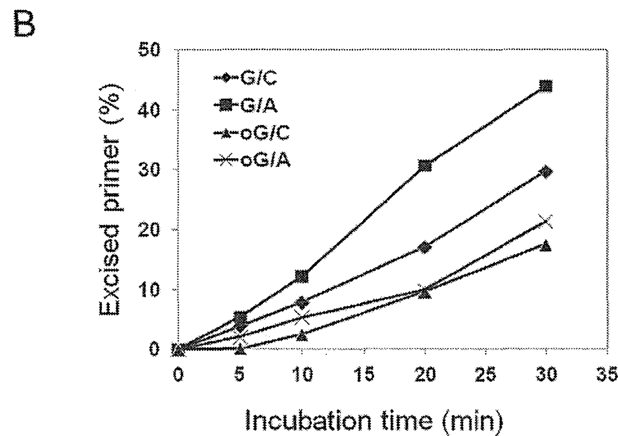
Extension from dG or 8-oxo-dG at the end of a primer

Primer DNA having incorporated 8-oxo-dGMP at the termini has to be extended. Otherwise, it will induce DNA strand breaks. Next, we examined whether primer DNA having 8-oxo-dGMP at the termini was extended by pol III* *in vitro*. We also examined the effects of addition of the β subunit to pol III* on the extension reactions. For this purpose, we prepared template DNA having biotin/streptavidin at both ends (Fig. S2). The terminal streptavidin prevents the β subunit from falling off from the template/primer DNA. In standing start experiments, pol III* extended primer DNA having 8-oxo-dG at the termini and the extension was substantially enhanced by the addition of the β subunit (Fig. 4). The primer/template DNA having 8-oxo-dGMP pairing with template A appeared to be slightly more effectively extended compared with the primer/template DNA having 8-oxo-dGMP pairing with template C. In running start experiments where 8-oxo-dGMP was incorporated into DNA during DNA synthesis, pol III* extended primer DNA upon

Table 1. Kinetic parameters for 8-oxo-dGTP insertion catalysed by pol III*.

Template base/dNTP	K_m (μM)	Relative V_{max}	V_{max}/K_m	f_{inc}
A/dTTP	1.6 ± 0.4	2.9 ± 0.23	1.8	1
A/8-oxo-dGTP	2.5 ± 0.82	0.26 ± 0.03	0.1	0.056
C/dGTP	3.2 ± 0.72	5.6 ± 0.47	1.75	1
C/8-oxo-dGTP	212 ± 45.7	1.1 ± 0.1	0.005	0.0029





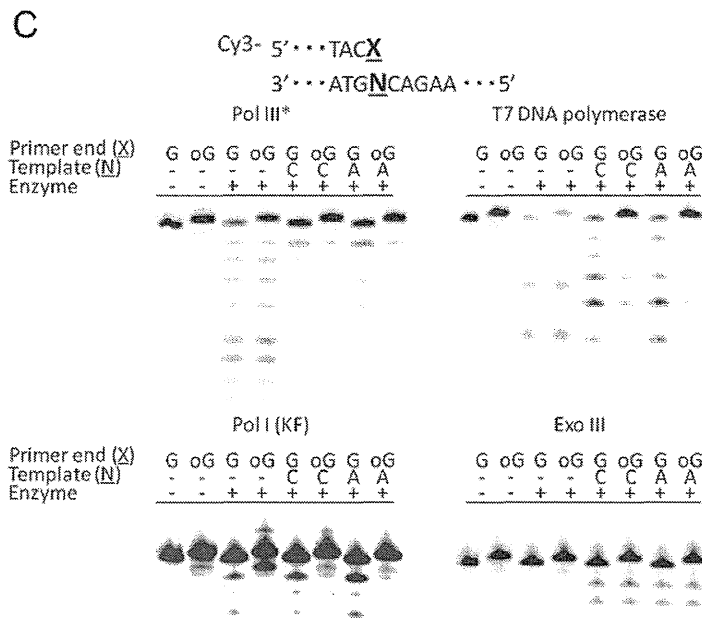


Fig. 3. A. Exonuclease digestion of primers by pol III*. The Cy3-labelled 19-mer primer having G or 8-oxo-G at the 3′-termini/36-mer template having C or A at the position N (0.1 μM) were incubated with pol III* (1 nM) for 5, 10, 20 or 30 min at 25°C. The products were analysed by denaturing polyacrylamide gel electrophoresis and visualized by the Molecular Imager. oG represents 8-oxo-G.
B. Time course of digestion of primers by pol III*. Four types of primer/template DNA having G/C, G/A, 8-oxo-G/C or 8-oxo-G/A at the termini were incubated with pol III* (1 nM) for 5, 10, 20 or 30 min at 25°C and the percentage of the digested primer DNA was plotted.
C. Excision of 8-oxo-dGMP at the end of the primer by pol III*, T7 pol, pol I (KF) and exo III. The 19-mer primer/36-mer template DNA (0.1 μM) or the 19-mer primer DNA alone (0.1 μM) was incubated with pol III* (1 nM) at room temperature, T7 pol (0.0001 unit μl^{-1}), pol I (KF)(0.001 unit μl^{-1}) or exo III (0.0001 unit μl^{-1}) at 37°C for 10 min without dNTP. The products were analysed as described in the legend to A.

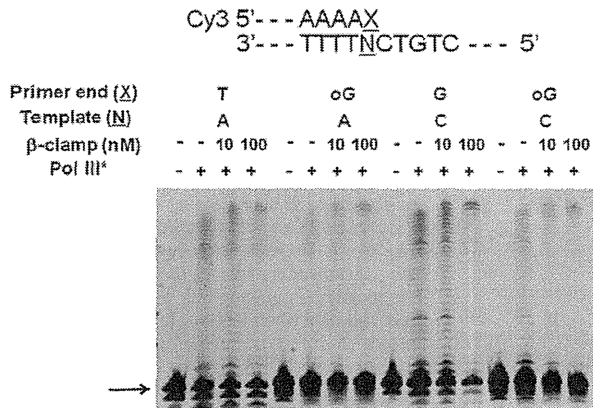


Fig. 4. Standing start extension of primers having 8-oxo-G at the termini by pol III* with or without β clamp. Cy3-labelled 35-mer primer (X = T, G or 8-oxo-G) with 100-mer template DNA (N = A or C) having biotin/streptavidin at both ends (20 nM) was incubated with pol III* (10 nM) and β clamp (0, 10 or 100 nM) in the presence of 100 μM dNTPs. The reaction mixtures were incubated at room temperature for 1 min. The samples were analysed by denaturing polyacrylamide gel electrophoresis and visualized by the Molecular Imager. The alphabets shown in the figure represent: X, primer terminal base; N, template base; A, adenine; C, cytosine; G, guanine; T, thymine; oG, 8-oxo-G. The arrow indicates the position of primer. Although we have purified the primer DNA, there appears shorter primer DNAs, which were present below the position of the primer.

incorporation of 8-oxo-dGMP opposite template A (Fig. 5). In these reactions, addition of the β subunit substantially enhanced the extension reactions. It should be noted, however, that the extension reactions upon incorporation of 8-oxo-dGMP opposite template A were less efficient compared with the reactions upon incorporation of dTMP opposite template A even in the presence of the β subunit. As controls, we also examined the extension activity of pol I (KF) and pol IV with primers having 8-oxo-dG at the termini (Fig. S3). Pol I (KF) displayed potent extension activity with primers having terminal 8-oxo-dGMP pairing with template A or C. The primer DNA having 8-oxo-dGMP pairing with template A was more effectively extended by pol I (KF) than primer DNA having normal G pairing with template A. Pol IV exhibited moderate extension activity with primer DNA having 8-oxo-dGMP pairing with template A or C.

Discussion

dNTP pool and DNA are continuously exposed to a variety of exogenous and endogenous genotoxic agents, including ROS, and incorporation of oxidized dNTPs into DNA is a source of spontaneous mutagenesis and carcinogenesis (Ames and Gold, 1991). Here, we provided genetic and biochemical evidence that a replicative pol of *E. coli*, i.e. pol III HE, may be involved in oxidative mutagenesis through misincorporation of an oxidized nucleotide, i.e. 8-oxo-dGTP, during DNA synthesis in the *mutT* background. Although deletion of the genes encoding pol I, pol II, pol IV and pol V reduced the mutation frequency by more than 50%, the resulting strain YG6376 having pol III alone exhibited more than 50 times higher spontaneous mutation frequency in the *mutT* background than the wild-type strain (Fig. 1 and Table S1). Pol III* incorporated 8-oxo-dGTP opposite template A about 20 times more effectively than opposite template C *in vitro* (Fig. 2 and Table 1). Genetic analyses also suggest that 8-oxo-dGTP is preferentially incorporated opposite template A in the *mutT* background *in vivo* (Fowler *et al.*, 2003). This biased specificity incorporating 8-oxo-dGTP opposite template A is reminiscent of that of X-family or Y-family pols involved in DNA repair and translesion DNA synthesis (Katafuchi and Nohmi, 2010). Mammalian pol β, a representative of X-family pols, incorporates 8-oxo-dGTP opposite template A and opposite template C at a ratio of 24:1 (Miller *et al.*, 2000). Human pol η, a Y-family pol, preferentially incorporates 8-oxo-dGTP opposite template A at 60% efficiency of normal dTTP incorporation (Shimizu *et al.*, 2007). Interestingly, α subunit, the catalytic subunit of pol III HE of *E. coli* and *Thermus aquaticus*, is structurally related to mammalian pol β, but not to mammalian replicative pols such as pol δ or pol ε, which are B-family enzymes (Bailey *et al.*, 2006; Lamers *et al.*, 2006). Therefore, we speculate that the structural similarity of the α subunit of pol III HE to mammalian pol β might be the

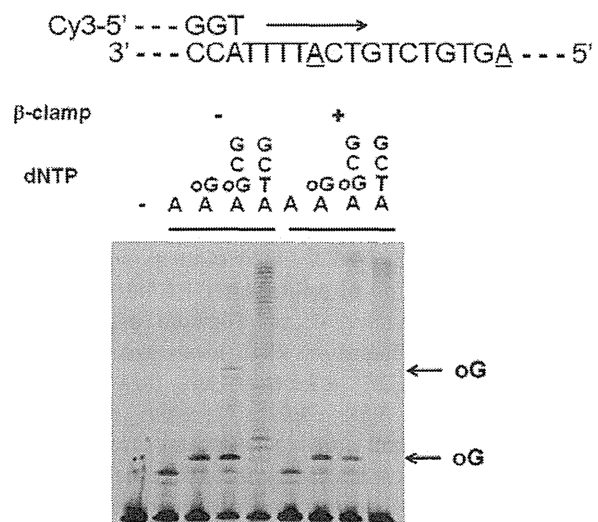


Fig. 5. Incorporation of 8-oxo-dGTP and extension by pol III* with or without β clamp under running start conditions. The 30-mer primer/100-mer streptavidin bound template (sequences 2, 20 nM) were incubated with pol III* (10 nM) with or without β clamp (100 nM) in the presence of indicated dNTPs (100 μM each) for 1 min at 25°C. The samples were analysed by denaturing polyacrylamide gel electrophoresis and visualized by the Molecular Imager. The alphabets shown in the figure represent: A, dATP; C, dCTP; G, dGTP; T, dTTP; oG, 8-oxo-dGTP.

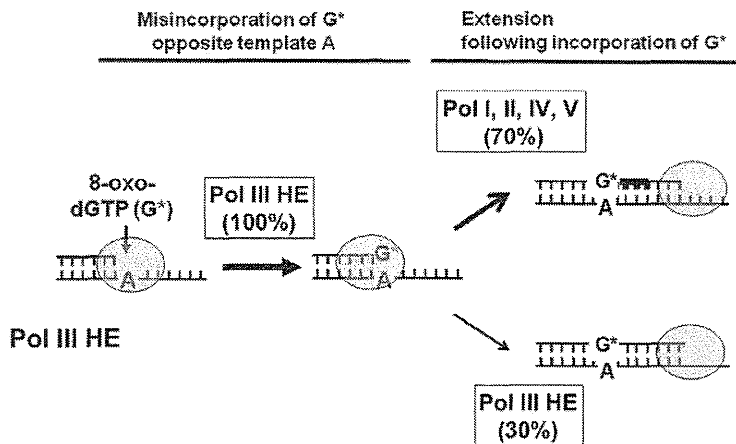


Fig. 6. MutT mutator phenotype: a multi-polymerase affair. Pol III HE (oval) incorporates 8-oxo-dGTP (G*) opposite template A during the chromosomal replication. We suggest that while pol III accounts for nearly all 8-oxo-dGTP incorporation opposite template A, it only extends ≈ 30% of them, the remaining 70% being extended by the combined action of pol I, pol II, pol IV or pol V (based on data from Fig. 1). We also speculate that the roles of the auxiliary pols might be redundant because the mutation frequency was significantly reduced only when three or four auxiliary pols are deleted (Fig. 1). Following a short patch (a thick line) of DNA synthesis by the auxiliary pols, pol III HE will resume chromosomal replication.

structural basis for the preferential incorporation of 8-oxo-dGTP opposite A in template DNA.

In general, 8-oxo-dGTP is a poor substrate for most of pols (Nohmi *et al.*, 2005; Katafuchi and Nohmi, 2010). For example, the efficiency of incorporation of 8-oxo-dGTP by pol δ involved in the chromosome replication in mammalian cells is more than 10^4-fold lower than that of incorporation of normal dGTP, and the enzyme prefers to incorporate 8-oxo-dGTP opposite template C (Einolf and Guengerich, 2001). 8-oxo-dGTP is poorly incorporated into DNA by T7 pol exo⁻, HIV reverse transcriptase, *E. coli* pol II and pol I (KF) exo⁻ as well (Einolf *et al.*, 1998). In contrast, pol III* incorporates 8-oxo-dGTP opposite template A at about 5% efficiency of normal dTTP incorporation (Table 1). We suggest that the erroneous and efficient incorporation of 8-oxo-dGTP opposite template A by pol III HE may account for the extremely high spontaneous mutations in the *mutT* mutants of *E. coli.*

At first, we expected that pol IV and pol V might be responsible for the misincorporation of 8-oxo-dGTP into DNA during DNA replication in the *mutT* background. This is because pol IV and pol V are involved in mutagenesis through incorporation of oxidized dNTPs into DNA in a Δ*sod*Δ*fur* background of *E. coli* (Yamada *et al.*, 2006). In the mutants, intracellular ROS levels are extremely elevated, and SOS responses are fully induced (Nunoshiba *et al.*, 1999; 2002). Therefore, expression levels of pol IV and pol V are highly elevated. In contrast, in the *mutT* mutants, no SOS responses are induced (Janion *et al.*, 2003). Thus, it is expected that the expression of pol IV and pol V is constitutive levels. The different status of SOS induction might explain the different contribution of SOS-inducible Y-family pols to oxidative mutagenesis in Δ*sod*Δ*fur* and *mutT* strains although oxidized dNTPs are deeply involved in the mutagenic processes.

It appears, however, that pols other than pol III play roles in the high spontaneous mutations in the *mutT* background. This is because the mutant lacking all four DNA polymerases (YG6376) exhibited a 70% reduction in mutability compared with *mutT* (Fig. 1 and Table S1). Single deletions of each of the genes encoding the auxiliary pols did not reduce the mutation frequency. Only in the cases where three or four pols are absent, there was a significant reduction of the *mutT* mutator phenotype. Thus, pol I, pol II, pol IV and pol V might have redundant roles in the mutagenesis. One possible explanation for the redundant role is that they might play roles in extension of primer DNA upon incorporation of 8-oxo-dGMP into DNA by pol III HE (Fig. 6). This speculation is based on the results that extension of primer upon incorporation of 8-oxo-dGMP opposite template A by pol III HE is less efficient compared with the incorporation of normal dTMP opposite template A (Fig. 5). In addition, pol I and pol IV possess ability to extend primer DNA having 8-oxo-dGMP opposite template A at the termini (Fig. S3). Hence, the auxiliary pols might play roles in the extension step, thereby enhancing the mutagenesis in the *mutT* background. Another, but not exclusive, alternative would be that the presence of pol I, pol II, pol IV and pol V might affect the efficiency of pol III HE incorporating 8-oxo-dGTP into DNA during the chromosome replication. It is well known that all five pols in *E. coli* interact with the β subunit and compete to take over the primer termini (Lopez de Saro *et al.*, 2003; Burnouf *et al.*, 2004). Thus, it seems possible that the absence of the auxiliary pols might affect the interactions of pol III with the β subunit, which in turn affects the efficiency of pol III HE incorporating 8-oxo-dGTP during DNA synthesis. It is reported that processivity factors such as eukaryotic PCNA and β subunit of *E. coli* affect the processivity and specificity of pols (Bloom *et al.*, 1997; Maga *et al.*, 2007).

Our biochemical results indicated that pol III*, T7 pol and pol I (KF) could not excise incorporated 8-oxo-dGMP from the primer effectively even when the oxidized dGMP was paired with template A (Fig. 3). Previous genetic analyses also suggest that the proofreading activity of pol III HE has little effect on the *mutT* mutator phenotype

(Fowler *et al.*, 1992). In pol III HE, the proofreading 3′ to 5′ exonuclease exists as a separate subunit ε, which binds to the catalytic subunit (McHenry, 2011). In contrast, exonucleases and polymerases exist in separate domains in single polypeptides of T7 pol and pol I (KF) (Beese *et al.*, 1993; Doublie *et al.*, 1998). Because pol III* and T7 pol can digest the single-stranded DNA having 8-oxo-dGMP and exonuclease III digested double-stranded DNA having 8-oxo-dGMP at the 3′-termini (Fig. 3), the exonucleases in the pols and exonucleases III appear to have the ability to hydrolyse phosphodiester bonds between the terminal 8-oxo-dGMP and the second terminal dNMP in the primer strands. Therefore, we speculate that the reason for the poor excision of 8-oxo-dGMP from the 3′-termini of primer strands by pol III* and T7 pol might be inefficient transfer of the primer strands having 8-oxo-dGMP from the polymerase domain (or subunit) to the exonuclease domain (or subunit) even when 8-oxo-dGMP pairs with template A. In contrast, pol I (KF) did not digest single-stranded DNA having 8-oxo-dGMP at the termini. Therefore, in the case of pol I (KF), the poor proofreading against 8-oxo-dGMP paired with template A or C may be due to its weak or defective nuclease activity against single-stranded DNA having 8-oxo-dGMP at the terminus. It might be interesting to investigate the structural and biochemical reasons for the poor excision of 8-oxo-dGMP at the 3′-termini of primers by the pols.

It may be counterintuitive that pol III HE, the replicative pol, which is supposed to be very accurate, positively produces errors during the chromosome replication if *mutT* is inactivated. One plausible explanation is that pol III HE incorporates the oxidized dGTP into DNA, thereby enhancing mutagenesis to generate progenies that can adapt to the stressful environments, when the bacteria are exposed to oxidative stress and the *mutT* gene is inactivated. The original habitat of *E. coli* is intestine, which is strictly anaerobic. Therefore, aerobic culture conditions may be somewhat stressful to *E. coli*. A precedent for such enhanced mutagenesis in stressful conditions is the SOS-induced mutagenesis where *E. coli* induces error-prone pol IV and pol V and enhances mutagenesis when DNA is damaged by ultraviolet light or other DNA-damaging agents (Echols, 1981; Friedberg *et al.*, 2002). High mutation rates may be detrimental to individual bacterium but may be beneficial for the whole population because the high mutation rates may lead to generation of mutant progenies that can survive under the stressful conditions. Other C-family pols from organisms originally living in anaerobic conditions might have evolved in a manner to incorporate 8-oxo-dGTP into DNA effectively as in the case of *E. coli* pol III HE.

In summary, we presented genetic and biochemical evidence that suggests that the replicative pol of *E. coli*, i.e. pol III HE, effectively and incorrectly incorporates oxidized dGTP opposite template A during the chromosome replication *in vivo*. The auxiliary pols appear to help the erroneous DNA replication by pol III HE in the *mutT* background. The specificity and efficiency of incorporation of 8-oxo-dGTP into DNA by pol III HE are marked contrast with those of replicative pols in mammals, e.g. pol δ, which incorporate 8-oxo-dGTP into DNA very poorly. The erroneous nature of pol III HE, which is uniquely present in eubacteria, might explain the extremely high spontaneous mutations of the *mutT* background in *E. coli* compared with the moderate mutator effects of mammalian counterparts lacking enzymes with 8-oxo-dGTP hydrolysing activities such as MTH1.

Experimental procedures

Materials

Pol III* was purified as described (Fujii and Fuchs, 2004). Deoxyribonucleoside triphosphates (ultrapure grade) and 8-oxo-dGTP were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK) and TriLink BioTechnologies (San Diego, CA, USA) respectively. Oligonucleotides were synthesized and purified twice by high-performance liquid chromatography (BEX, Tokyo, Japan). Primers were annealed to templates at a 1:1 molar ratio. For primer extension and kinetic studies of nucleotide incorporation, two types of DNA substrates were used. The set of the 18-mer primer/36-mer template named sequences 1 was as follows: 5′-CGCGC GAAGACCGGTTAC-3′ (18-mer primer) and 3′-GCGCGC TTCTGGCCAATGNCAGAATTCCTAGGGAAG-5′ (36-mer template, where N = A, C, G or T). The set of the 30-mer primer/100-mer template named as sequences 2 used for measuring the incorporation frequencies was as follows: 5′-GTACCGCCACCCTTAGAACCGGTGTTTGGT-3′ (primer; 30-mer) and 3′-GGCCTTATCCACATAGTGGCATGAGTCC TCCAAATCATGGCGGTGGGAATCTTGGCCACAAACCAT TTTXCTGTCTGTGACTCGTTCAGGCTATTACTGA-5′ [template; 100-mer, X (= A or C) is the target site]. The same 100-mer oligonucleotide with biotin was synthesized and purified twice by high-performance liquid chromatography (Tsukuba Oligo Service, Tsukuba, Japan).

Strain construction

All the strains and plasmids used in this study are listed in Table 2. P1 transduction was conducted at 37°C. When strains lacking pol I Klenow fragment (ΔKF) were used as either recipients or donors, the transduction was conducted at 30°C.

To construct *polA*$^-$ derivative, the Δ*klenow*::chloramphenicol resistance (Cm^R) gene (*cat*) allele of strain HRS7052 (Wagner and Nohmi, 2000), which was constructed by Dr H. Iwasaki (Tokyo Institute of Technology, Japan), was transferred to AB1157 by P1 transduction. The resultant strain was named as YG6343, which lacked pol and 3′ to 5′ exonuclease of pol I but retained 5′ to 3′ exonuclease activity. YG6371 (ΔKFΔ*dinB*) was constructed by P1 transduction using HRS7052 as a donor and YG6162 (Δ*dinB*, see below) as a

Table 2. Strains and plasmids used in this study.

Strains	Genetic characteristics	Sources
AB1157	F$^-$ *thr1 ara14 leuB6 proA2 lacG1 tsx33 supE44 galK2 hisG4 rfbD1 mgl51 rpsL31 xyl5 mtl1 argE3 thi1* λ$^-$ *rac*$^-$	Laboratory stock
V355	F$^-$ *lac-3350 galK2(O*^{c}) *galT22* λ$^-$ *recD1014*(Nuc$^-$) *IN(rrnD-rrnE)1 rpsL179*(str^R)	Shevell *et al.* (1988)
JW0059	F$^-$ Δ*polB770::kan* Δ(*araD–araB*)*567* Δ*lacZ4787*(::*rrnB-3*) λ$^-$ *rph-1* Δ(*rhaD–rhaB*)*568 hsdR514*; Km^R	Keio Collection, NBRP
YG2004	The same as V355, but deficient in *mutT* with Km^R cassette insertion; Km^R, Δ*mutT*	This study
YG6156	The same as AB1157, but deficient in *mutT* with Km^R cassette insertion; Km^R, Δ*mutT*	This study
HRS7052	Deficient in a part of *polA* gene, thus lacking both 3′–5′ exonuclease and polymerase activities; Cm^R, ΔKF	Wagner and Nohmi (2000)
YG6162	The same as AB1157, but deficient in *dinB* with in frame deletion; Δ*dinB*	Salem *et al.* (2009)
YG6167	The same as YG6162, but deficient in *mutT* with Km^R cassette insertion; Km^R, Δ*dinB* Δ*mutT*	This study
YG6168	The same as AB1157, but deficient in *umuDC* with *ermGT* insertion, Δ*umuDC*	Salem *et al.* (2009)
YG6174	The same as YG6168, but deficient in *mutT* with Km^R cassette insertion; Km^R, Δ*umuDC* Δ*mutT*	This study
YG6171	The same as YG6168, but deficient in *dinB* with in frame deletion, Δ*umuDC* Δ*dinB*	Salem *et al.* (2009)
YG6172	The same as YG6171, but deficient in *mutT* with Km^R cassette insertion; Km^R, Δ*umuDC* Δ*dinB* Δ*mutT*	This study
YG6343	The same as AB1157, but deficient in a part of *polA* gene, thus lacking both 3′–5′ exonuclease and polymerase activities *with* Cm^R cassette insertion; Cm^R, ΔKF	This study
YG6347	The same as YG6343, but deficient in *mutT* with Km^R cassette insertion; Cm^R; Km^R, ΔKF Δ*mutT*	This study
YG6371	The same as YG6162, but deficient in a part of *polA* gene, thus lacking both 3′–5′ exonuclease and polymerase activities *with* Cm^R cassette insertion; Cm^R, Δ*dinB* ΔKF	This study
YG6372	The same as YG6371, but deficient in *mutT* with Km^R cassette insertion; Cm^R; Km^R, Δ*dinB* ΔKF Δ*mutT*	This study
YG6375	The same as YG6171, but deficient in a part of *polA* gene with Cm^R cassette insertion as HRS7052 and deficient in *polB* gene with in frame deletion, Cm^R; Δ*umuDC* Δ*dinB* ΔKF Δ*polB*	This study
YG6376	The same as YG6375, but deficient in *mutT* with Km^R cassette insertion; Cm^R; Km^R, Δ*umuDC* Δ*dinB* Δ*polB* ΔKF Δ*mutT*	This study
YG6377	The same as AB1157, but deficient in *polB* with in frame deletion; Δ*polB*	This study
YG6378	The same as YG6377, but deficient in *mutT* with Km^R cassette insertion; Km^R, Δ*polB* Δ*mutT*	This study
YG6381	The same as YG6168, but deficient in *polB* with in frame deletion; Δ*umuDC* Δ*polB*	This study
YG6382	The same as YG6171, but deficient in *polB* with in frame deletion; Δ*umuDC* Δ*dinB* Δ*polB*	This study
YG6385	The same as YG6382, but deficient in *mutT* with Km^R cassette insertion; Km^R, Δ*umuDC* Δ*dinB* Δ*polB* Δ*mutT*	This study
YG6383	The same as YG6171, but deficient in a part of *polA* gene with Cm^R cassette insertion as HRS7052; Cm^R, Δ*umuDC* Δ*dinB* ΔKF	This study
YG6386	The same as YG6383, but deficient in *mutT* with Km^R cassette insertion; Cm^R, Km^R, Δ*umuDC* Δ*dinB* ΔKF Δ*mutT*	This study
YG6384	The same as YG6381, but deficient in a part of *polA* gene with Cm^R cassette insertion as HRS7052, Cm^R; Δ*umuDC* Δ*polB* ΔKF	This study
YG6387	The same as YG6384, but deficient in *mutT* with Km^R cassette insertion; Cm^R; Km^R, Δ*umuDC* Δ*polB* ΔKF Δ*mutT*	This study
YG6379	The same as YG6371, but deficient in *polB* with in frame deletion; Cm^R, Δ*dinB* ΔKF Δ*polB*	This study
YG6380	The same as YG6379, but deficient in *mutT* with Km^R cassette insertion; Cm^R, Km^R, Δ*dinB* ΔKF Δ*polB* Δ*mutT*	This study
Plasmids		
pYG703	Plasmid pUC19, but has the *mutT* gene	This study
pYG704	The same as pYG703, but has *mutT*::Km^R	This study
pCP20	pCP20 has the yeast Flp recombinase gene, *FLP*, chloramphenicol and ampicillin resistance genes, and temperature-sensitive replication; Ap^R, Cm^R	CGSC

recipient. Similarly, YG6383 (ΔKFΔ*dinB*Δ*umuDC*), YG6384 (ΔKFΔ*polB*Δ*umuDC*) and YG6375 (ΔKFΔ*polB*Δ*dinB*Δ*umuDC*) were constructed by P1 transduction with YG6171 (Δ*dinB*Δ*umuDC*), YG6381 (Δ*polB*Δ*umuDC*, see below) and YG6382 (Δ*polB*Δ*dinB*Δ*umuDC*, see below) respectively.

To construct *polB*$^-$ derivative, *polB*::kanamycin resistance (Km^R) gene (*kan*) was transferred from JW0059 into a recipient AB1157, YG6171 (Δ*dinB*Δ*umuDC*; Salem *et al.*, 2009), YG6371 (ΔKFΔ*dinB*) or YG6168 (Δ*umuDC*; Salem *et al.*, 2009) by P1 transduction, and the Km^R colonies were selected. The Km^R strains were transformed with pCP20 [ampicillin resistance (Ap^R), Cm^R], which encodes a yeast FLP recombinase. The Cm^R transformants were selected at 30°C since the replication origin of the plasmid was temperature-sensitive. In the case of YG6371 (ΔKFΔ*dinB*), Ap^R transformants were selected. A few colonies were picked up and

streaked on a fresh plate, and then incubated at 43°C. An expression of the FLP was induced, which resulted in removal of the Km^R cassette whose both ends had recognition sites for the FLP recombinase. The high temperature also makes pCP20 cured from the cells. The obtained colonies were confirmed for their sensitivity to Cm (except for ΔKF strain), Ap and Km and also the size of the bands by PCR analysis (Table S2). The resultant strains were designated as YG6377 (Δ*polB*), YG6382 (Δ*polB*Δ*dinB*Δ*umuDC*), YG6379 (ΔKFΔ*polB*Δ*dinB*) and YG6381 (Δ*polB*Δ*umuDC*) respectively. They had no drug resistance maker for the *polB* deletion.

To construct *mutT*-deficient strains, we cloned the *mutT* gene from Kohara library (Kohara *et al.*, 1987) into pUC19, then designated as pYG703. Inserting 1.3 kb EcoRI cassette carrying the Km^R gene into EcoRI site of pYG703, the clone in which transcription direction of *mutT* is opposite to the *kan* gene was selected and named as pYG704. After digestion of pYG704 with KpnI, the linear fragment was isolated and introduced into strain V355, which lacks *recD*, to obtain the clone whose *mutT* was replaced with Δ*mutT*::*kan*. The resultant strain was designated as YG2004, which was used to transfer Δ*mutT*::*kan* to strain AB1157 and the pol-defective derivatives by P1 transduction. The *mutT*::*kan* derivative of AB1157 was designated as YG6156 (AB1157Δ*mutT*). Other host strains for the P1 transduction were YG6162 (Δ*dinB*; Salem *et al.*, 2009), YG6168 (Δ*umuDC*), YG6171 (Δ*dinB* Δ*umuDC*), YG6377 (Δ*polB*) and YG6382 (Δ*polB*Δ*dinB* Δ*umuDC*). The resultant strains were designated as YG6167 (Δ*dinB*Δ*mutT*), YG6174 (Δ*umuDC*Δ*mutT*), YG6172 (Δ*dinB* Δ*umuDC*Δ*mutT*), YG6378 (Δ*polB*Δ*mutT*), YG6385 (Δ*polB*Δ*dinB*Δ*umuDC*Δ*mutT*) respectively. In addition, the Δ*mutT*::*kan* allele was transferred from YG2004 to strains YG6343 (ΔKF), YG6371 (ΔKFΔ*dinB*), YG6383 (ΔKFΔ*dinB* Δ*umuDC*), YG6384 (ΔKFΔ*polB*Δ*umuDC*), YG6375 (ΔKF Δ*polB*Δ*dinB*Δ*umuDC*) and YG6379 (ΔKFΔ*polB*Δ*dinB*) at 30°C, and the resultant strains were designated as YG6347 (ΔKFΔ*mutT*), YG6372 (ΔKFΔ*dinB*Δ*mutT*), YG6386 (ΔKF Δ*dinB*Δ*umuDC*Δ*mutT*), YG6387 (ΔKFΔ*polB*Δ*umuDC*Δ*mutT*), YG6376 (ΔKFΔ*polB*Δ*dinB*Δ*umuDC*Δ*mutT*) and YG6380 (ΔKFΔ*polB*Δ*dinB*Δ*mutT*) respectively.

To confirm proper replacements in the chromosome, each constructed strain was subject to polymerase chain reactions with primers designed to display different amplification sizes when gene replacements successfully occur (Table S2).

Mutation assay

To determine mutation frequency, acquisition of resistance to rifampicin was used in fluctuation analysis. A fresh single colony was picked from LB agar and grown overnight in a tube at 37°C with aeration in LB medium and 10 tubes were prepared for each strain. The culture was diluted 10^6 times in fresh LB medium to achieve no mutants in one culture. The cultures were grown at 37°C with aeration (on a roller drum) to saturation (16 h), and 50 μl of aliquot for each tube was plated on a LB agar plate supplemented with 100 μg ml^{-1} rifampicin (Sigma, OH, USA). For viable cell count, three randomly selected cultures were serially diluted in cold saline and plated in LB agar without antibiotics. All plates were incubated at 37°C, except for the Klenow-deficient strains for which 30°C was used, for 24 h before counting colonies. We repeated the experiments three times for the determination of mutation frequency, which was calculated with median for 10 cultures divided by a mean value of viable cell count (Hasegawa *et al.*, 2008).

Primer extension assay

The enzyme reaction buffer containing 20 mM Tris-HCl (pH 7.5), 4% glycerol, 8 mM DTT, 80 μg ml^{-1} BSA, 2.5 mM ATP, 8 mM $MgCl_2$, 100 μM 8-oxo-dGTP, 1 nM pol III* was incubated with 0.1 μM 5′-Cy3-primer/template (sequences 1) at room temperature (20 to 25°C) for 1 min. Reactions were terminated by adding 10 μl of stop solution (98% formamide, 10 mM EDTA, 10 mg ml^{-1} Blue Dextran). Samples were denatured at 100°C for 10 min, then applied to 15% denaturing polyacrylamide gel for electrophoresis and the patterns were visualized by the Molecular Imager FX Pro System (Bio-Rad, CA, USA).

Kinetics analysis

For incorporation kinetics, 1 nM pol III*, 0.1 μM substrate (sequences 2) and 0.05–500 μM dNTPs were incubated at room temperature for 1 to 3 min in the reaction buffer written above. The reaction samples were subjected to 15% denaturing polyacrylamide gel. The gel band intensities were measured using the Molecular Imager FX Pro System and Quantity One software (Bio-Rad). The nucleotide incorporation efficiency opposite the target site was obtained by measuring I_T^{Σ}/I_{T-1}, where I_T^{Σ} represents the integrated gel band intensities of primers extended to the target site and beyond, and I_{T-1} is the integrated gel band intensity of primers extended to the site just prior to the target site (Creighton and Goodman, 1995; Bloom *et al.*, 1997). For each DNA substrate, the rate of incorporation was plotted as a function of dNTP concentration, and the relative V_{max} and apparent K_m values were determined by nonlinear regression fitting using the SigmaPlot software (SigmaPlot Software Sciences, IL, USA). The relative V_{max} value is equal to the maximum value of I_T^{Σ}/I_{T-1}. The frequency of incorporation (f_{inc}) was calculated using the equation $f_{inc} = (V_{max}/K_m)_{incorrect} / (V_{max}/K_m)_{correct}$. All values are means (± standard error) of three experiments.

Assay for excision of 8-oxo-G-ended primer

The set of the 19-mer primer/36-mer template was basically the same as sequences 1 (see above). It was as follows: 5′-CGCGCGAAGACCGGTTACX-3′ (19-mer primer, where X = G or 8-oxo-G) and 3′-GCGCGCTTCTGGCCAATGNCA GAATTCCTAGGGAAG-5′ (36-mer template, where N = A or C). The primer whose 3′-terminus is 8-oxo-G was purchased from Tsukuba Oligo Service (Tsukuba, Japan). For the assay for excision of 8-oxo-G-ended primer, these primer/templates (0.1 μM) were incubated with 1 nM pol III* at 25°C for 5, 10, 20 or 30 min in the reaction buffer described above. The products were analysed by denaturing polyacrylamide gel electrophoresis and visualized by the Molecular Imager. Percentage of the amount of digested primers compared with that of the original primers was calculated. For comparison, the primer/template DNA or the primer DNA alone was incu-

bated pol III* (1 nM) at room temperature, T7 pol (0.0001 unit μl^{-1}), pol I (KF) (0.001 unit μl^{-1}) or exo III (0.0001 unit μl^{-1}, New England BioLabs) at 37°C for 10 min in a reaction mixture for each enzyme without dNTP. The products were analysed as described above.

Assay for primer extension with β clamp

Primer extension with the β subunit was examined under two different conditions, i.e. standing start and running start experiments. In the standing start experiments, the primer was the same as that of sequences 2, but had extra five bases at the 3′-end, i.e. 5′-GTACCGCCACCCTTAGAACC GGTGTTTGGTAAAAX-3′ (35-mer, where X = T, G or 8-oxo-G). The template was the same as that of sequences 2 (100-mer), but had biotin/streptavidin at both ends (see Fig. S2). The primer/template DNA (20 nM), pol III* (10 nM) and four normal dNTPs (100 μM) were incubated for 1 min at 25°C. When the β subunit (10 or 100 nM) was included, the reaction mixture without dNTPs was pre-incubated for 10 min at 25°C and the reaction was started by addition of dNTPs. The reaction buffer and the methods to analyse the reaction products were the same as those described in the primer extension assay. In running start experiments, the primer (30-mer)/template (100-mer) DNA was the same as those of sequences 2, but the template DNA had A at the position of N and biotin/streptavidin at both ends. The primer/template DNA (20 nM), pol III* (10 nM) and dNTP(s) (100 μM) were incubated in the presence or the absence of the β subunit (100 nM) for 1 min at 25°C. In these experiments, four types of dNTP solution were used. Each contained dATP alone, dATP and 8-oxo-dGTP, dATP, 8-oxo-dGTP, dCTP and dGTP, or four normal dNTPs.

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Supporting information

Additional supporting information may be found in the online version of this article.

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Meeting report

2nd International Symposium on Genotoxic and Carcinogenic Thresholds

Takehiko Nohmi[1,3], Masamitsu Honma[1], Masami Yamada[1], Kenichi Masumura[1], Manabu Yasui[1], Katsuyoshi Horibata[1] and Shoji Fukushima[2]

[1]Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo, Japan
[2]Japan Bioassay Research Center, Japan Industrial Safety and Health Association, Kanagawa, Japan

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Since the disaster at the Fukushima No. 1 nuclear power plant in March 2011, people became concerned about adverse effects of radiation, in particular those of low dose radiation strongly. The effects of radiation on chromosome DNA are stochastic events, and thus it is thought that radiation poses cancer risk to humans even at very low doses. Likewise, genotoxic compounds, which interact with DNA and induce mutations, are assumed to have no thresholds for their action. These compounds are used to be called "radiomimetic compounds". Hence, genotoxic carcinogens, which induce cancer via genotoxic mechanisms such as mutations, are regulated based on a paradigm that they have no thresholds for the cancer risk. Recently, however, the paradigm has been challenged by research on analyzes of carcinogenicity and genotoxicity of chemicals at low doses. In addition, organisms including humans possess various self-defense mechanisms, such as detoxication metabolism, DNA repair, error-free translesion DNA synthesis and apoptosis etc, which may suppress genotoxicity of chemicals at low doses and reduce the mutation frequency and cancer risk to spontaneous levels. These self defense mechanisms may constitute "apparent" or "practical" thresholds for genotoxic carcinogens. To discuss the low dose effects of genotoxic and carcinogenic compounds and the implication in regulatory toxicology, the second international symposium on genotoxic and carcinogenic thresholds was held on November 23, 2011 in Tokyo. In this symposium, six and four experts of genotoxicity and chemical carcinogenicity were invited from inside and outside of Japan, respectively, to discuss genotoxicity and carcinogenicity of chemicals at low doses and the regulatory policies. This symposium follows the precedent symposia "International symposium—threshold of carcinogenicity and genotoxicity" in Kobe in Japan in 2006, and "the 1st International symposium on genotoxic and carcinogenic thresholds" in Tokyo in 2008. Here, we summarize the presentations of the symposium to discuss future perspectives of research on genotoxic and carcinogenic thresholds.

Session 1 (Chaired by Shoji Fukushima and Samuel M. Cohen)

Opening Address

Takehiko Nohmi (National Institute of Health Sciences)

Nohmi declared the opening of the symposium and introduced basic concepts of regulation for chemical carcinogens. Currently, carcinogens are classified into genotoxic and non-genotoxic carcinogens. Genotoxic carcinogens are those that induce cancer through interaction with DNA and induction of mutations. Typical examples are aflatoxin B1 and dimethylnitrosamine. These compounds are positive in Ames Salmonella mutation assays and have structural alerts, e.g., epoxides, aromatic amino groups or nitroso groups, which are characteristics of DNA-interacting agents. They induce tumors in multiple organs of trans-species of rodents. Non-genotoxic carcinogens induce cancer through mechanisms other than DNA interactions or mutations. The mechanisms include cytotoxicity, hormonal effects and inflammation. Non-genotoxic carcinogens usually induce tumors in single organs in single species of rodents. The classification of carcinogens into genotoxic or non-genotoxic ones is important in administrative regulation because genotoxic carcinogens are regulated based on the assumption that they have no thresholds for cancer risk. Therefore, no acceptable daily intake (ADI) can be set for genotoxic carcinogens. In contrast, non-genotoxic carcinogens are regulated as other toxic compounds that they have thresholds for the action. Non-genotoxic carcinogens can be used safety below the threshold dose while genotoxic carcinogens impose cancer risk to humans even at very low doses. Nohmi ques-

[3]Correspondence to: Takehiko Nohmi, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel: +81-3-3700-1564, Fax: +81-3-3700-1622, E-mail: nohmi@nihs.go.jp
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tioned the assumption because humans possess multiple defense mechanisms, e.g., detoxication, DNA repair, error-free translesion DNA synthesis and apoptosis, which may suppress the cancer risk to spontaneous levels and constitute "practical" thresholds for genotoxic carcinogens. He emphasized the importance of understanding the mechanisms underlying the practical thresholds to solve the issue of genotoxic and carcinogens thresholds.

1. Genotoxic Thresholds: Identification of Mutations *in vivo* and Mechanistic Studies *in vitro*

Takehiko Nohmi (National Institute of Health Sciences, Japan)

First, Nohmi reported characteristics of *gpt* delta transgenic mice and rats for *in vivo* gene mutation assays. These transgenic rodents allow to detect mutagenicity of chemicals in any organs of rodents such as liver, stomach and testis. In addition, mutations can be identified at sequence levels. The transgenic rodents have been established in genetic backgrounds of C57BL/6J mice, Sprague-Dawley rats and Fischer 344 rats. At present, Ames Salmonella mutation assay is the most widely employed mutation assay and Ames positive compounds are interpreted as mutagenic compounds. However, there are cases where the results of Ames assay are inconsistent with those of cancer bioassays with rodents. Nohmi proposed that *in vivo* mutation assays with *gpt* delta transgenic mice may provide more reliable information whether the chemical is genotoxic or not because the *in vivo* assays can detect mutations in target organs of carcinogens. Second, Nohmi reported novel human cell lines expressing genetically modified specialized DNA polymerases, i.e., DNA polymerases ζ and κ, and showed results suggesting that error-free translesion DNA synthesis catalyzed by the polymerases might be a factor influencing the practical thresholds for genotoxicity of chemicals. He also introduced an endeavor to introduce single DNA adduct, i.e., 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), into a specific site of human chromosome. The results suggest that not all 8-oxo-dG formed in the chromosome induces mutations in human cells.

2. Threshold of Genotoxic Carcinogens: It Is Central Concerns of Carcinogenic Risk Assessment

Shoji Fukushima (Japan Bioassay Research Center, Japan)

Dr. Fukushima reported low-dose carcinogenicity data based on medium-term rat liver bioassays for three genotoxic hepatocarcinogens. Although administration of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) generated DNA-MeIQx adducts even at very low doses, higher doses are required to elevate levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) and frequencies of gene mutations. Induction of glutathione *S*-transferase placental form (GST-P) positive foci, a well-known preneoplastic lesion in rat hepatocarcinogenesis, was observed only at the highest dose. Similarly, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) caused an increase in the number of GST-P positive foci only at high doses. Induction of $p21^{Cip/WAF1}$ was observed at low doses where no IQ-mediated carcinogenic effects were observed. *N*-nitrosodiethylamine (NDEA) did not induce either GST-P positive foci or gene mutation at low doses. Concurrent administration of MeIQx and NDEA at no-effect doses did not induce GST-P positive foci. The no-effect doses for the biomarkers involved in the initiation and promotion stages of carcinogenesis suggest the existence of a threshold, at least a practical one, for the carcinogenic effects of these genotoxic carcinogens in the rat.

3. Lessons Learned from 40,000-animal Cancer Dose-response Studies

George S. Bailey (Oregon State University, USA)

Dr. Bailey conducted two 40,000-animal cancer dose-response studies. These experiments used rainbow trout, an animal model well suited to ultra low-dose carcinogenesis research, to explore dose-response down to a targeted 10 excess liver tumors per 10,000 animals (ED_{001}*). In Study 1, 42,000 trout were fed 0–225 ppm dibenzo(*def*, *p*)chrysene (DBC) for four weeks, sampled for biomarker analyses, and returned to control diet for nine months prior to gross and histologic examination. Suspect tumors were confirmed by pathology, and resulting incidences were modeled and compared to the default EPA LED_{10}** linear extrapolation method. Among nine statistical models explored, three were determined to fit the liver data well-linear probit, quadratic logit, and Ryzin-Rai. Two-order extrapolations below the modeled tumor data predicted DBC doses producing one excess cancer per million individuals ($ED_{10^{-6}}$) that were 500–1500-fold higher than that predicted by the five-order LED_{10} extrapolation. In Study 2, aflatoxin B1 (AFB1) was tested by using a similar design of Study 1 instead of DBC. Inclusion of all fish also yielded a sub-linear dose-response, with slope 1.31 (95%CI 1.13–1.50), and an extrapolated $ED_{10^{-6}}$ 17-fold greater than the LED_{10} default extrapolation. He concluded that two genotoxins with differing biological properties yielded ultra-low dose-response curves in the same animal model that are not compatible with the linear default as-

*ED_{001}: the effective dose of carcinogen that induces 10 excess tumors in 10,000 animals.

**LED_{10}: a lower confidence limit on dose estimate of carcinogen that achieves 10% incidence of tumors.

sumption.

4. Urinary Bladder Carcinogenesis by DNA Reactive and Non-Reactive Chemicals: Non-linearity's and Thresholds

Samuel M. Cohen (University of Nebraska Medical Center, USA)

Dr. Cohen categorized chemicals which can increase cancer risk into two groups: one can directly damage DNA and another can increase cell proliferation. He introduced that both types of chemicals have been shown to induce urinary bladder cancer in animal models and in humans. In animal models, cytotoxicity can be produced by formation of urinary solids or generation of reactive metabolites which are excreted and concentrated in the urine. In humans, arsenic is an example of a bladder carcinogen which acts by formation of reactive metabolites. The threshold is dependent on the presence in the urine of a cytotoxic concentration of the metabolite(s). The DNA reactive effect operates through formation of DNA adducts, and the dose response can be linear or non-linear, depending on metabolic activation processes. In contrast, for non-DNA reactive carcinogens, a threshold is present. Increased cell proliferation can occur either by cytotoxicity and regeneration, by direct mitogenesis, or by decreasing cell death. Dr. Cohen concluded that DNA reactive carcinogens have a non-linear dose response with respect to carcinogenicity and frequently have non-linear responses for DNA effects due to competing metabolic and repair processes. In contrast, non-DNA reactive carcinogens induce cancer as a consequence of a precursor toxic biologic effects which have thresholds.

Session 2 (Chaired by Teruhisa Tsuzuki and Elmar Gocke)

5. A Threshold for the Murine T-cell Lymphoma Induction by *N*-ethyl-*N*-nitrosourea and/or Radiation

Shizuko Kakinuma (National Institute of Radiological Sciences, Japan)

Dr. Kakinuma reported that current strategy for estimating the risk of genotoxic substances at low dose is a linear extrapolation based on the observed effects at high dose. It is generally considered that genotoxic carcinogens have no thresholds in exerting their potential for cancer induction. Here, she introduced her group's recent animal studies, which exhibited the existence of a threshold for carcinogenicities. They observed a threshold for both the *N*-ethyl-*N*-nitrosourea (ENU) and fractionated X-rays in induction of T-cell lymphoma in B6C3F1 mice. In addition, they found that ENU significantly increased point mutation frequency in a dose dependent manner, even below threshold dose for lymphomagenesis. Interestingly, co-exposure with sub-carcinogenic dose of X rays decreased incidence of ENU-induced lympomas, suggesting that threshold of ENU mutagenesis could be influenced by X-ray co-irradiation. Dr. Kakinuma concluded that threshold of ENU for lymphomagenesis is determined by the condition of thymic cells to be initiated and progressed into malignancy.

6. Exposure to Ethylating Agents: Where Do the Thresholds for Mutagenic/Clastogenic Effects Arise?

Elmar Gocke (Hoffmann-La Roche Ltd, Switzerland)

Dr. Gocke reported on the consequences of an accident in the production of tablets of a HIV medication (Viracept) leading to the presence of a low amount of EMS (ethyl methanesulfonate) as genotoxic contaminant. For alkylating agents like EMS it is generally assumed that the dose response for mutagenicity is linear—indicating that no 'safe' dose does exist. The ingestion of the genotoxic contaminant, thus, may confer some genotoxic/carcinogenic risk to the patients. This issue triggered non-clinical studies where the dose response for mutation was analyzed after chronic dosing of mice with EMS. His group's studies with dose levels ranging from 1.25 to 260 mg/kg/day up to 28 days provided evidence that daily doses of up to 25 mg/kg/day did not induce any increase of mutations in the *lacZ* gene in the four organs tested (bone marrow, liver, GI tract, liver) or of micronuclei in bone marrow. Their investigations unambiguously demonstrated thresholded dose relations for mutagenic/clastogenic effects by EMS. The evidence was accepted by the authorities as sufficient to show that the exposure to the contaminated Viracept tablets posed no risk to the patients. These findings have important implications for the risk assessment of low dose exposures to genotoxic agents, and should impact on impending new regulation, e.g., on the limitation of PGI's (potentially genotoxic impurities) in pharmaceuticals.

7. Oxidative Stress-induced Tumorigenesis in the Small Intestine of *Mutyh*-deficient Mice: the Effect of Low-level Exposure to $KBrO_3$

Teruhisa Tsuzuki (Kyushu University, Japan)

MUTYH is a DNA glycosylase that excises adenine or 2-hydroxyadenine (2-OH-A) incorporated opposite either 8-dihydroguanine (8-oxoG) or guanine, respectively, thus considered to prevent G:C to T:A transversions in mammalian cells. Dr. Tsuzuki reported *Mutyh*-deficient mice showed a marked predisposition to spontaneous tumorigenesis in various tissues including intestines when examined at 18 months of age. The incidence of adenoma/carcinoma in the intestine significantly in-

creased in *Mutyh*-deficient mice, as compared with wild-type mice. He also showed that the intestinal tumor susceptibility of *Mutyh*-deficient mice was further enhanced by treatment with $KBrO_3$, a known oxidative renal carcinogen associated with 8-oxo-G accumulations. Oral administration of $KBrO_3$ at a dose of 0.2% in drinking water dramatically increased the formation of intestinal tumors in *Mutyh*-deficient mice. Additionally, he reported a tumor-formation at a dose of 0.1% in the small intestines of *Mutyh*-deficient mice, but no tumor-formation was observed at a 0.05%. Based on these findings, he concluded that cells are able to correctly repair oxidative DNA lesions resulting from exposures to a certain level of low doses of endogenous and exogenous chemicals with oxidizing property, and thus are less likely to be transformed to the neoplastic phenotype.

8. How Do Thresholds for Mutagenicity and Clastogenicity Arise for DNA Damaging Agents?

George E. Johnson (Swansea University, U.K.)

Dr. Johnson introduced prospective observations about thresholds for mutagenicity and clastogenicity arise for DNA damaging agents. There has been a recent shift by the scientific and regulatory community, towards accepting genotoxic thresholds. Nevertheless, there are still many unanswered questions and the mechanisms responsible for 'genotoxic tolerance' at low doses are wide ranging but poorly understood. For DNA reactive genotoxins, non-linear dose responses can arise from many different biological mechanisms. These include lack of bioavailability and nuclear exclusion, detoxification/activation, DNA repair and other homeostatic defense enzymes. He showed the results about the roles of DNA repair in genotoxic thresholds for alkylating agents and pro-oxidant chemicals. Specific DNA repair enzymes have been shown to be up-regulated by low dose alkylating agents, and knocking down specific DNA repair enzymes *in vitro* alters the shape of the dose response, e.g., to EMS. Conversely, for pro-oxidants, he exhibited that antioxidant defenses and specifically the presence of glutathione, are perhaps more important in genotoxic tolerance at low doses of pro-oxidants. Other mechanisms that impact on the dose response are linked to secondary effects such as dose fractionation and metabolic activity. Based on these observations, he concluded that ascertainment of mechanism is essential before genotoxic thresholds can be accepted.

Session 3 (Chaired by Yasunobu Aoki and George S. Bailey)

9. Health Risk Assessment of Air Pollutants: Air Pollutant Genotoxicity and Its Enhancement on Suppression of Phase II Drug-metabolizing Enzymes

Yasunobu Aoki (National Institute for Environmental Studies, Japan)

In health risk assessment of environmental contaminates, such as air pollutants, carcinogenic chemicals are generally categorized according to whether or not they are genotoxic. The potency of genotoxicity is determined by not only the reactivity of the chemical to DNA but also the protective system against chemical toxicity. This protective system is governed by processes such as phase I and II drug-metabolism, excretion, and DNA repair. Dr. Aoki's research focused on phase II drug-metabolizing enzymes, whose constitutive and inducible gene expression is regulated by the essential transcription factor Nrf2. The genotoxic potency of air pollutants under Nrf2-deficient conditions was examined by using diesel exhaust (DE) and benzo[*a*]pyrene (BaP) as model pollutants. After exposing mice to DE for 4 weeks, the levels of bulky-DNA adduct and 8-OHdG in the lungs of Nrf2-knockout (KO) mice were higher than those of Nrf2-bearing control mice. Intratracheal administration of BaP elevated the *in vivo* mutation frequency (MF) in the lungs of both Nrf2-KO and Nrf2-bearing control mice, but the increase in MF induced by BaP was enhanced in Nrf2-KO mice. These results indicate that the level of phase II-drug metabolizing enzymes is a determinant of the genotoxic potency of air pollutants such as DE and BaP.

10. Toxicity Testing Strategy Based on the Concept of the Threshold of Toxicological Concern (TTC)

Akihiko Hirose (National Institute of Health Sciences, Japan)

Dr. Hirose introduced the concept of Threshold of Toxicological Concern (TTC). TTC is an exposure level below which there would be no appreciable risk to human health. The concept is practically useful to evaluate risk of a large number of chemicals such as those migrated from plastics of food containers, packaging and apparatus where only insufficient toxicity data are available. Regulatory authorities, i.e., U.S. Food and Drug Administration (FDA) and European Food Safety Authority (EFSA), and several industrial associations in Japan developed the guidelines for assessment of potential risk of food-contact materials based on the concept of TTC and the migration levels. In the guidelines, the lowest threshold level of exposure is 0.5 ppb (=1.5 μg/person/day). No safety tests are required if the ex-

posure levels are below it. This is based on the assumption that even genotoxic compounds have no adverse effects on humans below the level. The threshold value of 0.5 ppb is deduced from the carcinogenic potency database. Above 0.5 ppb but below 50 ppb, only *in vitro* genotoxicity tests are required. The level of 50 ppb is considered to be a threshold for non-genotoxic substances. Above 50 ppb but below 1 ppm, an *in vivo* genotoxicity test and two subchronic toxicity tests are additionally requested. However, scientific bases for the higher thresholds of 50 ppb or 1 ppm are unclear. Therefore, Dr. Hirose is replacing the threshold values of 50 ppb and 1 ppm with exposure levels below which no toxicity is actually observable. The concept of TTC has been expanded for general toxicity endpoints (Kroes *et al.* 2000, 2004) other than carcinogenicity. The concept may be helpful for establishment of thresholds for various toxicity indexes.

Closing Remarks

Shoji Fukushima (Japan Bioassay Research Center, Japan)

It has been accepted that no threshold for the genotoxic carcinogens exits in the risk assessment and management. However, this conclusion is based not on the scientifically obtained data but on the hypothetical presumption. Therefore, to solve this problem, and to investigate whether the threshold for the effects of genotoxic carcinogens exists or not, the accumulation of the scientific data is very important. This is the 2nd International Symposium on Genotoxic and Carcinogenic Thresholds organized under the leadership of Dr. Nohmi. The aim of this Symposium, in line with the previous one, is the meeting of genotoxicity, carcinogenicity and other field researchers, who do not usually contact, and make them to dispute, thus to come closer to the solution of the problem concerning the threshold. Just I want to ask you, what is your opinion on the dispute at the Symposium? I feel that the contour of the top of mountain covered with very heavy clouds is now become clearer seen. However, not completely, therefore it is still necessary to coordinate our efforts to make it more and more clear. The future suggestions from the participants of the Symposium on the topic are very welcomed. Please do not hesitate to contact and support this research field for right resolution. At last, I would like to say my gratitude to the staff from the Department of Dr. Nohmi for the wonderful organization of the Symposium.

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Review

in vivo Approaches to Identify Mutations and *in vitro* Research to Reveal Underlying Mechanisms of Genotoxic Thresholds

Takehiko Nohmi[1,2], Masami Yamada and Kenichi Masumura

Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo, Japan

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In regulatory toxicology, it is assumed that genotoxic carcinogens, which induce cancer through genotoxic mechanisms, have no threshold for their action. However, humans possess a number of defense mechanisms against DNA damaging agents, which may reduce the genotoxic and cancer risk at low doses to the spontaneous levels. The defense mechanisms may constitute practical thresholds for genotoxic carcinogens. In fact, accumulating evidence with rodent carcinogenicity and genotoxicity assays suggest that some genotoxic compounds clearly exhibit threshold-like dose responses *in vivo*. These results challenge the paradigm that cancer risk induced by genotoxic compounds at high doses can be linearly extrapolated into low doses where people are exposed in daily life (linear non-threshold model). Here, we discuss two issues regarding the practical thresholds for genotoxic carcinogens. The first issue is how to define "genotoxicity" of chemicals. There are a number of genotoxicity assays *in vitro* and *in vivo*. Therefore, it is unclear what genotoxicity assay(s) should be employed to define whether the compound is genotoxic or not. The second issue is possible mechanisms underlying the practical thresholds. In particular, we emphasize the importance of DNA repair and translesion DNA synthesis as the underlying mechanisms of the practical thresholds. Finally, we discuss issues associated with low dose exposure to genotoxic carcinogens, i.e., risk assessment of exposure to multiple genotoxic chemicals.

Key words: practical thresholds, genotoxic carcinogens, non-genotoxic carcinogens, DNA repair, translesion DNA synthesis

Introduction

Humans are exposed to a variety of chemicals that may induce damage in DNA. Although the damage may be repaired by multiple defense mechanisms against DNA damaging agents before DNA replication occurs, DNA that possesses modified bases or missing bases may be used as template for DNA replication. Such damaged DNA replication results in genetic alterations such as mutations and chromosome aberrations (1). It is widely accepted that cancer is a result of multiple genetic alterations in important genes such as those involved in maintenance of genome integrity, e.g., *p53*, or cell proliferation, e.g., *ras* (2–5). Therefore, risk assessment of genotoxicity of chemicals is critically important to protect humans from environmental carcinogens. In 1980s, a large number of chemicals have been examined for the carcinogenicity and genotoxicity at the National Toxicology Program in the United States (6). These chemicals are commercially important and may represent significant environmental and occupational hazards by virtue of their high volume of production and release to the environment. Although most of rodent carcinogens were expected to be positive in the bacterial mutation assay (Ames assay) and have structural alert to interact with DNA, about 40% of rodent carcinogens were negative in the genotoxicity assays and had no structural alerts to interact with DNA (6,7). Therefore, the term "non-genotoxic carcinogens" was coined to define the carcinogens that do not exhibit genotoxicity and have no structural alerts to interact with DNA (8–10). Carcinogens that were positive in genotoxicity assays were referred to as "genotoxic carcinogens". The former included carcinogens that may promote cancer via cell toxicity, cell proliferation, epigenetics or hormonal effects (9). Because these "non-genotoxic carcinogens" are neither supposed to interact with DNA nor induce mutations, they were expected to possess "thresholds" for their action as other toxic agents (8). No-observed-adverse-effect levels (NOAEL)

[1]Correspondence to: Takehiko Nohmi, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel: +81-3-3700-1564, Fax: +81-3-3700-1622, E-mail: nohmi@nihs.go.jp

[2]Present address: Department of Research and Development Promotion, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan. Tel: +81-72-641-9803, Fax: +81-72-641-9830, E-mail: tnoumi@nibio.go.jp

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can be set by chronic rodent bioassays and acceptable daily intake (ADI) is calculated for the non-genotoxic carcinogens (11). If the dose used in daily life is below the ADI, non-genotoxic carcinogens may be allowed to be used in the society. In contrast, genotoxic carcinogens are believed to have no thresholds because mutation is a stochastic event and even single molecules may induce mutations and cancer in humans (12). Hence, if candidate chemicals for pesticides, food additives or veterinary medicines are carcinogenic in rodents and genotoxicity is involved in the cancer mechanisms, no ADI can be set and the chemicals are not allowed to go to the markets. As shown in this special issue and other publications, however, the paradigm that genotoxic carcinogens have no thresholds has been challenged by experimentally (13). In fact, it seems plausible that at least some genotoxic carcinogens have "practical threshold" for their action (14–16). The term "practical thresholds" is defined as the doses below which no mutations are detected (12,17). In addition, self defense mechanisms such as detoxication and DNA repair may constitute the practical thresholds for genotoxicity (18).

In this report, we first discuss how we should identify genotoxicity of chemicals. As written in the next section, there are a number of genotoxicity assays *in vitro* and *in vivo*. The endpoints are diverged such as DNA damage, point mutations or chromosome aberrations. Ames test is the most widely accepted indicator for genotoxicity but has also some limitations, e.g., prokaryotes versus eukaryotes. We discuss the importance of *in vivo* gene mutations assays in rats and mice in terms of examination of possible involvement of genotoxicity in carcinogenesis in rodents (19). We then discuss possible mechanisms underlying genotoxic thresholds. In particular, we focus on DNA repair and translesion DNA synthesis (TLS) (20). Finally, we discuss other issues related to risk of genotoxic carcinogens at low doses where humans are actually exposed to the chemicals in daily life.

Pivotal Roles of *in vivo* Transgenic Gene Mutation Assays to Make a Distinction between Genotoxic and Non-genotoxic Carcinogens

Currently, there are more than 100 genotoxicity assays so far published. The organisms used for the assays include phage, bacteria, yeast, plants, insects, fish, fungi and rodents so on. In addition, cultured mammalian or human cells are frequently used for the assays. Therefore, it is not uncommon that one chemical exhibits different test results depending on the assays used, e.g., positive in one assay but negative in another assay. Of the various genotoxicity assays, bacterial mutation assays with *Salmonella typhimurium* and *Escherichia coli*, either chromosome aberration assays or gene mutation assays with cultured mammalian cells, and rodent micronucleus or chromosome aberration assays are regarded as a standard battery of genotoxicity assays (21). However, it is still not easy to define whether the chemical is genotoxic or not because each assay has its own merits and limitations to evaluate the genotoxic effects in human genome. The bacterial mutation assays could indicate the chemical or its metabolites have potential to interact with DNA and induce mutations or not. However, the prokaryotes lack the metabolism of mammals such as P450 drug metabolizing enzymes (22). The *in vitro* metabolism by S9 enzymes prepared from drug-induced rat liver does not necessarily represent the *in vivo* metabolism. Urethane and procarbazine are such examples (23,24). They are negative in Ames test but are positive in *in vivo* genotoxicity assays. Cultured mammalian genotoxicity assays have merits to detect chemicals that interact with proteins essential for chromosome segregation such as tubulines or topoisomerases (21). However, chromosome aberration assays *in vitro* give high percentage of false positive results partly because the aberrations are often induced associated with cellular toxicity (25). Rodent micronucleus assays could identify genotoxic agents *in vivo* and have low percentage of false positives. Nevertheless, the target organs for the micronucleus assays are bone marrow or peripheral blood cells. Thus, negative in the assays does not mean that the chemical dose not induce mutations in other organs such as liver. Diethylnitrosamine and 2,4-diaminotolune are such examples. These chemicals are negative in mouse micronucleus assays but are positive in gene mutation assays in the liver. In addition, they are hepatocarcinogens in rodents. A possible reason for the negative results in the micronucleus assays in the bone marrow is that the active metabolites generated in the liver are short lived and do not reach the bone marrow (26,27). Genotoxicity assays *in vivo* such as micronucleus assays are usually conducted in mice while two-year cancer bioassays are conducted in rats mainly. In fact, rats and mice are not the same and exhibit different sensitivity to a number of carcinogens. The most represent example is aflatoxin B1, which induces liver cancer in rats and humans but not in mice (28,29). Therefore, it is desirable to examine the genotoxicity in target organs for carcinogenicity in rodents used for two-year cancer bioassays.

Transgenic rodent mutation assays are *in vivo* genotoxicity assays that detect mutations in any organs of rodents. Transgenic mutation assays with *gpt* delta rats and mice are one of them and unlike other transgenic tests they allow to identify point mutations such as base substitutions and frameshift and deletion mutations by *gpt* selection and Spi^- selection, respectively (19). Initially, *gpt* delta mice have been established by microinjection of lambda EG10 DNA carrying reporter genes