18. Ogi, T., Shinkai, Y., Tanaka, K., and Ohmori, H. (2002) Polκ protects mammalian cells against the lethal and mutagenic effects of benzo[a] pyrene. *Proc. Natl. Acad. Sci. U.S.A. 99*, 15548–15553.
19. Suzuki, N., Ohashi, E., Kolbanovskiy, A., Geacintov, N. E., Grollman, A. P., Ohmori, H., and Shibutani, S. (2002) Translesion synthesis by human DNA polymerase κ on a DNA template containing a single stereoisomer of dG-(+)- or dG-(−)-*anti*-N^2-BPDE (7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene). *Biochemistry 41*, 6100–6106.
20. Zhang, Y., Yuan, F., Wu, X., Wang, M., Rechkoblit, O., Taylor, J. S., Geacintov, N. E., and Wang, Z. (2000) Error-free and error-prone lesion bypass by human DNA polymerase κ *in vitro*. *Nucleic Acids Res. 28*, 4138–4146.
21. Rechkoblit, O., Zhang, Y., Guo, D., Wang, Z., Amin, S., Krzeminsky, J., Louneva, N., and Geacintov, N. E. (2002) trans-Lesion synthesis past bulky benzo[a]pyrene diol epoxide N2-dG and N6-dA lesions catalyzed by DNA bypass polymerases. *J. Biol. Chem. 277*, 30488–30494.
22. Choi, J. Y., Angel, K. C., and Guengerich, F. P. (2006) Translesion synthesis across bulky N^2-alkyl guanine DNA adducts by human DNA polymerase κ. *J. Biol. Chem. 281*, 21062–21072.
23. Huang, X., Kolbanovskiy, A., Wu, X., Zhang, Y., Wang, Z., Zhuang, P., Amin, S., and Geacintov, N. E. (2003) Effects of base sequence context on translesion synthesis past a bulky (+)-*trans*-*anti*-B[*a*]P-N^2-dG lesion catalyzed by the Y-family polymerase pol κ. *Biochemistry 42*, 2456–2466.
24. Fischhaber, P. L., Gerlach, V. L., Feaver, W. J., Hatahet, Z., Wallace, S. S., and Friedberg, E. C. (2002) Human DNA polymerase κ bypasses and extends beyond thymine glycols during translesion synthesis in vitro, preferentially incorporating correct nucleotides. *J. Biol. Chem. 277*, 37604–37611.
25. Washington, M. T., Johnson, R. E., Prakash, L., and Prakash, S. (2002) Human DINB1-encoded DNA polymerase κ is a promiscuous extender of mispaired primer termini. *Proc. Natl. Acad. Sci. U.S.A. 99*, 1910–1914.
26. Lone, S., Townson, S. A., Uljon, S. N., Johnson, R. E., Brahma, A., Nair, D. T., Prakash, S., Prakash, L., and Aggarwal, A. K. (2007) Human DNA polymerase κ encircles DNA: Implications for mismatch extension and lesion bypass. *Mol. Cell 25*, 601–614.
27. Zhou, B. L., Pata, J. D., and Steitz, T. A. (2001) Crystal structure of a DinB lesion bypass DNA polymerase catalytic fragment reveals a classic polymerase catalytic domain. *Mol. Cell 8*, 427–437.
28. Ling, H., Boudsocq, F., Woodgate, R., and Yang, W. (2001) Crystal structure of a Y-family DNA polymerase in action: A mechanism for error-prone and lesion-bypass replication. *Cell 107*, 91–102.
29. Shimizu, M., Gruz, P., Kamiya, H., Kim, S. R., Pisani, F. M., Masutani, C., Kanke, Y., Harashima, H., Hanaoka, F., and Nohmi, T. (2003) Erroneous incorporation of oxidized DNA precursors by Y-family DNA polymerases. *EMBO Rep. 4*, 269–273.
30. Etchegaray, J. P., and Inouye, M. (1999) Translational enhancement by an element downstream of the initiation codon in *Escherichia coli*. *J. Biol. Chem. 274*, 10079–10085.
31. Johnson, F., Bonala, R., Tawde, D., Torres, M. C., and Iden, C. R. (2002) Efficient synthesis of the benzo[a]pyrene metabolic adducts of 2′-deoxyguanosine and 2′-deoxyadenosine and their direct incorporation into DNA. *Chem. Res. Toxicol. 15*, 1489–1494.
32. Zhang, Y., Wu, X., Guo, D., Rechkoblit, O., and Wang, Z. (2002) Activities of human DNA polymerase κ in response to the major benzo[a]pyrene DNA adduct: Error-free lesion bypass and extension synthesis from opposite the lesion. *DNA Repair 1*, 559–569.
33. Carlson, K. D., Johnson, R. E., Prakash, L., Prakash, S., and Washington, M. T. (2006) Human DNA polymerase κ forms nonproductive complexes with matched primer termini but not with mismatched primer termini. *Proc. Natl. Acad. Sci. U.S.A. 103*, 15776–15781.
34. Niimi, A., Limsirichaikul, S., Yoshida, S., Iwai, S., Masutani, C., Hanaoka, F., Kool, E. T., Nishiyama, Y., and Suzuki, M. (2004) Palm mutants in DNA polymerases α and η alter DNA replication fidelity and translesion activity. *Mol. Cell. Biol. 24*, 2734–2746.
35. Sakamoto, A. N., Stone, J. E., Kissling, G. E., McCulloch, S. D., Pavlov, Y. I., and Kunkel, T. A. (2007) Mutator alleles of yeast DNA polymerase ζ. *DNA Repair 6*, 1829–1838.
36. Kusumoto, R., Masutani, C., Shimmyo, S., Iwai, S., and Hanaoka, F. (2004) DNA binding properties of human DNA polymerase η: Implications for fidelity and polymerase switching of translesion synthesis. *Genes Cells 9*, 1139–1150.
37. Suzuki, N., Itoh, S., Poon, K., Masutani, C., Hanaoka, F., Ohmori, H., Yoshizawa, I., and Shibutani, S. (2004) Translesion synthesis past estrogen-derived DNA adducts by human DNA polymerases η and κ. *Biochemistry 43*, 6304–6311.
38. Kobayashi, S., Valentine, M. R., Pham, P., O'Donnell, M., and Goodman, M. F. (2002) Fidelity of *Escherichia coli* DNA polymerase IV. Preferential generation of small deletion mutations by dNTP-stabilized misalignment. *J. Biol. Chem. 277*, 34198–34207.

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Role of Parp-1 in suppressing spontaneous deletion mutation in the liver and brain of mice at adolescence and advanced age

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ABSTRACT

Poly(ADP-ribose) polymerase-1 knockout (*Parp-1*$^{-/-}$) mice show increased frequency of spontaneous liver tumors compared to wild-type mice after aging. To understand the impact of *Parp-1* deficiency on mutations during aging, in this study, we analyzed spontaneous mutations in *Parp-1*$^{-/-}$ aged mice. *Parp-1*$^{-/-}$ mice showed tendencies of higher mutation frequencies of the *red/gam* genes at 18 months of age, compared to *Parp-1*$^{+/+}$ mice, in the liver and brain. Complex-type deletions, accompanying small insertion were observed only in *Parp-1*$^{-/-}$ mice in the liver and brain. Further analysis in the liver showed that the frequency of single base deletion mutations at non-repeat or short repeat sequences was 5.8-fold higher in *Parp-1*$^{-/-}$ than in *Parp-1*$^{+/+}$ mice ($p < 0.05$). A 3.2-fold higher tendency of the deletion frequency of two bases or more was observed in *Parp-1*$^{-/-}$ mice compared to *Parp-1*$^{+/+}$ mice ($p = 0.084$). These results support the model that *Parp-1* is involved in suppressing imprecise repair of endogenous DNA damage leading to deletion mutation during aging. The mutation frequencies of the *gpt* gene in the brain were found to be 3-fold lower in *Parp-1*$^{-/-}$ than in *Parp-1*$^{+/+}$ mice at 4 months of age ($p < 0.01$), implying that Parp-1 may be positively involved in imprecise DNA repair in the brain. On the other hand, the frequencies of *gpt* mutation showed an increase at 18 months of age in the *Parp-1*$^{-/-}$ ($p < 0.05$) but not in *Parp-1*$^{+/+}$ brains, suggesting that *Parp-1* deficiency causes an increase of point mutations in the brain by aging.

1. Introduction

Poly(ADP-ribose) polymerase-1 (Parp-1) facilitates DNA strand break repair by binding to the end of DNA strand breaks and catalyzing transfer of ADP-ribose residues from NAD to itself and other nuclear proteins, including XRCC1 (X-ray cross-complementing factor 1) [1], WRN (Werner's syndrome protein) [2,3] and Ku70/80 [4,5]. PolyADP-ribosylation results in recruitment of DNA repair proteins to DNA damage sites [6,7]. Accumulating studies have indicated that Parp-1 is involved in base excision repair (BER) and single strand break (SSB) repair by interacting with XRCC1 through poly(ADP-ribose) residues, as well as DNA polymerase β [8] and DNA ligase IIIα [9] using the BRCT domain in Parp-1. We previously demonstrated that *Parp-1*$^{-/-}$ mice show higher susceptibility to carcinogenesis induced by alkylating agents such as *N*-nitrosobis(2-hydroxypropyl)amine (BHP) [10] and azoxymethane [11] but not with 4-nitroquinoline 1-oxide [12]. *Parp-1*$^{-/-}$ mice develop normally, and spontaneous tumor incidences in all organs are not elevated at least until 9 months old [11]. However, the incidences of hepatocellular adenomas and carcinomas in *Parp-1*$^{-/-}$ mice are increased at 18–24 months old compared to *Parp-1*$^{+/+}$ mice [13]. *Parp-1*$^{-/-}$*p53*$^{-/-}$ mice also show spontaneous medulloblastomas in *p53* knockout (*p53*$^{-/-}$) mice at a higher incidence compared to *Parp-1*$^{+/+}$*p53*$^{-/-}$ mice [14,15].

In wild-type mice, age-related increases of mutant frequencies are observed in the liver, spleen, heart and small intestine, whereas mutant frequencies in the brain and germ cells are only slightly increased [16–18]. Age-related increases in genome rearrangement as well as point mutations are reported in the liver but not observed in the brain [19]. Therefore, the effects of aging on spontaneous mutation frequency might be different among tissues.

To analyze the impact of aging on spontaneous mutant frequency and its spectra in *Parp-1*$^{-/-}$ mice, we performed mutation analysis in *Parp-1*$^{-/-}$ mice at advanced age using progeny of

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intercross with *gpt* delta transgenic mice harboring about 80 copies of tandemly integrated lambda EG10 DNA as a transgene [20,21]. The rescued phage was analyzed by the Spi$^-$ (sensitive to P2 interference) assay, which preferentially detects deletion mutations in the *red/gam* genes. The deletion mutations of a single base to approximately 10 kb or several copies of EG10 DNA could be detected. The *gpt* assay detects point mutations in the guanine phosphoribosyl transferase (*gpt*) gene. The spontaneous mutant frequency of the *gpt* gene in the liver of mice is around $2–6 \times 10^{-6}$ [23] in tissues including the liver and brain [24]. The frequency of mutation in the *red/gam* genes in the liver of mice is also reported to be around $1–5 \times 10^{-6}$ [23,24].

Analysis of deletion mutation with a Spi$^-$ assay using *gpt* delta transgenic mice has been shown to be useful in detecting deletion mutations after treatment with various types of chemicals or irradiation with γ-rays or heavy ions [23,25–26].

The results in this study suggest that Parp-1 suppresses spontaneous deletion mutations, especially at non-repeat or short repeat sequences in the liver and brain during aging. Complex-type deletions accompanying small insertion and microhomology at deletion junctions observed in *Parp-1*$^{-/-}$ livers and brains are also discussed. Additionally, we observed that the mutant frequencies of the *gpt* gene in the brains were found to be 3-fold lower in *Parp-1*$^{-/-}$ than in *Parp-1*$^{+/+}$ mice at 4 months of age but increased in *Parp-1*$^{-/-}$ mice to the level of *Parp-1*$^{+/+}$ mice at 18 months of age.

2. Materials and methods

2.1. Genomic DNA extraction and rescue of the transgene

Parp-1$^{-/-}$/*gpt* delta and *Parp-1*$^{+/+}$/*gpt* delta animals were previously established by intercrossing *Parp-1*$^{+/-}$/*gpt* delta mice [20]. The mice possess mixed genetic background of C57BL/6, ICR and 129Sv. Male *Parp-1*$^{-/-}$ and *Parp-1*$^{+/+}$ mice were fed a basal diet (CE-2, Clea Japan), and these mice were anaesthetized and sacrificed at the ages of 4 months ($n=5$ for each genotype) and 18 months ($n=6$ (*Parp-1*$^{-/-}$) and $n=4$ (*Parp-1*$^{+/+}$)). The livers and brains were immediately frozen in liquid nitrogen, and stored at −80 °C until DNA extraction. Genomic DNA was extracted by a RecoverEase DNA isolation kit (Stratagene). Two out of six *Parp-1*$^{-/-}$ mice (mouse ID, G60 and G94) of 18 months of age harbored tumors in the liver, and genomic DNA was extracted from areas containing no tumors. A lambda phage *in vitro* packaging reaction was performed with Transpack Packaging Extract (Stratagene). Part of the tissues were also fixed with formalin solution, routinely processed and sections were stained with hematoxyline-eosine. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the National Cancer Center Research Institute.

2.2. Spi$^-$ assay

A Spi$^-$ assay [21] was carried out with a modification as described previously [27]. The frequencies of background mutants were less than 10^{-8} in the Spi$^-$ assay and were negligible [28]. The data for Spi$^-$ mutant frequencies were therefore presented without subtracting the background mutant frequencies. To narrow down the deleted region, the structure of each mutation was analyzed by a Southern blot hybridization method that uses oligonucleotide DNA probes [29]. DNA sequencing of the mutated region was performed with a CEQ™ DTCS Quick Start Kit (Beckman Coulter).

2.3. gpt assay

The *gpt* assay was performed as described previously [21]. Briefly, the phages rescued from genomic DNA were transfected into *E. coli* YG6020 expressing Cre recombinase. Infected cells were cultured at 37 °C on plates containing chloramphenicol (Cm) and 6-thioguanine (6-TG) for 3 days until 6-TG resistant colonies appeared. To confirm the 6-TG resistant phenotype, colonies were restreaked on plates containing Cm and 6-TG. A 739 bp DNA fragment encompassing the *gpt* gene was amplified by PCR [30]. DNA sequencing of the target 456 bp in the *gpt* gene was performed with a CEQ™ DTCS Quick Start Kit (Beckman Coulter).

2.4. Statistical analysis

The statistical significance of differences in mutant or mutation frequencies between the two groups was analyzed by using the Mann–Whitney *U* test. When *p* value is less than 0.05, the difference was considered significant. Because the individual differences in mutant frequency became larger at advanced ages, "tendency of ≥1.5 fold increase or reduction" in the mutant frequency is also mentioned with *p* value in the text, when *p* value is equal to or larger than 0.05.

3. Results

3.1. Analysis of spontaneous mutant frequency of the red/gam genes and the gpt genes in the livers of Parp-1$^{-/-}$ mice at 4 and 18 months of age

There was no difference in the mutant frequencies of the *red/gam* genes in the liver between *Parp-1*$^{-/-}$ and *Parp-1*$^{+/+}$ mice at 4 months of age. The liver of *Parp-1*$^{-/-}$ mice at 18 months of age showed a 1.7-fold higher tendency of the *red/gam* mutant frequencies than those in *Parp-1*$^{+/+}$ mice ($p=0.34$, Fig. 1A). The tendency of age-dependent 1.5-fold increase in mutant frequency was observed in *Parp-1*$^{-/-}$ but not in *Parp-1*$^{+/+}$ mice.

On the other hand, in the case of the *gpt* gene (Fig. 1B), in which point mutations are mostly detected, the mutant frequencies in *Parp-1*$^{+/+}$ mice showed a higher elevation at 18 months than that at 4 months ($p=0.037$). In *Parp-1*$^{-/-}$ mice, a tendency of higher mutant frequency was noticed at 18 months compared to that at 4 months ($p=0.14$). There was no significant difference in the mutant frequency of *gpt* gene between *Parp-1*$^{-/-}$ and *Parp-1*$^{+/+}$ mice at either 4 or 18 months (Fig. 1B).

3.2. Structural analysis of deletion mutations in the red/gam genes of Parp-1$^{-/-}$ mice at 18 months of age

The mutations in the *red/gam* genes could be categorized into deletion, base substitution and single base insertion. As shown in Fig. 1C, deletion mutation frequencies in the liver of *Parp-1*$^{-/-}$ mice showed a tendency of 1.7-fold increase compared to those in *Parp-1*$^{+/+}$ mice ($p=0.20$). The deletion mutations could be classified into single base deletion and deletion of two bases or more (Fig. 1C). Fig. 1D shows the distribution of single base deletions of the *gam* gene in the liver of *Parp-1*$^{-/-}$ and *Parp-1*$^{+/+}$ mice at 18 months of age. Single nucleotide repeats, –AAAAA– at 227–231, –AAAAAA– at 295–300 and –GGGG– at 286–289, are known as hot spots of single base deletions in the *gam* gene of wild-type mice [28]. The frequency of single base deletions at hot spots, namely at 4–6 bp mononucleotide repeats was not increased in *Parp-1*$^{-/-}$ mice compared to *Parp-1*$^{+/+}$ mice (Fig. 1C). In contrast, the frequency of single base deletions at non-repeat sequences or short repeats of 2–3 bp mononucleotides showed a 5.8-fold increase in *Parp-1*$^{-/-}$ mice ($p=0.031$, Fig. 1C). The single base deletions at non-repeat sequences were only observed in *Parp-1*$^{-/-}$ mice at a frequency of 4.3×10^{-7} and showed a higher frequency than that in *Parp-1*$^{+/+}$ mice ($p=0.023$). The specific deletion mutation frequencies of two bases or more in the liver showed a 3.2-fold (Fig. 1C) higher tendency in *Parp-1*$^{-/-}$ mice than those in *Parp-1*$^{+/+}$ mice, although there was no statistical significance ($p=0.084$). Deletions of both 2 bp–1 kb and deletions larger than 1 kb were observed in the liver of *Parp-1*$^{-/-}$ mice, whereas all three mutants in *Parp-1*$^{+/+}$ mice (Table 1) had deletions larger than 1 kb (data not shown).

The deletion mutations of two bases or more were also categorized into those that occurred at non-repeat and short repeat sequences of mononucleotides. Frequencies of deletion mutations of two bases or more at non-repeat and short repeats of mononucleotides showed a higher tendency in *Parp-1*$^{-/-}$ than *Parp-1*$^{+/+}$ mice ($p=0.28$) at 18 months old (Fig. 1C). There was no deletion mutation of two bases or more that occurred on a mononucleotide repeat larger than 4 bp in both genotypes.

We further categorized deletion mutations of two bases or more into simple or complex types (Table 1). Complex-type deletions were defined as accompanying small insertions or recombination with deletions [20]. Complex-type deletions were found in

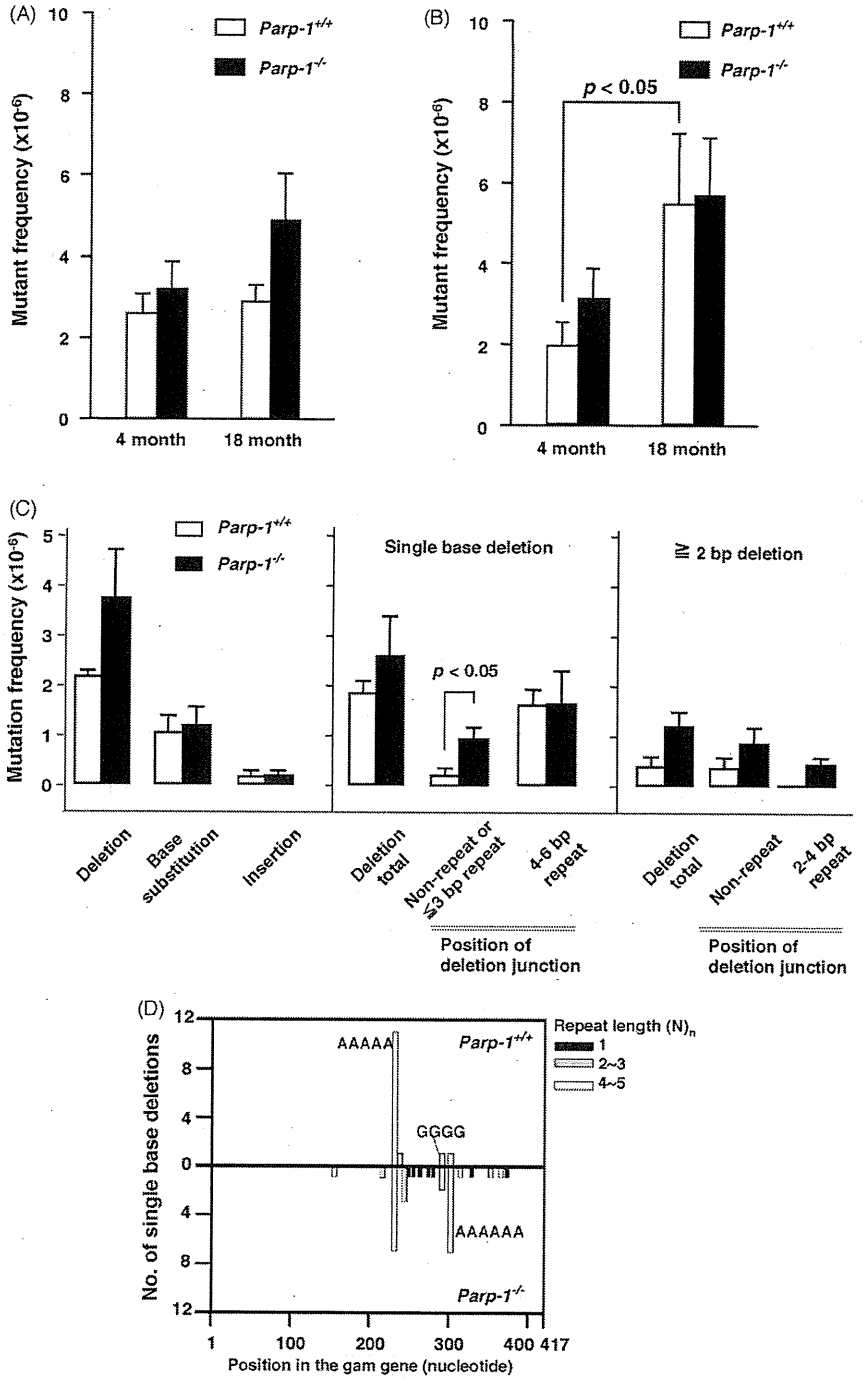

Fig. 1. Spontaneous mutant frequencies of the *red/gam* and *gpt* genes in the liver of *Parp-1*$^{-/-}$ and *Parp-1*$^{+/+}$ mice at 4 and 18 months of age. (A) Spontaneous mutant frequencies of the *red/gam* genes in the livers. (B) Spontaneous mutant frequencies in the *gpt* genes of the livers. Error bars represent standard error values. (C) Effect of *Parp-1* deficiency on the mutation spectrum of the *red/gam* genes in the liver at 18 months of age. Specific mutation frequencies in the *red/gam* genes of the liver are shown. Mean values and standard error values are presented for *Parp-1*$^{-/-}$ and *Parp-1*$^{+/+}$ mice ($n = 6$ and 4, respectively). (D) Distribution of single base deletion mutations in the *gam* gene of the livers at 18 months of age. Single base deletions were observed on non-repeat, or 2–3 base repeats, or 4–6 base repeats as indicated in the figure as repeat length $(N)_n$ of 1, 2, 4–6, respectively.

Parp-1$^{-/-}$ mice, but not in *Parp-1*$^{+/+}$ mice in the liver at 18 months old. As shown in Table 1, the frequencies of complex-type deletions in *Parp-1*$^{-/-}$ mice showed a higher tendency than those in *Parp-1*$^{+/+}$ mice, although it is not statistically significant ($p = 0.224$). The structures of complex-type mutations of *Parp-1*$^{-/-}$ mice observed at 18 months of age are shown in Table 2. Two complex-type deletions observed in *Parp-1*$^{-/-}$ mice accompanied both small insertions and microhomologous sequences at deletion junctions (Table 2). It is of note that complementary nucleotides AAA (G61-1-3) or TT (G93-2-3) (marked with upper lines in Table 2) are present at the 5′ position to these microhomologous deletion junctions in each case.

Table 1
Spectrum of the mutations of two bases or more in the *red/gam* genes in the liver and brain of *Parp-1*$^{-/-}$ mice at 18 months old.

Tissue	Deletion	*Parp-1*$^{+/+}$		*Parp-1*$^{-/-}$	
		Mutation frequency ($\times 10^{-6}$)	No. of mutants (MEJ/Non-MEJ)	Mutation frequency ($\times 10^{-6}$)	No. of mutants (MEJ/Non-MEJ)
Liver	Simple	0.34 ± 0.21	3 (2/1)	0.96 ± 0.27	13 (6/7)
	Complex	<0.16	0	0.13 ± 0.08	2 (2/0)
	with small insertion[a]	<0.16	0	0.13 ± 0.08	2 (2/0)
	with recombination	<0.16	0	<0.13	0
Brain	Simple	0.15 ± 0.15	1 (0/1)	0.32 ± 0.14	3 (2/1)
	Complex	<0.18	0	0.32 ± 0.14	3 (1/1)*
	with small insertion	<0.18	0	0.19 ± 0.12	2 (1/1)
	with recombination	<0.18	0	0.12 ± 0.12	1

MEJ; microhomology-mediated end joining. Non-MEJ; non-microhomology-mediated end joining.
[a] Small insertion represents 4–9 bp insertion.
* One of the mutants could not be classified into MEJ or non-MEJ type.

3.3. Mutation frequencies of the red/gam gene in the brains at 4 and 18 months of age

Parp-1$^{-/-}$ mice showed 1.5-fold higher mutant frequencies compared to *Parp-1*$^{+/+}$ mice ($p = 0.047$) in the brains at 4 months of age (Fig. 2A). The brains of *Parp-1*$^{-/-}$ mice showed a 2.2-fold higher tendency of mutant frequencies than those in *Parp-1*$^{+/+}$ mice ($p = 0.088$) at 18 months of age (Fig. 2A). The tendency of age-dependent slight increase in the mutant frequency in the brain was observed in *Parp-1*$^{-/-}$ but not in *Parp-1*$^{+/+}$ mice, as mentioned earlier in the case with the liver. Analysis of the mutation spectrum in the brain (Fig. 2C) revealed some differences from that of the livers. In the brain, a tendency of increase in base substitution and deletion mutations of two bases or more was observed in *Parp-1*$^{-/-}$ mice compared to *Parp-1*$^{+/+}$ mice (base substitution: $p = 0.055$, deletion mutation: $p = 0.11$). Different from the cases in the liver, the frequency of single base deletions at non-repeat or 2–3 bp repeats is not increased in the brain of *Parp-1*$^{-/-}$ mice at 18 months of age compared to *Parp-1*$^{+/+}$ mice (Fig. 2C).

3.4. Lower mutation frequencies of the gpt gene in the brains of Parp-1$^{-/-}$ than Parp-1$^{+/+}$ mice at 4 months of age and age-dependent increase

Of note, mutant frequencies of the *gpt* gene in the brains of *Parp-1*$^{-/-}$ mice were lower than those of *Parp-1*$^{+/+}$ mice ($p = 0.009$) at 4 months of age (Fig. 2B). No pathological changes in the brains were observed in *Parp-1*$^{-/-}$ and *Parp-1*$^{+/+}$ mice. Mutation spectra in the brains of *Parp-1*$^{-/-}$ mice showed a lower frequency of G:C to A:T base transition mutations ($p = 0.047$) as well as deletion mutations ($p = 0.034$) compared to *Parp-1*$^{+/+}$ mice at 4 months old (Fig. 2D).

The *gpt* mutant frequency showed an increase at 18 months of age in the *Parp-1*$^{-/-}$ but not in *Parp-1*$^{+/+}$ mice ($p = 0.011$, Fig. 2B). There was no difference in the mutant frequencies of the *gpt* gene in the brain between *Parp-1*$^{-/-}$ and *Parp-1*$^{+/+}$ mice at 18 months of age (Fig. 2B).

Comparison of the mutation spectra between 4 and 18 months of age in *Parp-1*$^{-/-}$ mice suggests a tendency of age-dependent increase in the frequencies of deletion mutations ($p = 0.068$, Fig. 2D). A tendency of increase of point mutation ($p = 0.144$) is also noticed, suggesting that Parp-1 may be involved in suppressing age-dependent introduction of point mutations in the brain.

4. Discussion

Spontaneous *gpt* and *red/gam* mutant frequencies are reported to be around 2–6 × 10^{-6} and 1–5 × 10^{-6}, respectively, in *gpt* delta mice of C57BL/6 genetic background [23,24]. In this study, the spontaneous mutation frequencies of *gpt* and *red/gam* mutant frequencies in the liver and the brain of *Parp-1*$^{+/+}$ are both around 2 × 10^{-6} at 4 months of age and thus consistent with the previous reports. The mutant frequency of the *gpt* gene in the small intestine

Table 2
Junctional sequences of complex-type mutations in the liver and brain of *Parp-1*$^{-/-}$ mice at 18 months old.

Tissue	Mutant ID[a]	Original sequence in lambdaEG10		Junctional sequence of mutation	Deletion/insertion size (nucleotide position in lambdaEG10)
Liver	G61-1-3	5′-GTCATCAAACgcac 3′-CAGTAGTTTGcgtg	ttttGCTGGCCCCG-3′ aaaaCGACCGGGGC-5′	5′-GTCATCAAACacacGCTGGCCCCG-3′ 3′-CAGTAGTTTGtgtgCGACCGGGGC-5′	20 bp deletion + 4 bp insertion (25021 - 25040)
	G93-2-3	5′-CCGTGGCGTTgcaa 3′-GGCACCGCAAcgtt	ataaGCGTTCATGG-3′ tattCGCAAGTACC-5′	5′-CCGTGGCGTTttgctgGCGTTCATGG-3′ 3′-GGCACCGCAAaacgacCGCAAGTACC-5′	149 bp deletion + 6 bp insertion (25058 - 25206)
Brain	G61-1-1	5′-TTCATTAGACttat 3′-AAGTAATCTGaata	tagtGAATGCTTTT-3′ atcaCTTACGAAAA-5′	5′-TTCATTAGACaaattaGAATGCTTTT-3′ 3′-AAGTAATCTGtttaatCTTACGAAAA-5′	3694 bp deletion + 6 bp insertion (21600 - 25293)
	G94-1-1	5′-TGTCTGCATGgaga 3′-ACAGACGTACctct	aatcGATTTTCCCT-3′ ttagCTAAAAGGGA-5′	5′-TGTCTGCATGagaccagaaGATTTTCCCT-3′ 3′-CGTACCTCTGtctggtcttCTAAAAGGGA-5′	3805 bp deletion + 9 bp insertion (21682 - 25486)
	G93-2-4		acgcGCCCAGCTCT-3′ tgcgCGGGTCGAGA-5′	5′-taagagtcagGCCCAGCTCT-3′ 3′-attctcagtcCGGGTCGAGA-5′	Recombination with unknown sequence

[a]ID; Identification number. Red and blue letters indicate deleted and inserted sequences, respectively. Letters in the box are microhomologous sequences. Upperlines show complementary mononucleotide sequences at 5′ positions of the microhomologous sequences.

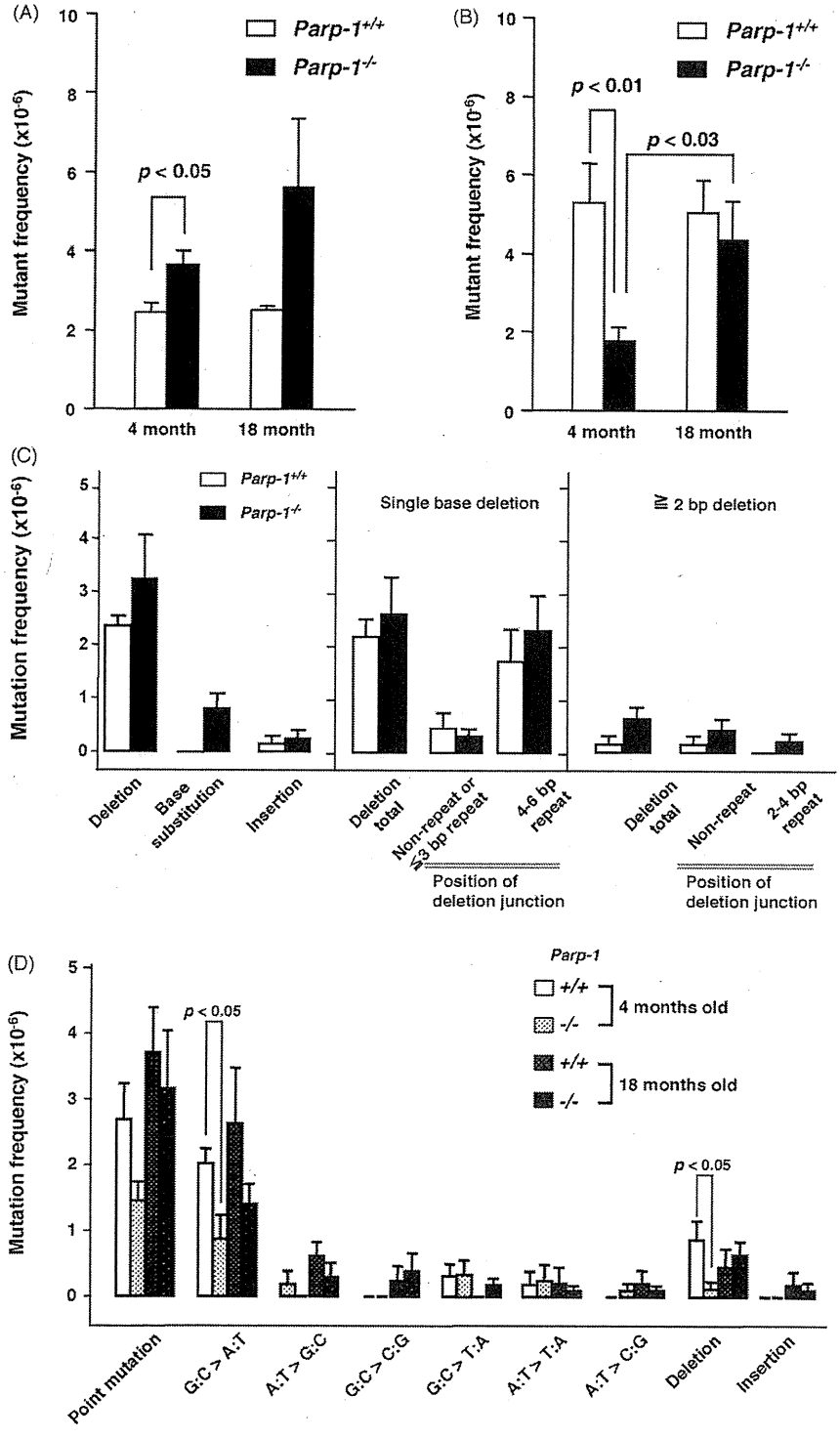

Fig. 2. Spontaneous mutant frequencies of the *red/gam* and *gpt* genes in the brain of *Parp-1*$^{-/-}$ and *Parp-1*$^{+/+}$ mice at 4 and 18 months of age. (A) Spontaneous mutant frequencies of the *red/gam* genes. (B) Spontaneous mutant frequencies in the *gpt* genes. Error bars represent standard error values. (C) Mutation spectra of the *red/gam* genes in the brain of *Parp-1*$^{-/-}$ and *Parp-1*$^{+/+}$ mice at 18 months of age. (D) Mutation spectra of the *gpt* genes in the brain of *Parp-1*$^{-/-}$ and *Parp-1*$^{+/+}$ mice at 4 and 18 months of age.

of *gpt* delta transgenic mice of mixed genetic background of SWR and C57BL/6 is reported to be 2.5×10^{-5} [22], which is higher compared to other reports on *gpt* delta mice [23,24]. This difference could be due to the mouse strain, tissues or other factors. From 4 to 18 months of age, the mutant frequency of the *gpt* gene in *Parp-1*$^{+/+}$ mice increased 2-fold. The mutant frequency of the *lacZ* marker gene in the liver is around 5×10^{-6} at 4–6 months of age and 1.2×10^{-5} at 24–34 months of age in wild-type mice [19]. Therefore age-dependent 2-fold increase in mutant frequency is consistently observed both in the *gpt* and *lacZ* [19] genes. On the other hand, size change mutations in the liver detected by the *lacZ* gene system did not significantly increase before 25–27 months [19] but

increased thereafter. Increase of mutant frequency in the *red/gam* gene in *Parp-1*$^{+/+}$ mice at 18 months of age, which detects deletion mutation, was not observed in the liver, being consistent with the results in the *lacZ* gene [19]. In the *lacZ* gene system, the target size is around 3000 bp, whereas that in the *gpt* and *red/gam* gene (Spi$^-$ assay) are around 456 and 417 bp, respectively. The smaller size of the target sequences of the *gpt* and *red/gam* genes could be also responsible for the lower spontaneous mutant frequencies.

In this study, *Parp-1*$^{-/-}$ mice showed a tendency of higher frequencies of spontaneous deletion mutations in the *red/gam* gene, including complex-type deletions in the liver ($p = 0.20$) and brain ($p = 0.29$) at 18 months of age.

The single base deletion mutations at non-repeat or short repeat sequences of the *red/gam* gene showed a 5.8-fold increase ($p = 0.031$) in the liver of *Parp-1*$^{-/-}$ mice compared to *Parp-1*$^{+/+}$ mice at 18 months of age. The frequency of deletion mutations of two bases or more also showed a 3.2-fold higher tendency in the *Parp-1*$^{-/-}$ than in the *Parp-1*$^{+/+}$ liver ($p = 0.084$). We observed complex-type deletions in the livers and brains of *Parp-1*$^{-/-}$ but not in *Parp-1*$^{+/+}$ mice at 18 months old.

8-Oxodeoxyguanosine (8-oxodG) is one outcome of major oxidative DNA damage [31]. The 8-oxodG levels in DNA of the liver, lungs, and small intestine in double knockout mice lacking both 8-oxoguanine DNA glycosylase 1 (*Ogg1*) and Mut Y homologue (*Myh*) genes increased linearly between 4 and 14 months of age [32]. 8-OxodG and SSB, which are expected outcomes of major endogenous DNA damage, are preferentially repaired by BER. Parp-1 is shown to be involved in BER and deletion mutations of single base and larger sizes of deletion as well as complexed-type were increased in *Parp-1*$^{-/-}$ mice after treatment with an alkylating agent, BHP [20]. The frequency of single base deletion mutations at non-repeat or short repeat sequences of the *red/gam* gene also increased 2.9-fold in *Parp-1*$^{-/-}$ mice compared to *Parp-1*$^{+/+}$ mice ($p = 0.043$) in the liver after treatment of the alkylating agent, whereas no difference in the frequency of single base deletion at 4–6 bp of mononucleotide repeats was observed between genotypes [20]. Therefore the spectra of single base deletions in the liver of *Parp-1*$^{-/-}$ mice at advanced age and after treatment with the alkylating agent are similar to each other. Stalled BER in the absence of Parp-1 at a SSB introduced step may further cause deletion mutations after treatment with an alkylating agent [20]. Therefore, there is a possibility that deletion mutation is also caused through BER induced by endogenous DNA damage during aging in *Parp-1*$^{-/-}$ mice. After introduction of SSB during BER, lack of Parp-1 may induce stall or delay in BER and terminal nucleotides may be destabilized and lost under *Parp-1* deficiency by exonuclease activity (Fig. 3). Collision between SSB and replication forks induces double strand breaks (DSBs) [33]. Two SSBs on opposite strands within at least 30 nt could resolve into a DSB [34]. Therefore, an increase of spontaneous DSBs might also be caused by the presence of SSBs during replication fork progression or defective BER under *Parp-1* deficiency.

Deletion mutations including single base deletions may be also produced during imprecise non-homologous end joining (NHEJ). In NHEJ reconstituted systems that utilize DSB substrates, it is shown that deletion or insertion of single bases as well as larger sizes occurs during the NHEJ process [35–37]. In chicken DT-40 cells, Parp-1 negatively regulates the NHEJ process by inhibiting Ku70/Ku80 action, and *Parp-1* deficiency causes an increase of NHEJ frequency [38]. However, DT-40 cells are known to have high HR levels compared to typical mammalian somatic cells. Using mouse embryonic fibroblast or CHO cells, it is demonstrated that Parp-1 competes with Ku for DSB binding and is shown to be involved in a backup pathway of classical NHEJ pathway with DNA ligase III [39]. Therefore, as shown in Fig. 3, during a NHEJ process of DSB, terminal nucleotides may be destabilized in the absence of Parp-1, and resection of bases by the exonuclease may lead to deletion mutation.

It is also notable that the frequency of single base deletions at 4–6 bp mononucleotide repeats did not show a difference between either genotypes in the livers and brains. Single base deletion mutations at 4–6 bp of mononucleotide repeats, namely at run sequences, might be caused by slippage error during DNA replication or repair reaction. The results suggest that Parp-1 is not essential to suppress these slippage type errors induced during aging.

Two complex-type deletions observed in *Parp-1*$^{-/-}$ mice accompanied small insertions as well as microhomologous sequences at deletion junctions, suggesting that these mutations could be

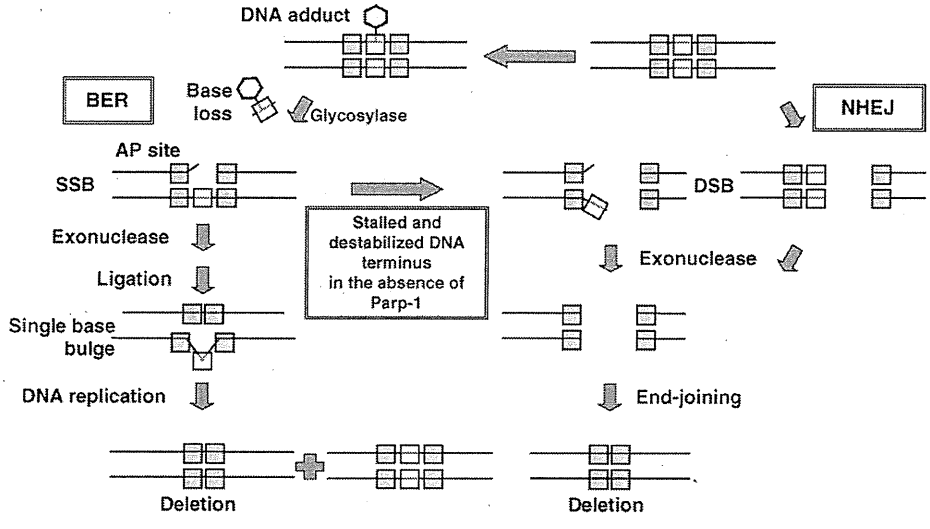

Fig. 3. A model for augmented development of deletion mutation through imprecise BER or NHEJ process in the absence of Parp-1. During BER, after single strand breaks are introduced following damaged base removal, the DNA terminus may be destabilized in the absence of Parp-1. Base loss could occur by the DNA exonuclease activity. When misannealing and ligation occur, the deletion will be fixed by subsequent DNA replication. Stalled BER reaction in the absence of Parp-1 on single strand breaks may also cause DSB and may induce switching to a NHEJ reaction and subsequently base loss will be fixed by end-joining process. During DSB repair process by NHEJ, base loss frequency might be augmented at the destabilized DNA terminus in the absence of Parp-1.

caused by insertion of a few nucleotides during microhomologous end-joining (MEJ)-type reactions. A few complementary bases are present at the 5′ position of the microhomologous sequences (marked with upper lines in Table 2). During the end-joining process, after resection of strand ends, transient base-pairing at microhomologous sequences may occur and a few complementary bases at the 5′ position may also form base-pairing. In the absence of Parp-1, these base-pairings may be destabilized and resection and insertion of a few bases may tend to occur in the livers. Consistently of all seven simple-type deletions of two bases or more observed in the livers of *Parp-1*$^{-/-}$ mice (Table 1), none harbored a few complementary bases at the 5′ position of the microhomologous sequences (data not shown). On the other hand, in two simple-type deletions of two bases or more in *Parp-1*$^{+/+}$ mice, one deletion harbored a few complementary bases at the 5′ position of the microhomologous deletion junctions (Table 1).

In the brain, one out of three complex-type deletions of *Parp-1*$^{-/-}$ mice harbored microhomologous deletion junctions but did not harbor complementary bases at 5′ positions of the microhomologous deletion junctions. This point should be further evaluated by analyzing deletion mutations induced after treatment with various types of DNA damaging agents in different tissues.

The xeroderma pigmentosum complementation group A (Xpa) plays an important role in nucleotide excision repair (NER) and *Xpa*-deficient mice also show higher spontaneous mutant frequencies in the liver at advanced ages [40]. In fact, *Xpa*-deficient mice show an increased frequency of hepatocellular adenomas at older ages [34]. It is thus possible that endogenous DNA damage repairable by NER may occur during aging. However, no increase in the susceptibility to carcinogenesis induced in *Parp-1*$^{-/-}$ mice by 4-nitrosoquinoline1-oxide [41], which induces bulky DNA adducts, suggests that Parp-1 is not involved in NER.

Most liver cells stay in the G0 phase and they usually enter the cell division cycle after various stimulating events. An augmented frequency of DNA replication, like that in preneoplastic lesions, can also increase the chance of DSBs and may increase the frequency of deletions. Two of six *Parp-1*$^{-/-}$ mice used in the mutation analysis harbored tumors in the liver and the tumor regions were not included for DNA isolation. Because the frequencies and spectrum of mutations in the *gpt* or *red/gam* genes were unbiased in each mouse, we can exclude the possibility that the tissues used for isolation of DNA contained monoclonally proliferating preneoplastic lesions or other cycling cells.

It is also possible that an increased frequency of cell division may be causative of augmented frequency of DSBs and may result in a higher frequency of deletion mutation. However, if this is true, the observed mutation spectrum is expected to be the same between the genotypes. We could rule out this possibility because we observed different spectra of deletion mutations between the genotypes.

Unexpectedly we also found a 3-fold lower frequency of point mutations in adolescent *Parp-1*$^{-/-}$ compared to *Parp-1*$^{+/+}$ mice in the brain ($p = 0.009$). An age-dependent increase in the mutant frequency in *Parp-1*$^{-/-}$ mice was also shown ($p = 0.011$). Lower frequencies of G:C to A:T type mutation and deletion mutation in *Parp-1*$^{-/-}$ mice suggest that Parp-1 may be positively involved imprecise repair pathways which cause base substitution mutation of G:C to A:T and deletion mutation in the brain.

In conclusion, this result supports the view that Parp-1 is involved in suppressing imprecise repair of endogenous DNA damage leading to deletion mutation during aging in the liver and brain. *Parp-1*$^{-/-}$ mice show increased incidence of hepatocellular tumors at 18–24 months of ages [13]. The present results suggest a substantial role of Parp-1 in the maintenance of genomic stability and suppression of carcinogenesis during aging.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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References

[1] M. Masson, C. Niedergang, V. Schreiber, S. Muller, J. Menissier-de Murcia, G. de Murcia, XRCC1 is specifically associated with poly(ADP-ribose) polymerase and negatively regulates its activity following DNA damage, Mol. Cell. Biol. 18 (1998) 3563–3571.
[2] C. von Kobbe, J.A. Harrigan, V. Schreiber, P. Stiegler, J. Piotrowski, L. Dawut, V.A. Bohr, Poly(ADP-ribose) polymerase 1 regulates both the exonuclease and helicase activities of the Werner syndrome protein, Nucleic Acids Res. 32 (2004) 4003–4014.
[3] C. von Kobbe, J.A. Harrigan, A. May, P.L. Opresko, L. Dawut, W.H. Cheng, V.A. Bohr, Central role for the Werner syndrome protein/poly(ADP-ribose) polymerase 1 complex in the poly(ADP-ribosyl)ation pathway after DNA damage, Mol. Cell. Biol. 23 (2003) 8601–8613.
[4] S. Galande, T. Kohwi-Shigematsu, Poly(ADP-ribose) polymerase and Ku autoantigen form a complex and synergistically bind to matrix attachment sequences, J. Biol. Chem. 274 (1999) 20521–20528.
[5] B. Li, S. Navarro, N. Kasahara, L. Comai, Identification and biochemical characterization of a Werner's syndrome protein complex with Ku70/80 and poly(ADP-ribose) polymerase-1, J. Biol. Chem. 279 (2004) 13659–13667.
[6] L. Lan, S. Nakajima, Y. Oohata, M. Takao, S. Okano, M. Masutani, S.H. Wilson, A. Yasui, In situ analysis of repair processes for oxidative DNA damage in mammalian cells, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 13738–13743.
[7] S. Okano, L. Lan, K.W. Caldecott, T. Mori, A. Yasui, Spatial and temporal cellular responses to single-strand breaks in human cells, Mol. Cell. Biol. 23 (2003) 3974–3981.
[8] F. Le Page, V. Schreiber, C. Dherin, G. De Murcia, S. Boiteux, Poly(ADP-ribose) polymerase-1 (PARP-1) is required in murine cell lines for base excision repair of oxidative DNA damage in the absence of DNA polymerase beta, J. Biol. Chem. 278 (2003) 18471–18477.
[9] J.B. Leppard, Z. Dong, Z.B. Mackey, A.E. Tomkinson, Physical and functional interaction between DNA ligase IIIalpha and poly(ADP-Ribose) polymerase 1 in DNA single-strand break repair, Mol. Cell. Biol. 23 (2003) 5919–5927.
[10] M. Tsutsumi, M. Masutani, T. Nozaki, O. Kusuoka, T. Tsujiuchi, H. Nakagama, H. Suzuki, Y. Konishi, T. Sugimura, Increased susceptibility of poly(ADP-ribose) polymerase-1 knockout mice to nitrosamine carcinogenicity, Carcinogenesis 22 (2001) 1–3.
[11] T. Nozaki, H. Fujihara, M. Watanabe, M. Tsutsumi, K. Nakamoto, O. Kusuoka, N. Kamada, H. Suzuki, H. Nakagama, T. Sugimura, M. Masutani, *Parp-1* deficiency implicated in colon and liver tumorigenesis induced by azoxymethane, Cancer Sci. 94 (2003) 497–500.
[12] A. Gunji, A. Uemura, M. Tsutsumi, T. Nozaki, O. Kusuoka, K. Omura, H. Suzuki, H. Nakagama, T. Sugimura, M. Masutani, *Parp-1* deficiency does not increase the frequency of tumors in the oral cavity and esophagus of ICR/129Sv mice by 4-nitroquinoline 1-oxide, a carcinogen producing bulky adducts, Cancer Lett. 241 (2005) 87–92.
[13] W.M. Tong, U. Cortes, M.P. Hande, H. Ohgaki, L.R. Cavalli, P.M. Lansdorp, B.R. Haddad, Z.Q. Wang, Synergistic role of Ku80 and poly(ADP-ribose) polymerase in suppressing chromosomal aberrations and liver cancer formation, Cancer Res. 62 (2002) 6990–6996.
[14] W.M. Tong, U. Cortes, Z.Q. Wang, Poly(ADP-ribose) polymerase: a guardian angel protecting the genome and suppressing tumorigenesis, Biochim. Biophys. Acta 1552 (2001) 27–37.
[15] W.M. Tong, H. Ohgaki, H. Huang, C. Granier, P. Kleihues, Z.Q. Wang, Null mutation of DNA strand break-binding molecule poly(ADP-ribose) polymerase causes medulloblastomas in p53(−/−) mice, Am. J. Pathol. 162 (2003) 343–352.
[16] M.E. Dolle, W.K. Snyder, J.A. Gossen, P.H. Lohman, J. Vijg, Distinct spectra of somatic mutations accumulated with age in mouse heart and small intestine, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 8403–8408.
[17] K.A. Hill, V.L. Buettner, A. Halangoda, M. Kunishige, S.R. Moore, J. Longmate, W.A. Scaringe, S.S. Sommer, Spontaneous mutation in Big Blue mice from fetus to old age: tissue-specific time courses of mutation frequency but similar mutation types, Environ. Mol. Mutagen. 43 (2004) 110–120.
[18] T. Ono, H. Ikehata, S. Nakamura, Y. Saito, Y. Hosoi, Y. Takai, S. Yamada, J. Onodera, K. Yamamoto, Age-associated increase of spontaneous mutant frequency and

molecular nature of mutation in newborn and old lacZ-transgenic mouse, Mutat. Res. 447 (2000) 165–177.
[19] M.E. Dolle, H. Giese, C.L. Hopkins, H.J. Martus, J.M. Hausdorff, J. Vijg, Rapid accumulation of genome rearrangements in liver but not in brain of old mice, Nat. Genet. 17 (1997) 431–434.
[20] A. Shibata, N. Kamada, K. Masumura, T. Nohmi, S. Kobayashi, H. Teraoka, H. Nakagama, T. Sugimura, H. Suzuki, M. Masutani, *Parp-1* deficiency causes an increase of deletion mutations and insertions/rearrangements in vivo after treatment with an alkylating agent, Oncogene 24 (2005) 1328–1337.
[21] T. Nohmi, M. Katoh, H. Suzuki, M. Matsui, M. Yamada, M. Watanabe, M. Suzuki, N. Horiya, O. Ueda, T. Shibuya, H. Ikeda, T. Sofuni, A new transgenic mouse mutagenesis test system using Spi- and 6-thioguanine selections, Environ. Mol. Mutagen. 28 (1996) 465–470.
[22] R.R. Swiger, L. Cosentino, K.I. Masumura, T. Nohmi, J.A. Heddle, Further characterization and validation of gpt delta transgenic mice for quantifying somatic mutations in vivo, Environ. Mol. Mutagen. 37 (2001) 297–303.
[23] K. Masumura, K. Kuniya, T. Kurobe, M. Fukuoka, F. Yatagai, T. Nohmi, Heavy-ion-induced mutations in the *gpt* delta transgenic mouse: comparison of mutation spectra induced by heavy-ion, X-ray, and gamma-ray radiation, Environ. Mol. Mutagen. 40 (2002) 207–215.
[24] K. Masumura, T. Nohmi, Spontaneous mutagenesis in rodents: spontaneous gene mutations identified by neutral reporter genes in *gpt* delta transgenic mice and rats, J. Health Sci. 55 (2009) 40–49.
[25] K. Masumura, K. Matsui, M. Yamada, M. Horiguchi, K. Ishida, M. Watanabe, O. Ueda, H. Suzuki, Y. Kanke, K.R. Tindall, K. Wakabayashi, T. Sofuni, T. Nohmi, Mutagenicity of 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) in the new *gpt* delta transgenic mouse, Cancer Lett. 143 (1999) 241–244.
[26] F. Yatagai, T. Kurobe, T. Nohmi, K. Masumura, T. Tsukada, H. Yamaguchi, K. Kasai-Eguchi, N. Fukunishi, Heavy-ion-induced mutations in the gpt delta transgenic mouse: effect of p53 gene knockout, Environ. Mol. Mutagen. 40 (2002) 216–225.
[27] A. Shibata, M. Masutani, T. Nozaki, N. Kamada, H. Fujihara, K. Masumura, H. Nakagama, T. Sugimura, S. Kobayashi, H. Suzuki, T. Nohmi, Improvement of the Spi-assay for mutations in *gpt* delta mice by including magnesium ions during plaque formation, Environ. Mol. Mutagen. 41 (2003) 370–372.
[28] T. Nohmi, M. Suzuki, K. Masumura, M. Yamada, K. Matsui, O. Ueda, H. Suzuki, M. Katoh, H. Ikeda, T. Sofuni, Spi(-) selection: an efficient method to detect gamma-ray-induced deletions in transgenic mice, Environ. Mol. Mutagen. 34 (1999) 9–15.
[29] A. Shibata, M. Masutani, N. Kamada, K. Masumura, H. Nakagama, S. Kobayashi, H. Teraoka, H. Suzuki, T. Nohmi, Efficient method for mapping and characterizing structures of deletion mutations in *gpt* delta mice using Southern blot analysis with oligo DNA probes, Environ. Mol. Mutagen. 43 (2004) 204–207.
[30] K. Masumura, M. Matsui, M. Katoh, N. Horiya, O. Ueda, H. Tanabe, M. Yamada, H. Suzuki, T. Sofuni, T. Nohmi, Spectra of *gpt* mutations in ethylnitrosourea-treated and untreated transgenic mice, Environ. Mol. Mutagen. 34 (1999) 1–8.
[31] H. Kasai, P.F. Crain, Y. Kuchino, S. Nishimura, A. Ootsuyama, H. Tanooka, Formation of 8-hydroxyguanine moiety in cellular DNA by agents producing oxygen radicals and evidence for its repair, Carcinogenesis 7 (1986) 1849–1851.
[32] M.T. Russo, G. De Luca, P. Degan, E. Parlanti, E. Dogliotti, D.E. Barnes, T. Lindahl, H. Yang, J.H. Miller, M. Bignami, Accumulation of the oxidative base lesion 8-hydroxyguanine in DNA of tumor-prone mice defective in both the *Myh* and *Ogg1* DNA glycosylases, Cancer Res. 64 (2004) 4411–4414.
[33] T. Furuta, H. Takemura, Z.Y. Liao, G.J. Aune, C. Redon, O.A. Sedelnikova, D.R. Pilch, E.P. Rogakou, A. Celeste, H.T. Chen, A. Nussenzweig, M.I. Aladjem, W.M. Bonner, Y. Pommier, Phosphorylation of histone H2AX and activation of Mre11, Rad50, and Nbs1 in response to replication-dependent DNA double-strand breaks induced by mammalian DNA topoisomerase I cleavage complexes, J. Biol. Chem. 278 (2003) 20303–20312.
[34] S. Vispe, M.S. Satoh, DNA repair patch-mediated double strand DNA break formation in human cells, J. Biol. Chem. 275 (2000) 27386–27392.
[35] F. Liang, M. Han, P.J. Romanienko, M. Jasin, Homology-directed repair is a major double-strand break repair pathway in mammalian cells, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 5172–5177.
[36] M. Honma, M. Sakuraba, T. Koizumi, Y. Takashima, H. Sakamoto, M. Hayashi, Non-homologous end-joining for repairing I-SceI-induced DNA double strand breaks in human cells, DNA Repair (Amst.) 6 (2007) 781–788.
[37] Y. Ma, H. Lu, B. Tippin, M.F. Goodman, N. Shimazaki, O. Koiwai, C.L. Hsieh, K. Schwarz, M.R. Lieber, A biochemically defined system for mammalian nonhomologous DNA end joining, Mol. Cell 16 (2004) 701–713.
[38] H. Hochegger, D. Dejsuphong, T. Fukushima, C. Morrison, E. Sonoda, V. Schreiber, G.Y. Zhao, A. Saberi, M. Masutani, N. Adachi, H. Koyama, G. de Murcia, S. Takeda, Parp-1 protects homologous recombination from interference by Ku and Ligase IV in vertebrate cells, EMBO J. 25 (2006) 1305–1314.
[39] M. Wang, W. Wu, W. Wu, B. Rosidi, L. Zhang, H. Wang, G. Iliakis, PARP-1 and Ku compete for repair of DNA double strand breaks by distinct NHEJ pathways, Nucleic Acids Res. 34 (2006) 6170–6182.
[40] H. Giese, M.E. Dolle, A. Hezel, H. van Steeg, J. Vijg, Accelerated accumulation of somatic mutations in mice deficient in the nucleotide excision repair gene *XPA*, Oncogene 18 (1999) 1257–1260.
[41] A. de Vries, C.T. van Oostrom, P.M. Dortant, R.B. Beems, C.F. van Kreijl, P.J. Capel, H. van Steeg, Spontaneous liver tumors and benzo[a]pyrene-induced lymphomas in *XPA*-deficient mice, Mol. Carcinogen. 19 (1997) 46–53.

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Mutagenicity testing for chemical risk assessment: update of the WHO/IPCS Harmonized Scheme

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Since the publication of the International Programme on Chemical Safety (IPCS) Harmonized Scheme for Mutagenicity Testing, there have been a number of publications addressing test strategies for mutagenicity. Safety assessments of substances with regard to genotoxicity are generally based on a combination of tests to assess effects on three major end points of genetic damage associated with human disease: gene mutation, clastogenicity and aneuploidy. It is now clear from the results of international collaborative studies and the large databases that are currently available for the assays evaluated that no single assay can detect all genotoxic substances. The World Health Organization therefore decided to update the IPCS Harmonized Scheme for Mutagenicity Testing as part of the IPCS project on the Harmonization of Approaches to the Assessment of Risk from Exposure to Chemicals. The approach presented in this paper focuses on the identification of mutagens and genotoxic carcinogens. Selection of appropriate *in vitro* and *in vivo* tests as well as a strategy for germ cell testing are described.

Introduction

Since the publication of the International Programme on Chemical Safety (IPCS) Harmonized Scheme for Mutagenicity Testing (1), there have been a number of publications addressing test strategies for mutagenicity (2–6) and reviews thereof (7). In addition, analyses of test batteries and their correlation with carcinogenicity (8–11) have indicated that an optimal solution to this issue has not yet been found. The 2005 International Workshop on Genotoxicity Testing (IWGT) meeting in San Francisco, USA, discussed many of these problems, and reports of this meeting (10,12) and companion papers (13–16) have recently been published.

Safety assessments of substances with regard to genotoxicity are generally based on a combination of tests to assess effects on three major end points of genetic damage associated with human disease: gene mutation (i.e. point mutations or deletions/insertions that affect single or blocks of genes), clastogenicity (i.e. structural chromosome changes) and aneuploidy (i.e. numerical chromosome aberrations). It is now clear from the results of international collaborative studies and the large databases that are currently available for the assays evaluated that no single assay can detect all genotoxic substances. This is not surprising, as a wide variety of possible genetic events can occur. For example, some mutagens preferentially induce gene mutations by either base pair substitutions or frameshift mechanisms, whereas others induce chromosome mutations but show little or no evidence of inducing gene mutations.

The World Health Organization (WHO) therefore decided to update the IPCS Harmonized Scheme for Mutagenicity Testing (1) as part of the IPCS project on the Harmonization of Approaches to the Assessment of Risk from Exposure to Chemicals. A public review draft paper was prepared by an International Drafting Group Meeting of experts, held at the Fraunhofer Institute for Toxicology and Experimental Medicine in Hanover, Germany, on April 11–12, 2007, and revised, following peer and public review, by an expert review meeting hosted by the University of Bradford, Bradford, UK, on June 30 to July 1, 2008. The present paper is the product of the expert review meeting.

Strategy for mutagenicity testing

The approach presented in this paper (see Figure 1) focuses on the identification of mutagens and genotoxic carcinogens. The term 'mutation' as understood in this paper (a glossary of terms used in this paper is available on the IPCS website at http://www.who.int/ipcs/publications/methods/harmonization/en/index.html) refers to permanent changes in the structure and/or amount of the genetic material of an organism that can lead to heritable changes in its function, and it includes gene mutations as well as structural and numerical chromosome alterations. The group is aware of other mechanisms leading to carcinogenicity and other heritable diseases, but their identification requires additional types of mechanistic studies. 'Genotoxicity' refers to the capability of substances to damage DNA and/or cellular components regulating the fidelity of the genome—such as the spindle apparatus, topoisomerases, DNA repair systems and DNA polymerases (4)—and includes all adverse effects on genetic information. These potentially harmful effects on genetic material may be mediated directly or

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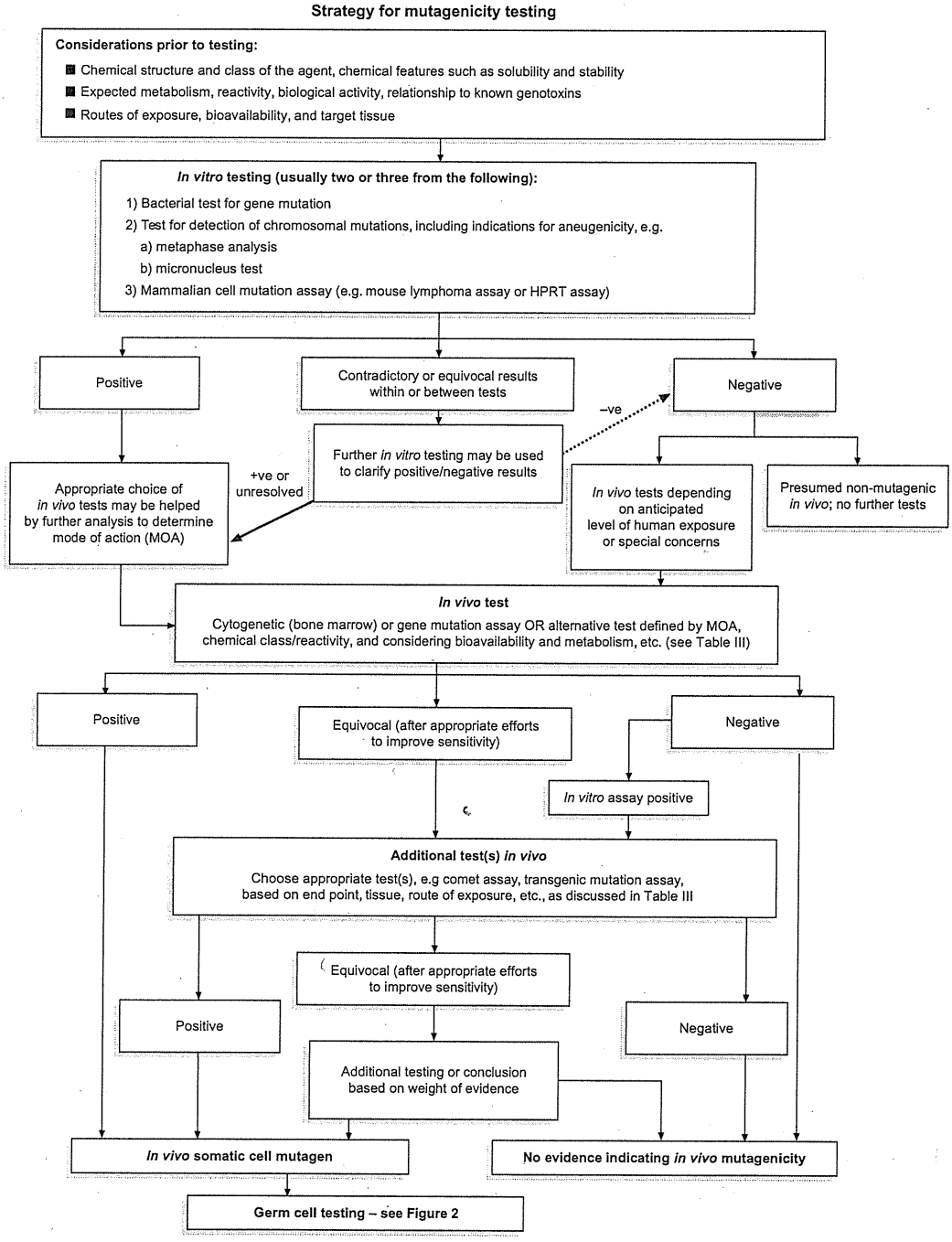

Fig. 1. Strategy for mutagenicity testing.

indirectly and are not necessarily associated with mutagenicity. Genotoxicity is therefore a broader term than 'mutagenicity', which refers to the capacity to give rise to mutations.

Because of the wide range of genetic damage that can occur, test batteries are designed to include complementary tests evaluating different mechanisms of mutagenicity. At all stages of the outlined testing strategy, a weight of evidence approach and scientific judgement should be used. Multiple negative results may not be sufficient to remove concern for mutagenicity raised by a clear positive result in a single mutagenicity assay.

Most short-term tests in bacteria and mammalian cell cultures have been designed primarily for hazard identification and thus can represent only the starting point in the process of risk assessment. Whether or not the observed effects are relevant for humans under anticipated exposure conditions depends on pharmacokinetic, pharmacodynamic and other factors that require investigation *in vivo*.

Especially when choosing *in vivo* assays and when proceeding into germ cell mutagenicity studies (see Strategy for germ cell testing), expert judgement is required to select the

appropriate test systems and to avoid uninformative and thus unnecessary animal experiments.

Development of a testing strategy

Before initiating mutagenicity testing on a particular substance (or mixture of substances), the following aspects should be considered, when available:

(i) Chemical structure and class of the substance (possible structure–activity relationships) and physicochemical properties, such as solubility and stability;
(ii) Expected pathways of metabolism, chemical and biological reactivity/activity and relationship to known genotoxic substances and
(iii) Routes of exposure, bioavailability and target tissues for genotoxicity.

Critical evaluation of available data prior to testing usually provides important information for choosing the appropriate *in vitro* assays, but even more so for the selection of appropriate *in vivo* studies.

Distinction needs to be made between 'mutagenicity tests' in the strict sense and 'indicator tests' that provide evidence of interaction with DNA that may or may not lead to mutations (e.g. DNA adducts, DNA strand breaks and sister chromatid exchanges). Preference should be given to mutagenicity tests whenever possible.

In vitro testing

Usually two or three different tests in bacteria and mammalian cells are selected to cover the end points of gene mutations, clastogenicity (structural chromosome aberrations) and aneuploidy (numerical chromosome aberrations), taking into account physicochemical properties of substances under consideration.

In vitro tests. Screening should be based on a limited number of tests that are well validated and informative. Genotoxicity test batteries generally include the following:

(i) A test for gene mutation in bacteria (bacterial reverse mutation assay): Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 recommends the use of at least five strains of bacteria: (a) *Salmonella typhimurium* TA1535, (b) *S.typhimurium* TA1537 or TA97 or TA97a, (c) *S.typhimurium* TA98, (d) *S.typhimurium* TA100 and (e) *Escherichia coli* WP2 or *E.coli* WP2*uvrA* or *S.typhimurium* TA102. The choice of additional tests depends on the chemical structure and class of the substance (see Development of a testing strategy). Table I describes the most commonly used bacterial mutagenicity tests.
(ii) *In vitro* mammalian assays: These assays should evaluate the potential of a substance to induce point mutations, clastogenicity and/or aneugenicity, by using either mammalian cell lines or primary human cell cultures such as fibroblasts or lymphocytes (e.g. mouse lymphoma thymidine kinase assay, hypoxanthine guanine phosphoribosyltransferase assay or cytogenetic evaluation of chromosomal damage in mammalian cells via either the *in vitro* chromosome aberration or the *in vitro* micronucleus test) (see Table II).

Evaluation of in vitro testing results. In the evaluation, results are classified into (i) positive, (ii) negative and (iii) contradictory or equivocal:

(i) Positive: Substance is positive at one or more end points of mutagenicity.
(ii) Negative: Substance is negative in all test systems under appropriate *in vitro* test conditions; the substance is not mutagenic (or genotoxic) *in vitro* and is anticipated not to be mutagenic *in vivo* [for exceptions, see refs (37,38)].
(iii) Contradictory or equivocal (e.g. borderline biological or statistical significance): All other substances.

Follow-up to in vitro testing.

(i) Positive *in vitro* results
In vivo test; selection of an appropriate end point; if necessary, further *in vitro* studies to optimize *in vivo* testing (e.g. kinetochore staining as an addition in the micronucleus assay of *in vitro* aneugens). Follow-up tests *in vitro* may also provide additional mechanistic information to enable interpretation of a positive finding.
(ii) Negative *in vitro* results
In vivo testing is recommended in the case of 'high' or 'moderate and sustained' human exposure or for substances otherwise of high concern. In limited cases, metabolic considerations may trigger *in vivo* testing (38).
(iii) Contradictory or equivocal *in vitro* results
Further *in vitro* testing to clarify positive or negative results; depending on whether the situation is resolved by further *in vitro* testing, proceed according to 'positive' or 'negative'.

In vivo testing

In vivo tests. In vivo tests (see Tables III and IV) should be chosen carefully to avoid an uninformative outcome and with concern for animal welfare. Therefore, toxicokinetics, metabolism and chemical reactivity have to be considered carefully. *In vivo* tests may also be used for evaluation of a dose–response, species differences or mode of action determination. The use of such tests needs to be considered on a case-by-case basis for risk assessment purposes.

The choice of an *in vivo* follow-up test should be guided by the spectrum of genotoxic events observed in the *in vitro* studies as well as knowledge of the bioavailability, distribution, metabolism and target organ specificity of the substance. Typically, a bone marrow micronucleus or clastogenicity test is conducted. However, if there are indications that point to a more appropriate assay, then this assay should be conducted instead (e.g. mutagenicity study with transgenic animals and/or comet assay in potential target tissues).

Follow-up to in vivo testing.

(i) Positive *in vivo* results
Substance is considered an '*in vivo* somatic cell mutagen'. Testing for germ cell mutagenicity (see Strategy for germ cell testing) may be required.

Table I. Common *in vitro* bacterial assays

Assay	Strain	End point	Comments	Published guidelines	References
Salmonella typhimurium reverse mutation assay	TA1535, TA1537 (or TA97 or TA97a), TA98, TA100	Primarily detects G/C base pair and frameshift mutations	Contain specific mutations in one of several genes involved in histidine biosynthesis that must be reverted to function normally. Testing with and without appropriate exogenous metabolic activation system. May not detect some oxidizing mutagens and cross-linking agents.	OECD Test Guideline 471 (replaces old OECD Test Guidelines 471 and 472)	(17–19)
S.typhimurium	TA102	Primarily detects A/T base pair damage and small deletions	Detects oxidizing mutagens and cross-linking agents	OECD Test Guideline 471	(19,20)
Other *S.typhimurium* mutants	YG1021, YG1026 (NR overexpression); YG1024, YG1029 (NAT overexpression)		For detection of mutagenicity of nitroaromatic and aminoaromatic substances that are bioactivated by NR and NAT. More sensitive than conventional strains. Used for detecting mutagenicity of toxic pollutants in air, water and food.		(21,22)
Escherichia coli reverse mutation assay	WP2, WP2*uvrA*	Primarily detects A/T base pair damage	Detects oxidizing mutagens and cross-linking agents	OECD Test Guideline 471	(19)

A, adenine; C, cytosine; G, guanine; NAT, *N*-acetyltransferase; NR, nitroreductase; T, thymine.

Table II. Common *in vitro* mammalian assays

Assay	Method/end point	Main attributes	Comments	Published guidelines	References
Mouse lymphoma TK gene mutation assay	L5178Y mouse lymphoma cell line; using a selective medium, mutant frequencies are determined	Detects not only point mutations but also various sizes of chromosome deletions and other effects that can lead to loss of heterozygosity (e.g. mitotic recombination, gene conversion and translocations)	Use of positive controls and colony sizing essential for quality control. Evaluation and interpretation changed over the years. Recent protocol updates recommendations. Can be used as alternative to metaphase analysis.	OECD Test Guideline 476; IWGT guidelines	(3,23–26)
HPRT gene mutation assay	Chinese hamster ovary, AS52 or other suitable cell line; using a selective medium, mutant frequencies are determined	Detects not only point mutations but also small deletions; larger deletions may be detected in AS52 cells	Use of positive controls essential for quality control	OECD Test Guideline 476	(23,27)
Metaphase analysis (*in vitro* mammalian chromosome aberration test)	A metaphase-arresting substance (e.g. colchicine) is applied; metaphase cells are analysed for the presence of structural chromosome aberrations	Detects clastogenicity; some information on aneugenicity can be obtained with extended culture times	A variety of cell lines, strains or primary cell cultures, including human cells, may be used (e.g. Chinese hamster fibroblasts, human or other mammalian peripheral blood lymphocytes) (28)	OECD Test Guideline 473	(29–31)
Micronucleus test	Detects micronuclei in the cytoplasm of cultured mammalian cells during interphase	Detects both aneugenic and clastogenic substances; established mammalian lines, cultured human peripheral blood lymphocytes or Syrian hamster embryo cells may be used	Several developments in updating the protocol. Immunochemical labelling of kinetochores or hybridization with general or chromosome-specific centromeric/telomeric probes gives information on the nature and mechanism of formation of micronuclei induced (whole chromosomes or fragments).	Draft OECD Test Guideline 487	(13,32–36)

HPRT, hypoxanthine guanine phosphoribosyltransferase; TK, thymidine kinase.

Table III. Common *in vivo* genotoxicity assays

Assay	End point	Main attributes	Comments	Published guidelines	References
Micronucleus test in erythropoietic cells	Structural and numerical chromosome alterations	Long history, regulatory acceptance, high relevance of end point	Has potential for application to other tissues	OECD Test Guideline 474	(15,28), and references cited therein
Metaphase analysis *in vivo*	Structural and numerical chromosome aberrations	Long history, regulatory acceptance, high relevance of end point	Has potential for application to other tissues	OECD Test Guideline 475	(39)
Transgenic animal models	Gene mutation	Can be applied to many tissues. Gene specific. No selective pressure on mutations. Relevant end point.	Need to optimize protocols overall and for each tissue. *lacI*, *lacZ*, *gpt* systems not sensitive to the detection of large deletions. Spi^- system detects large deletions.	IWGT, IPCS guidance	(40–44)
Chemically modified DNA	Covalent DNA adducts, oxidative lesions (e.g. 8-OH-dG)	Can be applied to many tissues. Can be highly sensitive (^{32}P-postlabelling or AMS) or chemically specific (MS). Other methods include immunochemical techniques, fluorescence, ECD (for 8-OH-dG).	Indicator test detecting premutagenic lesions. Interpretation of results can be complicated.	IWGT guidance	(45)
DNA strand breakage assays (e.g. comet assay)	DNA strand breaks, alkali-labile lesions	Can be applied to many tissues. Incorporation of enzymes can improve specificity. Cell division not required.	Indicator tests. Need to optimize protocols for different tissues. May be unable to detect mutagens that do not induce strand breaks or alkali-labile lesions, but may detect repair-induced breaks. Apoptosis/necrosis need to be controlled.	IWGT guidance	(14,46–49)
Liver UDS	Thymidine incorporation outside S phase	Long history of use; useful for some classes of substances.	Indicator test detecting repair activity. Uncertain acceptability and questionable sensitivity. Limited use in other tissues.	OECD Test Guideline 486	(50,51)

8-OH-dG, 8-hydroxy-2′-deoxyguanosine; AMS, accelerator mass spectrometry; ECD, electrochemical detection; MS, mass spectrometry; UDS, unscheduled DNA synthesis.

(ii) Negative *in vivo* results
Further *in vivo* testing is recommended in the case of positive *in vitro* studies. Again, the second *in vivo* test is chosen on a case-by-case basis, as stated above. If the test is negative, it is concluded that there is no evidence for *in vivo* mutagenicity.

(iii) Equivocal *in vivo* results
Equivocal results may be due to low statistical power, which can be improved by increasing the number of treated animals and/or scored cells.
If the situation is unresolved, a second *in vivo* test is recommended, chosen on a case-by-case basis (ordinarily on a different end point or in a different tissue, depending on toxicokinetics, metabolism and mode of action); proceed according to 'positive' or 'negative'.

Strategy for germ cell testing

When information on the risk to the offspring of exposed individuals is important, the following germ cell testing strategy is recommended.

For substances that give positive results for mutagenic effects in somatic cells *in vivo*, their potential to affect germ cells should be considered. If there is toxicokinetic or toxicodynamic evidence that germ cells are actually exposed to the somatic mutagen or its bioactive metabolites, it is reasonable to assume that the substance may also pose a mutagenic hazard to germ cells and thus a risk to future generations.

Where germ cell testing is indicated, judgement should be used to select the most appropriate test strategy. There are a number of tests available (summarized in Table IV), which fall into two classes:

(i) Tests in germ cells *per se* (class 1)
(ii) Tests to detect effects in the offspring (or potential offspring) of exposed animals (class 2)

Three tests that are available for such studies have established OECD test guidelines:

(i) Clastogenicity in rodent spermatogonial cells (class 1): OECD Test Guideline 483 (65)

Table IV. Germ cell assays

Assay	End point	Main attributes[a]	Comments	Published guidelines	References
Class 1: tests in germ cells *per se*					
Transgenic animal models	Gene mutation	Gene specific. No selective pressure on mutations. Relevant end point.	See Table III	See Table III	See Table III
ESTR assay	Non-coding tandem repeat DNA mutation	Potentially relevant end point. Detects heritable mutations at ambient exposure levels. Uses relatively few animals. Can be conducted in humans.	Some tandem repeat mutations also occur in, or near, coding genes. Although there are parallels with mutations in coding genes, the human health outcomes require further study.		(52–55)
Mammalian spermatogonial chromosome aberration test	Structural chromosome aberrations	Relevant end point		OECD Test Guideline 483	(56)
FISH assays	Structural chromosome aberrations; sperm aneuploidy	Relevant end points. Can be conducted in humans.	See Table III	See Table III	(57,58)
Comet assay	DNA strand breaks or alkali-labile sites	See Table III. Can be conducted in humans.	See Table III	See Table III	(59)
Chemically modified DNA	DNA adducts	See Table III. Can be conducted in humans.	See Table III	See Table III	(60)
Class 2: tests to detect effects in the offspring (or potential offspring)					
ESTR assay	As above for class 1 tests	As above for class 1 tests	As above for class 1 tests		As above for class 1 tests
Dominant lethal test	Reduction in viable embryos attributed to chromosome or gene mutations	Relevant end point. Provides data for quantification of pregnancy loss.		OECD Test Guideline 478	(61)
Mouse visible specific locus test	Gene mutation	Provides data for quantification of inherited mutation frequency. Relevant end point.	Uses large number of animals	EPA OPPTS 870.5200	(62)
Mouse biochemical specific locus test	Gene mutation	Provides data for quantification of inherited mutation frequency. Relevant end point.	Uses large number of animals	EPA OPPTS 870.5195	(63)
Mouse heritable translocation assay	Structural chromosome aberrations	Provides data for quantification of inherited mutation frequency. Relevant end point.	Uses large number of animals	OECD Test Guideline 485	(64)

EPA OPPTS, United States Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances; ESTR, Expanded Simple Tandem Repeat; FISH, fluorescence *in situ* hybridization.
[a]'Relevant end point' means relevant to the estimation of human heritable health risk.

(ii) The dominant lethal test (class 2): OECD Test Guideline 478 (66)
(iii) The mouse heritable translocation assay (class 2): OECD Test Guideline 485 (67)

The above-mentioned class 2 tests usually require large numbers of animals. Thus, in order to minimize the use of animals in germ cell testing, it is advisable to start with tests that detect effects in germ cells *per se* (class 1). Other methods include (but are not limited to) gene mutation tests in transgenic animals [see ref. (41) for IWGT guidance], gene mutations in the more recent Expanded Simple Tandem Repeat (ESTR) assay, chromosomal assays (including those using fluorescence *in situ* hybridization), comet assay and DNA adduct analysis.

Following the use of such tests, if quantification of heritable effects is required (class 2), an assay for ESTR mutations can be performed with the offspring of a low number of exposed animals. Tests used historically to investigate transmitted effects (e.g. the heritable translocation test and the specific locus test) can also be performed; however, they use large numbers of animals.

Class 1 and class 2 germ cell assays are summarized in Table IV. The strategy used in germ cell mutagenicity testing is outlined in Figure 2.

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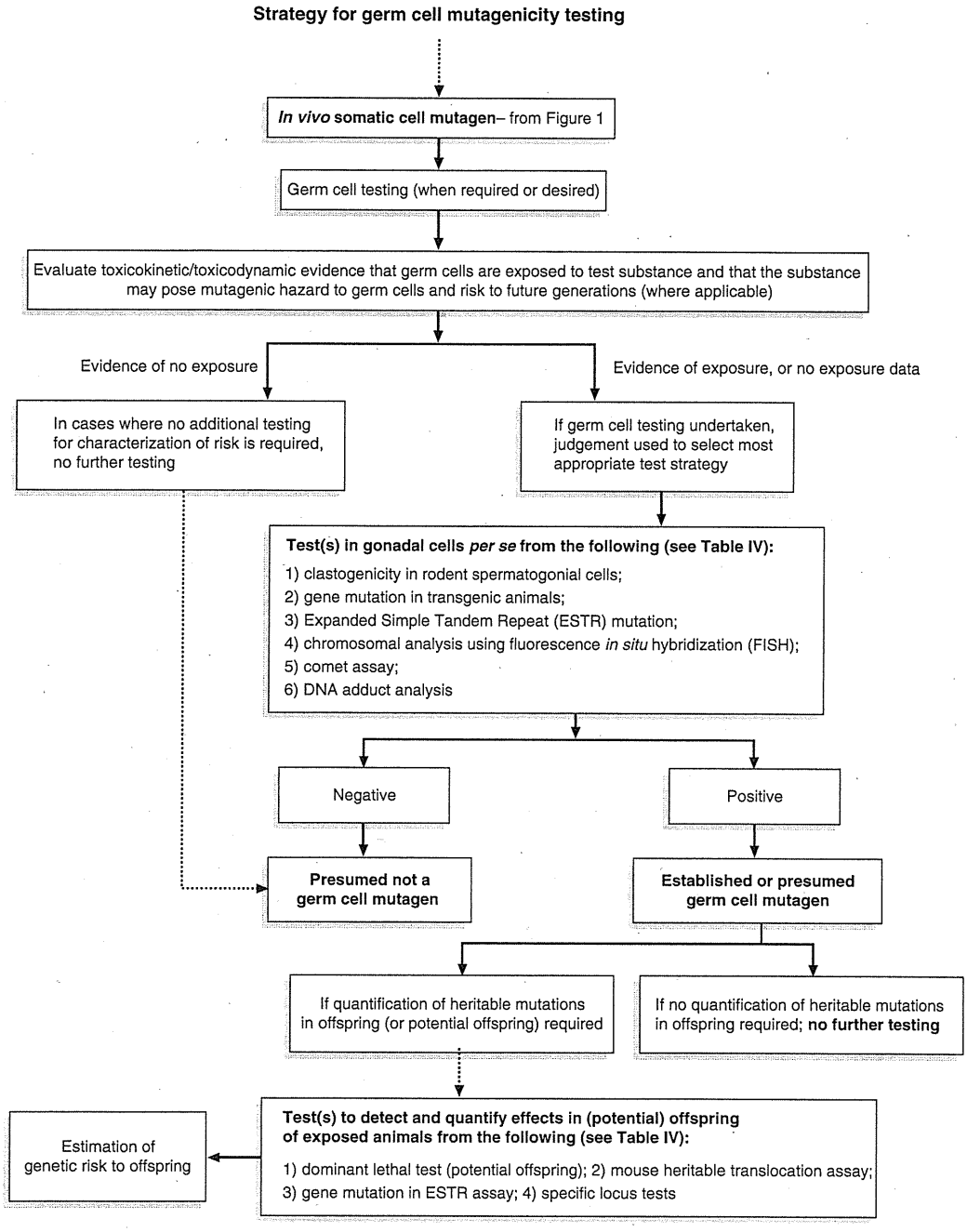

Fig. 2. Strategy in germ cell mutagenicity testing.

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References

1. Ashby, J., Waters, M. D., Preston, J. *et al.* (1996) IPCS harmonization of methods for the prediction and quantification of human carcinogenic/mutagenic hazard, and for indicating the probable mechanism of action of carcinogens. *Mutat. Res.*, **352**, 153–157.
2. ICH Steering Committee (1997) *Guidance for Industry. S2B Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals.* International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. pp. 1–8.
3. Müller, L., Kikuchi, Y., Probst, G., Schechtman, L., Shimada, H., Sofuni, T. and Tweats, D. (1999) ICH-harmonised guidances on genotoxicity testing of pharmaceuticals: evolution, reasoning and impact. *Mutat. Res.*, **436**, 195–225.
4. United Kingdom Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (2000) *Guidance on a Strategy for Testing of Chemicals for Mutagenicity.* pp. 1–36. (http://www.dh.gov.uk/assetRoot/04/07/71/96/04077196.pdf) (last accessed 14 August 2008).
5. United States Food and Drug Administration (2000) *Toxicological Principles for the Safety Assessment of Food Ingredients. Redbook 2000. IV.C.1. Short-Term Tests for Genetic Toxicity.* Center for Food Safety and Applied Nutrition, Office of Food Additive Safety, College Park, MD, pp. 1–5.
6. Dearfield, K. L. and Moore, M. M. (2005) Use of genetic toxicology information for risk assessment. *Environ. Mol. Mutagen.*, **46**, 236–245.
7. Cimino, M. C. (2006) Comparative overview of current international strategies and guidelines for genetic toxicology testing for regulatory purposes. *Environ. Mol. Mutagen.*, **47**, 362–390.
8. Brambilla, G. and Martelli, A. (2003) Failure of the standard battery of short-term tests in detecting some rodent and human genotoxic carcinogens. *Toxicology*, **196**, 1–19.
9. Kirkland, D., Aardema, M., Henderson, L. and Müller, L. (2005) Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens. I. Sensitivity, specificity and relative predictivity. *Mutat. Res.*, **584**, 1–256.
10. Kirkland, D., Aardema, M., Müller, L. and Makoto, H. (2006) Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens. II. Further analysis of mammalian cell results, relative predictivity and tumour profiles. *Mutat. Res.*, **608**, 29–42.
11. Matthews, E. J., Kruhlak, N. L., Cimino, M. C., Benz, R. D. and Contrera, J. F. (2006) An analysis of genetic toxicity, reproductive and developmental toxicity, and carcinogenicity data: I. Identification of carcinogens using surrogate endpoints. *Regul. Toxicol. Pharmacol.*, **44**, 83–96.
12. Kirkland, D. J., Hayashi, M., Jacobson-Kram, D., Kasper, P., MacGregor, J. T., Müller, L. and Uno, Y. (2006) Summary of major conclusions from the 4th IWGT, San Francisco, 9–10 September, 2005. *Mutat. Res.*, **627**, 5–9.
13. Lorge, E., Thybaud, V., Aardema, M. J., Oliver, J., Wakata, A., Lorenzon, G. and Marzin, D. (2006) SFTG international collaborative study on *in vitro* micronucleus test. I. General conditions and overall conclusions of the study. *Mutat. Res.*, **607**, 13–36.
14. Burlinson, B., Tice, R. R., Speit, G. *et al.* (2007) Fourth international workgroup on genotoxicity testing: results of the *in vivo* comet assay workgroup. *Mutat. Res.*, **627**, 31–35.
15. Hayashi, M., MacGregor, J. T., Gatehouse, D. G. *et al.* (2007) *In vivo* erythrocyte micronucleus assay. III. Validation and regulatory acceptance of automated scoring and the use of rat peripheral blood reticulocytes, with discussion of non-hematopoietic target cells and a single dose-level limit test. *Mutat. Res.*, **627**, 10–30.
16. Thybaud, V., Aardema, M., Clements, J. *et al.* (2007) Strategy for genotoxicity testing: hazard identification and risk assessment in relation to *in vitro* testing. *Mutat. Res.*, **627**, 41–58.
17. Ames, B., McCann, J. and Yamasaki, E. (1975) Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. *Mutat. Res.*, **31**, 347–364.
18. Maron, D. M. and Ames, B. N. (1983) Revised methods for the *Salmonella* mutagenicity test. *Mutat. Res.*, **113**, 173–215.
19. Organisation for Economic Co-operation and Development (1997) *Bacterial Reverse Mutation Test.* OECD, Paris (Test Guideline 471).
20. Levin, D., Hollstein, M., Christman, M. F., Schwiers, E. A. and Ames, B. N. (1982) A new *Salmonella* tester strain (TA102) with A*T base pairs at the site of mutation detects oxidative mutagens. *Proc. Natl Acad. Sci. USA*, **79**, 7445–7449.
21. Josephy, P. D., Gruz, P. and Nohmi, T. (1997) Recent advances in the construction of bacterial genotoxicity assays. *Mutat. Res.*, **386**, 1–23.
22. Nohmi, T. (2007) Novel DNA polymerases and novel genotoxicity assays. *Genes Environ.*, **29**, 75–88. (http://www.jstage.jst.go.jp/article/jemsge/29/3/29_75/_article) (last accessed 14 August 2008).
23. Organisation for Economic Co-operation and Development (1997) *In Vitro Mammalian Cell Gene Mutation Test.* OECD, Paris (Test Guideline 476).
24. Moore, M., Honma, M., Clements, J. *et al.* (2002) Mouse lymphoma thymidine kinase gene mutation assay: follow-up International Workshop on Genotoxicity Test Procedures, New Orleans, Louisiana, April 2000. *Environ. Mol. Mutagen.*, **40**, 292–299.
25. Moore, M., Honma, M., Clements, J. *et al.* (2003) Mouse lymphoma thymidine kinase gene mutation assay: International Workshop on Genotoxicity Tests Workgroup report—Plymouth, UK 2002. *Mutat. Res.*, **540**, 127–140.
26. Moore, M. M., Honma, M., Clements, J. *et al.* (2007) Mouse lymphoma thymidine kinase gene mutation assay: meeting of the International Workshop on Genotoxicity Testing—San Francisco, 2005, recommendations for 24-h treatment. *Mutat. Res.*, **627**, 36–40.
27. Aaron, C. S., Bolcsfoldi, G., Glatt, H. R., Moore, M., Nishi, Y., Stankowski, L., Theiss, J. and Thompson, E. (1994) Mammalian cell gene mutation assays working group report. *Mutat. Res.*, **312**, 235–239.
28. Organisation for Economic Co-operation and Development (1997) *Mammalian Erythrocyte Micronucleus Test.* OECD, Paris (Test Guideline 474).
29. Parry, J. M. (ed.) (1996) Molecular cytogenetics. *Mutat. Res.*, **372**, 151–294.
30. Organisation for Economic Co-operation and Development (1997) *In Vitro Mammalian Chromosome Aberration Test.* OECD, Paris (Test Guideline 473).
31. Aardema, M. J., Albertini, S., Arni, P., Henderson, L. M., Kirsch-Volders, M., Mackay, J. M., Sarrif, A. M., Stringer, D. A. and Taalman, R. D. (1998) Aneuploidy: a report of an ECETOC task force. *Mutat. Res.*, **410**, 3–79.
32. Kirsch-Volders, M., Elhajouji, A., Cundari, E. and Van Hummelen, P. (1997) The *in vitro* micronucleus test: a multi-endpoint assay to detect simultaneously mitotic delay, apoptosis, chromosomal breakage, chromosome loss and non-disjunction. *Mutat. Res.*, **392**, 19–30.
33. Kirsch-Volders, M., Sofuni, T., Aardema, M. *et al.* (2003) Report from the *in vitro* micronucleus assay working group. *Mutat. Res.*, **540**, 153–163.
34. Fenech, M. (2000) The *in vitro* micronucleus technique. *Mutat. Res.*, **455**, 81–95.
35. Lorge, E., Lambert, C., Gervais, V., Becourt-Lhote, N., Delongeas, J. L. and Claude, N. (2007) Genetic toxicity assessment employing the best science for human safety evaluation. Part II: performances of the *in vitro* micronucleus test compared to the mouse lymphoma assay and the *in vitro* chromosome aberration assay. *Toxicol. Sci.*, **96**, 214–217.
36. Organisation for Economic Co-operation and Development (2008) *OECD Guideline for the Testing of Chemicals. Draft Proposal for a New Guideline 487: In Vitro Mammalian Cell Micronucleus Test (MNvit).* OECD, Paris, pp. 1–28.
37. Tweats, D. J., Blakey, D., Heflich, R. H. *et al.* (2007) Report of the IWGT working group on strategies and interpretation of regulatory *in vivo* tests. I. Increases in micronucleated bone marrow cells in rodents that do not indicate genotoxic hazards. *Mutat. Res.*, **627**, 78–91.
38. Tweats, D. J., Blakey, D., Heflich, R. H. *et al.* (2007) Report of the IWGT working group on strategy/interpretation for regulatory *in vivo* tests. II. Identification of *in vivo*-only positive compounds in the bone marrow micronucleus test. *Mutat. Res.*, **627**, 92–105.
39. Organisation for Economic Co-operation and Development (1997) *Mammalian Bone Marrow Chromosome Aberration Test.* OECD, Paris (Test Guideline 475).
40. Heddle, J. A., Dean, S., Nohmi, T. *et al.* (2000) *In vivo* transgenic mutation assays. *Environ. Mol. Mutagen.*, **35**, 253–259.
41. Thybaud, V., Dean, S., Nohmi, T. *et al.* (2003) *In vivo* transgenic mutation assays. *Mutat. Res.*, **540**, 141–151.
42. Lambert, I. B., Singer, T. M., Boucher, S. E. and Douglas, G. R. (2005) Detailed review of transgenic rodent mutation assays. *Mutat. Res.*, **590**, 1–280.
43. Nohmi, T. and Masumura, K. (2005) Molecular nature of intrachromosomal deletions and base substitutions induced by environmental mutagens. *Environ. Mol. Mutagen.*, **45**, 150–161.

44. International Programme on Chemical Safety (2006) *Transgenic Animal Mutagenicity Assays*. World Health Organization, Geneva (Environmental Health Criteria 233).
45. Phillips, D., Farmer, P. B., Beland, F. A., Nath, R. G., Poirier, M. C., Reddy, M. V. and Turteltaub, K. W. (2000) Methods of DNA adduct determination and their application to testing compounds for genotoxicity. *Environ. Mol. Mutagen.*, **35**, 222–233.
46. Tice, R. R., Agurell, E., Anderson, D. *et al.* (2000) Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. *Environ. Mol. Mutagen.*, **35**, 206–221.
47. Hartmann, A., Agurell, E., Beevers, C. *et al.* (2003) Recommendations for conducting the *in vivo* alkaline comet assay. 4th International Comet Assay Workshop. *Mutagenesis*, **18**, 45–51.
48. Speit, G. and Hartmann, A. (2005) The comet assay: a sensitive genotoxicity test for the detection of DNA damage. *Methods Mol. Biol.*, **291**, 85–95.
49. Smith, C. C., Adkins, D. J., Martin, E. A. and O'Donovan, M. R. (2008) Recommendations for design of the rat comet assay. *Mutagenesis*, **23**, 233–240.
50. Madle, S., Dean, S. W., Andrae, U. *et al.* (1994) Recommendations for the performance of UDS tests *in vitro* and *in vivo*. *Mutat. Res.*, **312**, 263–285.
51. Organisation for Economic Co-operation and Development (1997) *Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells In Vivo*. OECD, Paris (Test Guideline 486).
52. Dubrova, Y. E., Plumb, M., Brown, J., Fennelly, J., Bois, P., Goodhead, D. and Jeffreys, A. J. (1998) Stage specificity, dose response, and doubling dose for mouse minisatellite germ-line mutation induced by acute radiation. *Proc. Natl Acad. Sci. USA*, **95**, 6251–6255.
53. Yauk, C. L. (2004) Advances in the application of germline tandem repeat instability for in situ monitoring. *Mutat. Res.*, **566**, 169–182.
54. Singer, T. M., Lambert, I. B., Williams, A., Douglas, G. R. and Yauk, C. L. (2006) Detection of induced male germline mutation: correlations and comparisons between traditional germline mutation assays, transgenic rodent assays and expanded simple tandem repeat instability assays. *Mutat. Res.*, **598**, 164–193.
55. Gomes-Pereira, M. and Monckton, D. G. (2006) Chemical modifiers of unstable expanded simple tandem repeats: what goes up, must come down. *Mutat. Res.*, **598**, 15–34.
56. Adler, I. D. (1986) Clastogenic potential in mouse spermatogonia of chemical mutagens related to their cell-cycle specifications. In Ramel, C., Lambert, B. and Magnusson, J. (eds), *Genetic Toxicology of Environmental Chemicals, Part B: Genetic Effects and Applied Mutagenesis*. Liss, New York, pp. 477–484.
57. Wyrobek, A. J. and Adler, I. D. (1996) Detection of aneuploidy in human and rodent sperm using FISH and applications of sperm assays of genetic damage in heritable risk evaluation. *Mutat. Res.*, **352**, 173–179.
58. Hill, F. S., Marchetti, F., Liechty, M., Bishop, J., Hozier, J. and Wyrobek, A. J. (2003) A new FISH assay to simultaneously detect structural and numerical chromosomal abnormalities in mouse sperm. *Mol. Reprod. Dev.*, **66**, 172–180.
59. Haines, G. A., Hendry, J. H., Daniel, C. P. and Morris, I. D. (2002) Germ cell and dose-dependent DNA damage measured by the comet assay in murine spermatozoa after testicular X-irradiation. *Biol. Reprod.*, **67**, 854–861.
60. Horak, S., Polanska, J. and Widlak, P. (2003) Bulky DNA adducts in human sperm: relationship with fertility, semen quality, smoking, and environmental factors. *Mutat. Res.*, **537**, 53–65.
61. Ehling, U. H., Machemer, L., Buselmaier, W. *et al.* (1978) Standard protocol for the dominant lethal test on male mice set up by the work group "Dominant Lethal Mutations of the ad hoc Committee Chemogenetics". *Arch. Toxicol.*, **39**, 173–185.
62. Russell, L. B., Selby, P. B., von Halle, E., Sheridan, W. and Valcovic, L. (1981) The mouse specific locus test with agents other than radiations: interpretation of data and recommendations for future work. *Mutat. Res.*, **86**, 329–354.
63. Lewis, S. E., Barnett, L. B., Felton, C., Johnson, F. M., Skow, L. C., Cacheiro, N. and Shelby, M. D. (1986) Dominant visible and electrophoretically expressed mutations induced in male mice exposed to ethylene oxide by inhalation. *Environ. Mutagen.*, **8**, 867–872.
64. Léonard, A. and Adler, I. D. (1984) Test for heritable translocations in male mammals. In Kilbey, B. J., Legator, M., Nichols, W. and Ramel, C. (eds), *Handbook on Mutagenicity Test Procedures*. 2nd edn. Elsevier Biomedical Press, Amsterdam, pp. 485–494.
65. Organisation for Economic Co-operation and Development (1997) *Mammalian Spermatogonial Chromosome Aberration Test*. OECD, Paris (Test Guideline 483).
66. Organisation for Economic Co-operation and Development (1984) *Genetic Toxicology: Rodent Dominant Lethal Test*. OECD, Paris (Test Guideline 478).
67. Organisation for Economic Co-operation and Development (1986) *Genetic Toxicology: Mouse Heritable Translocation Assay*. OECD, Paris (Test Guideline 485).

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Genetic Analysis of Repair and Damage Tolerance Mechanisms for DNA-Protein Cross-Links in *Escherichia coli*▽§

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DNA-protein cross-links (DPCs) are unique among DNA lesions in their unusually bulky nature. We have recently shown that nucleotide excision repair (NER) and RecBCD-dependent homologous recombination (HR) collaboratively alleviate the lethal effect of DPCs in *Escherichia coli*. In this study, to gain further insight into the damage-processing mechanism for DPCs, we assessed the sensitivities of a panel of repair-deficient *E. coli* mutants to DPC-inducing agents, including formaldehyde (FA) and 5-azacytidine (azaC). We show here that the damage tolerance mechanism involving HR and subsequent replication restart (RR) provides the most effective means of cell survival against DPCs. Translesion synthesis does not serve as an alternative damage tolerance mechanism for DPCs in cell survival. Elimination of DPCs from the genome relies primarily on NER, which provides a second and moderately effective means of cell survival against DPCs. Interestingly, Cho rather than UvrC seems to be an effective nuclease for the NER of DPCs. Together with the genes responsible for HR, RR, and NER, the mutation of genes involved in several aspects of DNA repair and transactions, such as *recQ*, *xth nfo*, *dksA*, and *topA*, rendered cells slightly but significantly sensitive to FA but not azaC, possibly reflecting the complexity of DPCs or cryptic lesions induced by FA. UvrD may have an additional role outside NER, since the *uvrD* mutation conferred a slight azaC sensitivity on cells. Finally, DNA glycosylases mitigate azaC toxicity, independently of the repair of DPCs, presumably by removing 5-azacytosine or its degradation product from the chromosome.

The DNA molecules of living organisms continuously suffer from various types of damage resulting from exposure to endogenous and environmental genotoxic agents. Damage to DNA impairs the faithful propagation of genetic information during replication and transcription, exerting deleterious effects on cells (20). DNA-protein cross-links (DPCs) are unique among DNA lesions in that they are extremely bulky compared to conventional bulky lesions, such as pyrimidine photodimers and the base adducts of aromatic compounds. DPCs are produced by a number of chemical agents, such as aldehydes and heavy metal ions, and also by physical agents such as ionizing radiation and UV light (reviewed in reference 3). DPCs have also been identified in cells or nuclei treated with antitumor agents (4, 10, 44, 62). In addition, we have shown that oxanine, which is produced by nitrosative damage to guanine, mediates the formation of DPCs and polyamine cross-link adducts (49, 50, 52). Thus, understanding the repair and/or damage tolerance mechanism of this ubiquitous and unique class of DNA lesions will provide further insight into how cells maintain genetic integrity and ensure survival in the face of genomic insults. However, the repair and damage tolerance mechanisms of DPCs have long remained elusive, partly because many but not all DPC-inducing agents produce other types of DNA lesions simultaneously, making it rather difficult to elucidate the repair and tolerance mechanisms associated with DPCs alone. Although preceding studies of the sensitivities of repair-deficient *Escherichia coli* mutants to DPC-inducing agents such as formaldehyde (FA) and 5-azacytidine (azaC) provided intriguing insights into the mechanisms of DPC processing in cells (5, 40, 54, 70), a unified mechanism has not yet been established.

We have recently shown in vivo and in vitro evidence that nucleotide excision repair (NER) and homologous recombination (HR) cooperate closely to mitigate the genotoxic effect of DPCs in *E. coli* cells (51). NER removes DPCs with cross-linked proteins smaller than 12 to 14 kDa, whereas oversized DPCs are processed exclusively by RecBCD-dependent HR. The upper size limit of DPCs amenable to NER is determined by the loading efficiency of UvrB, the damage recognition protein in NER, onto DPCs. Consistent with this mechanism, the NER incision efficiency for DNA containing DPCs varies significantly with the size of cross-linked proteins and peptides in vitro (2, 46, 47, 51, 58). Interestingly, no chromosome breakage was observed in cells following FA treatment, although the HR of DPCs proceeded through the RecBCD pathway (51), which is specific to recombination initiated at DNA double-strand breaks. Taken together, these results indicate that *E. coli* cells employ both repair and damage tolerance mechanisms for DPCs. However, a number of repair and damage tolerance genes still remain to be examined for obtaining an entire picture of the repair and tolerance mechanisms of DPCs.

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In the present work, we have systematically assessed the sensitivities of *E. coli* mutants defective in HR, replication restart (RR), translesion synthesis (TLS), NER, base excision repair (BER), transcription, and topological changes of chromosomes to DPC-inducing agents, including FA and azaC. FA is a relatively nonspecific DPC-inducing agent that produces DPCs containing various proteins of sizes greater than 7 kDa in *E. coli* (51). Thus, FA treatment of *E. coli* results in chromosomal DPCs that are processed by both NER (small DPCs) and HR (large DPCs). Conversely, azaC is a specific DPC-inducing agent. azaC is metabolized and incorporated into DNA, covalently trapping DNA cytosine methyltransferases (Dcm) (21, 65). Accordingly, azaC treatment of *E. coli* likely results in chromosomal DPCs containing 53-kDa Dcm, which is the sole Dcm in *E. coli* K-12. Due to the large size of the Dcm protein, Dcm-DPCs are processed by HR. The present results reveal differential or cryptic roles of repair and miscellaneous genes in the processing of DPCs and provide further insights into how cells survive when chromosomal DNA takes on the burden of unusually bulky lesions. Our data also suggest that DNA glycosylases mitigate azaC toxicity, presumably by removing 5-azacytosine or its degradation product from the chromosome.

MATERIALS AND METHODS

Strains, plasmids, and media. All strains used in this study are derivatives of *E. coli* K-12 (1, 6–8, 13, 17, 19, 27, 29, 30, 34–37, 48, 53, 60, 63, 67, 68, 71, 72, 76, 78, 79). The relevant genotypes of the strains and the properties of plasmids are listed in Table 1. A portion of the strains in Table 1 was constructed in this study. The strains deficient in *recQ* (NKJ1514) and *recJ* (NKJ1515) were made via P1 transduction of *recQ*::Tn*3* from KD2250 (53) and *recJ*::Tn*10* from BIK814 (71) into the AB1157 recipient strain, respectively. Transductants were selected for ampicillin resistance (Amp^r) with NKJ1514 and for tetracycline resistance (Tet^r) with NKJ1515. To construct NKJ1500 (*uvrC*), *uvrC279*::Tn*10* from CAG12156 (68) was transduced into AB1157 using P1 phage, and UV-sensitive and Tet^r derivatives were selected. Strains YG2238 (*polA*) and YG6341 (*polB*) were obtained by P1 transduction using AB1157 as the recipient and HRS7052 (chloramphenicol resistant [Cam^r]) (35, 76) and HRS6700 (kanamycin resistant [Kan^r]) (67) as donors, respectively. The *dinB* gene was disrupted as an in-frame deletion without a selection marker as described previously (14). Briefly, using the primers *dinB* W-F (5′-CAAACCCTGAAATCACTGTATACTTT ACCAGTGTTGAGAGGTGAGCAATGATTCCGGGGATCCGTCGACC-3′) and *dinB* W-R (5′-GCACACCAGAATATACATAATAGTATACATCATAATC CCAGCACCAGTTGTGTAGGCTGGAGCTGCTTCG-3′), the Kan^r cassette on pKD13 was amplified and the resultant fragment was flanked upstream and downstream of the *dinB* gene. The fragment was introduced into AB1157 harboring pKD46, and then recombination events generated Kan^r colonies, which are expected to carry Δ*dinB*::Kan^r. Cultivation of the colonies at 43°C cured the temperature-sensitive (Ts) plasmid pKD46, which had been introduced to allow AB1157 to keep the linear PCR fragments intact with its encoding genes derived from lambda phage. The Δ*dinB*::Kan^r strain without pKD46 was designated YG6158. After introducing another Ts plasmid (pCP20) into YG6158, the strain was incubated at 43°C again. The high temperature induced a recombinase FLP and also removed the Ts plasmid from the strain. The FLP specifically acts on the FLP recombination target (FRT) sequences which had been introduced between the *dinB* flanking regions and the Kan^r cassette in advance (Table 1). The recombination removed the Kan^r cassette and produced the Δ*dinB* strain (YG6162). To construct YG6168, Δ(*umuDC*)*596*::*ermGT* was transferred to AB1157 using the DE2302 and EC8 strains as described previously for two-step P1 transduction (19, 78). YG6171 (a *dinB umuDC* double mutant) was constructed by introduction of Δ*dinB*::Kan^r into YG6168, followed by removal of the Kan^r cassette as described above. To introduce Δ*polB*::Kan^r into YG6171, P1 transduction was carried out with HRS6700 as the donor. The resultant *polB dinB umuDC* triple mutant was designated YG6342. Finally, YG6344, which has deletions of *polA*, *polB*, *dinB*, and *umuDC*, was constructed by P1 transduction using YG6342 as the recipient and HRS7052 as the donor. RFM445 [*gyrB203*(Ts)] and RFM475 [*gyrB203*(Ts) Δ(*topA cysB*)204)] are the Ts mutants of topoisomerases. In keeping with the reported Ts properties (17), RFM445 and RFM475 exhibited heat and cold sensitivities, respectively (see Fig. S1 in the supplemental material). pNTR-SD is a set of mobile plasmid clones of *E. coli* open reading frames, and the expression of the open reading frames is strictly controlled by P_{tac} and *lacI*^{q} (61). pNTR-SD containing the *dcm* gene was designated pNTR-SD-Dcm (Table 1).

For FA and azaC treatment, cells were grown in LB, minimal A, or M9 medium. Minimal A medium was comprised of 60 mM K_2HPO_4, 33 mM KH_2PO_4, 7.5 mM $(NH_4)_2SO_4$, 1.7 mM sodium citrate dihydrate, 1 mM $MgSO_4 \cdot 7H_2O$, and 0.2% glucose, and supplemented with 1 μg/ml thiamine and 0.2% Casamino Acids (5). The composition of M9 medium was 47 mM $Na_2HPO_4 \cdot 12H_2O$, 22 mM KH_2PO_4, 8.6 mM NaCl, 19 mM NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.4% glucose, supplemented with 2 μg/ml thiamine, 20 μg/ml thymine, and 100 μg/ml each of arginine, leucine, tryptophan, histidine, and proline (36).

Cell survival assays with FA and azaC. The working solution of FA was prepared freshly from the 37% FA solution (Nacalai Tesque) for every experiment. azaC (Sigma) was dissolved in 50% acetic acid and stored at −20°C until use. Except for the *priA* and other related primosomal mutants (*priB*, *priC*, and *rep*), cells were grown to an optical density at 600 nm (OD_{600}) of 0.3 at 37°C in LB medium for FA treatment or in minimal A medium for azaC treatment. A total of 0.2 ml of cell culture was diluted with 4.8 ml of 66-mM phosphate buffer (pH 6.8) containing different concentrations of FA (indicated in the figures) or with 4.8 ml of minimal A medium containing different concentrations of azaC (indicated in the figures), incubated at 37°C for 30 min with shaking. The *priA*, *priB*, *priC*, and *rep* mutants were similarly grown and treated with FA and azaC in M9 medium, since the *priA* mutant is sensitive to rich media (36). After FA or azaC treatment, cells were diluted and plated on LB, minimal A, or M9 agar plates, and colony formation was typically analyzed after overnight incubation at 37°C. Some strains used in this study grew slowly on M9 plates {AQ10459 [wild type (wt)] and AQ10479 [*priA*]) or LB plates (RFM445 [*gyrB*(Ts)] and RFM475 [*gyrB*(Ts) *topA*]} (see Table S1 in the supplemental material) so that colony formation was analyzed after a few days of incubation. Cells transformed with pNTR-SD-Dcm (Table 1) were grown to an OD_{600} of 0.2 and incubated with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 10 min to induce Dcm. The subsequent FA and azaC treatments were performed as described above for cells without a plasmid. Unless otherwise noted, the survival data are based on three to five independent experiments. Statistical significance was determined by a two-sided unpaired Student *t* test. A *P* value of less than 0.05 was defined as significant.

Measurement of UV and MMC sensitivity. For the measurement of UV sensitivity, cells were grown to an OD_{600} of 0.3 at 37°C in LB medium, serially diluted and plated on LB agar plates, and exposed to the UV light at doses indicated in the figures. After overnight incubation at 37°C, the number of growing colonies was counted. The sensitivity to mitomycin C (MMC; Sigma) was measured as described above for FA.

RESULTS

RR following HR of DPCs depends on PriA but not Rep helicase. In *E. coli*, the PriA, PriB, and PriC proteins play vital roles in restarting chromosome replication through two PriA-dependent mechanisms, i.e., the PriA-PriB and PriA-PriC pathways (Fig. 1A) (24, 36). The RR proteins recognize forked DNA structures, such as arrested replication forks and D-loops, and sequentially load the replicative helicase DnaB (41, 64). To determine the roles of PriA, PriB, and PriC in RR following the HR of DPCs, the sensitivities of *priA*, *priB*, and *priC* mutants to DPC-inducing agents were assayed. The wt and mutant cells were treated with various concentrations of FA and azaC for 30 min, and the cell survival was analyzed. The *priA* mutant was hypersensitive to both FA and azaC (Fig. 1B and E), indicating that RR following the HR of DPCs proceeds through PriA-dependent mechanisms. Compared to wt, the *priB* mutant exhibited a moderate sensitivity to azaC at high concentrations (Fig. 1F), although it was not sensitive to FA (Fig. 1C). The *priC* mutant also showed a slight sensitivity to azaC at high concentrations (Fig. 1F), but the sensitivity increase was not statistically significant. The differential azaC