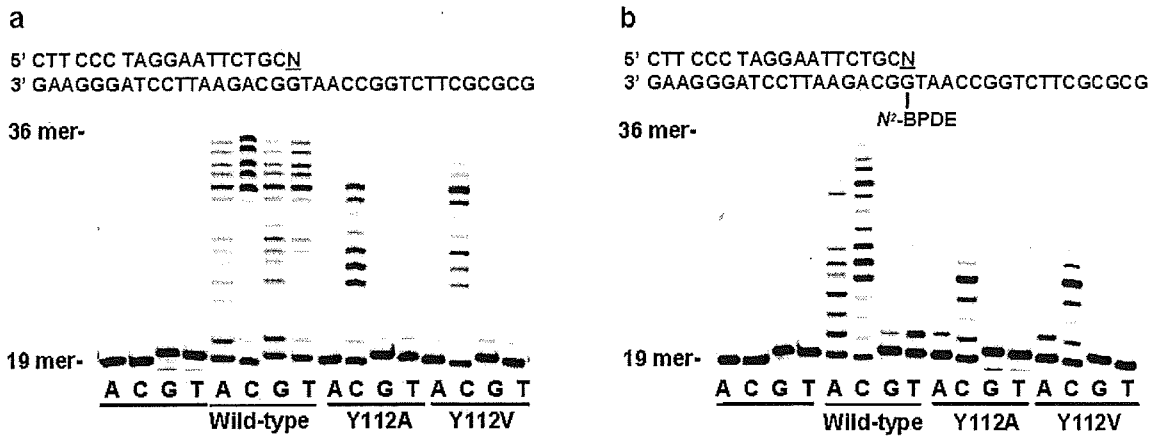

FIGURE 4: Extension from mismatched and matched DNA substrates by wild-type hPolκ, Y112A, and Y112V. Each of four 5′-Cy3-labeled primers (19mers) with either A, C, G, or T at the terminus (N) was annealed to a 36mer undamaged template (a) or a template with BPDE-N^2-dG at position 19 (b). Reactions were conducted for 20 min at 37 °C. The products were resolved by 15% polyacrylamide gel electrophoresis and visualized using a Molecular Imager FX (Bio-Rad) equipped with Quantity One software.

Table 2: Steady-State Kinetic Parameters for Mismatch Extension by WT and Y112A[a]

	k_{cat} (min^{-1})		K_m (μM)		k_{cat}/K_m (μM^{-1} min^{-1})		
base pair at 3′-primer termini (primer-template)	WT[b]	Y112A	WT	Y112A	WT	Y112A	Y112A/WT
C/G	18 ± 1.1	15 ± 0.73	3.5 ± 0.40	65 ± 6.1	5.2	2.3×10^{-1}	1/23
A/G	3.1 ± 0.26	0.11 ± 0.020	89 ± 16	1400 ± 450	3.5×10^{-2}	8.2×10^{-5}	1/420
G/G	4.3 ± 0.40	0.24 ± 0.024	73 ± 16	1700 ± 300	5.9×10^{-2}	1.4×10^{-4}	1/430
T/G	3.6 ± 0.24	0.34 ± 0.039	44 ± 8.3	1500 ± 320	8.2×10^{-2}	2.2×10^{-4}	1/360

[a] Steady-state kinetic parameters were determined for incorporation of dAMP opposite template thymine (T) adjacent to matched (C/G) or mismatched (A/G, G/G, or T/G) primer termini. Exact sequences of matched and mismatched primer-template DNA, i.e., 19C/36G, 19A/36G, 19G/36G, and 19T/36G, are described in Table 1 of the Supporting Information. Primer-template DNA (100 nM) and the proteins (5 nM) were incubated at 37 °C. [b] WT, wild-type hPolκ.

Crucial Role of Y112 in Extension Reactions from Mismatched Termini. Because hPolκ efficiently extends from primer-terminal mismatches, we next examined the possibility that the steric gate amino acid might play a role in mismatch extension reactions. Remarkably, the ability of both Y112A and Y112V to extend primers from mismatched termini was severely reduced compared with extension from matched termini (Figure 4). Reduced extension from mismatched termini was observed when the template DNA had either BPDE-N^2-dG or undamaged dG. To further analyze the effects of the amino acid changes on the mismatched extension reactions, we carried out steady-state kinetic analyses with an undamaged template (36mer) annealed to a 19mer primer with mismatched (A/G, G/G, and T/G) or matched (C/G) termini (Table 2). Y112A exhibited a severely reduced ability to extend primers from mismatched termini; the extension efficiency (k_{cat}/K_m) was 360−430 times lower than that of the wild-type enzyme. In contrast, the extension efficiency from matched termini was only 23-fold lower than that of the wild-type enzyme. Therefore, the amino acid change has an effect that is ~20 times greater on the ability of hPolκ to extend primers from mismatched termini, compared to the extension of primers with matched termini. The reduction in extension efficiency from mismatched termini was due to a reduction in the catalytic constant, i.e., k_{cat}. The k_{cat} values for mismatch extension were reduced 10−30-fold by the Y112A amino acid change, whereas the value for matched-primer extension was not affected by the amino acid change. In contrast, the K_m values were increased 20−30-fold by the Y112 to A change in both mismatched and matched primer termini. From these results, we conclude that the steric gate amino acid is crucial to the chemistry of extension reactions catalyzed by hPolκ from mismatched primer termini.

DISCUSSION

In this study, we report for the first time, to the best of our knowledge, that the steric gate residue of hPolκ plays a critical role in extension from mismatched DNA substrates. Mismatch extension reactions catalyzed by hPolκ were substantially compromised by the replacement of the Y112 amino acid with A or V (Figure 4). The steric gate residue appears to be critical to the chemistry of mismatch extension, i.e., the nucleophilic attack of the misaligned primer 3′-OH group upon the α-phosphate of dNTP, because the replacement of Y112 with A specifically reduced the k_{cat} values for mismatch extension reactions (Table 2). No reduction was observed in the k_{cat} values for matched-primer extension (18 ± 1.1 and 15 ± 0.73 min^{-1} in the wild-type and Y112A enzymes, respectively). Interestingly, amino acid replacement had similar distinct effects on the k_{cat} values for extension from the primer-template with thymine glycol/dAMP or BPDE-N^2-dG/dCMP at the termini (Table 1). The k_{cat} value for extension from primer-template DNA with thymine glycol/dAMP was reduced more than 20-fold by the amino acid replacement (4.4 ± 0.18 and 0.20 ± 0.026 min^{-1} in the wild-type and Y112A enzymes, respectively), while virtually no reduction was seen for extension from primer-template DNA with BPDE-N^2-dG/dCMP (11 ± 0.69 and 9.3 ± 1.7 min^{-1} in the wild-type and Y112A enzymes, respectively). Therefore, we propose that, at least partially, the termini of primer-template DNA with

thymine glycol/dAMP or BPDE-N^2-dG/dCMP lesions structurally resemble "mismatched" and "matched" termini, respectively, in the active site of hPolκ.

The first 18 N-terminal residues of hPolκ also play an important role in mismatch extension (*26*). hPolκ missing the first 18 amino acids has a severely reduced ability to extend mismatched primer termini, although it retains primer extension activity from matched termini. Because the first 18 amino acids contribute to the proficiency of DNA binding by hPolκ, it is surmised that the 18 amino acids and the subsequent N-clasp domain may contribute to the encirclement of DNA and may increase the time during which the mismatched primer 3′-OH group can acquire proper alignments for nucleophilic attack (*26*). Although the catalytic core of hPolκ_{19-526} in a ternary complex with DNA and an incoming nucleotide has been crystallized and the structure has been determined, it lacks the first 18 amino acids (*26*). Therefore, the exact position adopted by these N-terminal amino acids in the complete structure is unclear. We suggest that Y112 may play a critical role in mismatch extension reactions in a manner distinct from that of the action of the 18 N-terminal amino acids. This is because replacing Y112 with A did not reduce the affinity of hPolκ for primer-template DNA (Figure 2 and Table 3 of the Supporting Information), while removing the 18 N-terminal amino acids strongly affects the enzyme's ability to bind to DNA. In addition, the steric gate amino acid is close to primer termini and incoming dNTPs, whereas the 18 N-terminal amino acids may not be. Given the location of Y112, we speculate that Y112 may directly interact with a mismatched terminal base in the primer, thereby preventing the terminal base from moving from the position of the cognate Watson−Crick pairing terminal bases (Figure 5a). This may be a prerequisite for extension from mismatched primer termini, because the mismatched terminal bases, which are otherwise moved from the normal base pairing positions, should be in the proximal normal positions in the active site to acquire proper alignment. The presence of the extended N-terminal domain of hPolκ may enable the steric gate amino acid to interact with the terminal base in the primer, which thereby plays a decisive role in mismatch primer extension. Carlson et al. (*33*) reported that hPolκ forms productive complexes with mismatched primer termini but not with matched primer termini. Both the 18 N-terminal amino acids and Y112 may play roles in the formation of the productive complexes with the mismatched termini. In addition, the steric gate may guide incoming dNTPs to a particular position, where the chemistry between the primer 3′-OH group and α-phosphate of the dNTP can take place. Y112A may have a less constrained active site, which decreases the probability of achieving proper alignment for the phosphoryl transfer reactions, resulting in a decrease in k_{cat} values for mismatch primer extension (Figure 5b).

Immediately adjacent to the steric gate in hPolκ is the highly conserved amino acid F111. Replacing the corresponding amino acid with certain other amino acids in several B-family Pols such as yeast Polζ results in a decrease in the fidelity of DNA synthesis and an increase in the efficiency of mismatch extension (*34*, *35*). Although the exact mechanisms by which this conserved amino acid plays a role in the extension of mismatched termini are not known, amino acid substitutions may alter the geometry of the nascent base pair binding pocket and/or the chemistry of the reaction (*35*). To examine whether hPolκ F111 affects the efficiency of extension reactions from mismatched termini, we changed F111 to A and purified the protein. However, F111A displayed significantly reduced DNA synthesis activity (data not shown). Replacing both F111 and Y112 with A resulted in greatly reduced activity. Thus, we could not determine the efficiency of extension reactions from mismatched termini with F111A or F111A/Y112A. Interestingly, F34L of yeast Polη, which is located next to the steric gate amino acid F35, exhibits substantially reduced DNA synthesis activity (*34*). Therefore, we suggest that unlike B-family Pols, the conserved amino acids adjacent to the steric gate in Y-family Pols, i.e., F111 in hPolκ and F34 in yeast Polη, may primarily play roles in DNA synthesis activity.

Although Y112 was nonessential for TLS across BPDE-N^2-dG in DNA, replacing Y112 with A weakened the ability of hPolκ to bypass several other lesions such as thymine glycol (Figure 2 and Table 1). The efficiency (k_{cat}/K_m) of incorporation of dAMP opposite thymine glycol was ~350 times lower than that of the wild-type enzyme, while the efficiency of incorporation of dAMP opposite undamaged thymine was only 13 times lower than that of the wild-type enzyme. In addition, the k_{cat}/K_m for incorrect incorporation of dNMP opposite an undamaged base was 40−60 times lower in Y112A than in the wild-type enzyme (Table 4 of the Supporting Information), while the k_{cat}/K_m for correct incorporation of dCMP opposite template guanine was ~9 times lower than that of the wild-type enzyme. We propose therefore that replacing Y112 with A may alter the geometry of the nascent base pairing binding pocket, which in turn increases selectivity against both incorporation of dNTP

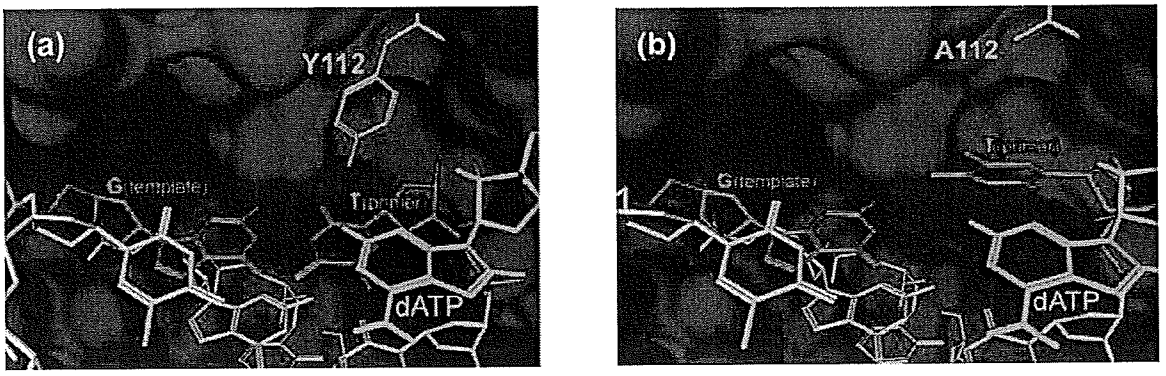

FIGURE 5: Images of the active site of wild-type hPolκ (a) and Y112A (b). The steric gate Y112 may interact with primer T, thereby locking T in a position close to template G (a). A112 has no interactions with primer T, and thus, primer T is positioned far from template G (b). The distance between A112 and primer T is more than 5 Å greater than that between Y112 and primer T. Y112 (a) and A112 (b) are highlighted in orange. A mismatched base pairing of template G with primer T (red) was modeled in the active site with incoming dATP (light blue). The remaining template and primer strands are colored light brown. The active site of hPolκ is displayed as a Connolly surface (blue).

opposite thymine glycol and incorrect incorporation of dNTP opposite an undamaged template base. In this respect, BPDE-N^2-dG is exceptional, because changing Y112 to A or V enhanced the efficiency of incorporation of dCMP opposite the lesion (Figure 2 and Table 1). Template guanine base modified with BPDE might better fit the less constrained active site in Y112A or Y112V than in the native one.

hPolκ strongly binds to primer-template DNA with BPDE-N^2-dG when the primer possesses dCMP opposite the lesion (Figure 3 and Table 2 of the Supporting Information). No such strong binding was observed with primer-template DNA with any of the other lesions that were examined in this study. Structural analysis of the hPolκ catalytic core suggests a model in which there is a cleft in the active site, through which the long chain of *trans*-4-hydroxy-2-nonenal-dG (HNE-dG) in the template extends into the solvent (*26*). By analogy with the position of the HNE-dG adduct, we postulate that the BPDE adduct also fits into the cleft, thereby reducing the level of obstruction of DNA synthesis mediated by hPolκ. Similar strong binding to primer-template DNA with a thymine dimer/dAMP base pair is observed with hPolη (*36*). Intriguingly, the strong binding by hPolη requires the presence of the correct nucleotide, i.e., dAMP, opposite the lesion. The strong binding disappears after Polη has inserted two nucleotides beyond the lesion, which is remarkably similar to the mode of binding of hPolκ to DNA with BPDE-N^2-dG. Because hPolκ and hPolη bypass BPDE-N^2-dG and thymine dimer, respectively, in an error-free manner, it is tempting to speculate that they are cognate lesions for these two Pols. Alternatively, structurally similar but endogenous DNA lesions such as steroid hormone DNA adducts (*37*) could be the cognate lesions for hPolκ. It has been proposed that the strong binding of hPolη may have implications for Pol switching and the restriction of error-prone Pols to damaged sites (*36*). Likewise, the strong binding of hPolκ may contribute to the mechanism of transient access to primer-template DNA with BPDE-N^2-dG by this intrinsically error-prone Pol.

In summary, we found that the steric gate amino acid Y112 was crucial to mismatch extension reactions catalyzed by hPolκ. Y112 appears to play an important role in the chemistry of mismatch extension. It may directly interact with the mismatched terminal base in the primer strand and prevent movement from the normal matched base pairing position in the active site (Figure 5). Unlike bacterial and archaeal orthologues, in which the steric gates are essential for TLS across N^2-dG adducts in DNA (*12*), hPolκ Y112 may be unnecessary in bypass reactions across BPDE-N^2-dG in DNA. *E. coli* DinB, which lacks the N-terminal clasp domain (Figure 1 of the Supporting Information), does not display high efficiency in mismatch extension, although it has a steric gate (*38*). We speculate, therefore, that the steric gate amino acid may have evolved into a major fidelity factor that regulates mismatch extension in hPolκ when the ancestral bacterial DinB and archaeal orthologue gained the extra N-terminal domain.

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SUPPORTING INFORMATION AVAILABLE

Detailed experimental protocols, sequences of primer-template DNA, affinity of hPolκ and the mutants for primer-template DNA with or without BPDE-N^2-dG, kinetic parameters for incorporation of dNTP opposite template G by hPolκ and Y112A, and purification of hPolκ and the mutants. This material is available free of charge via the Internet at http://pubs.acs.org.

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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

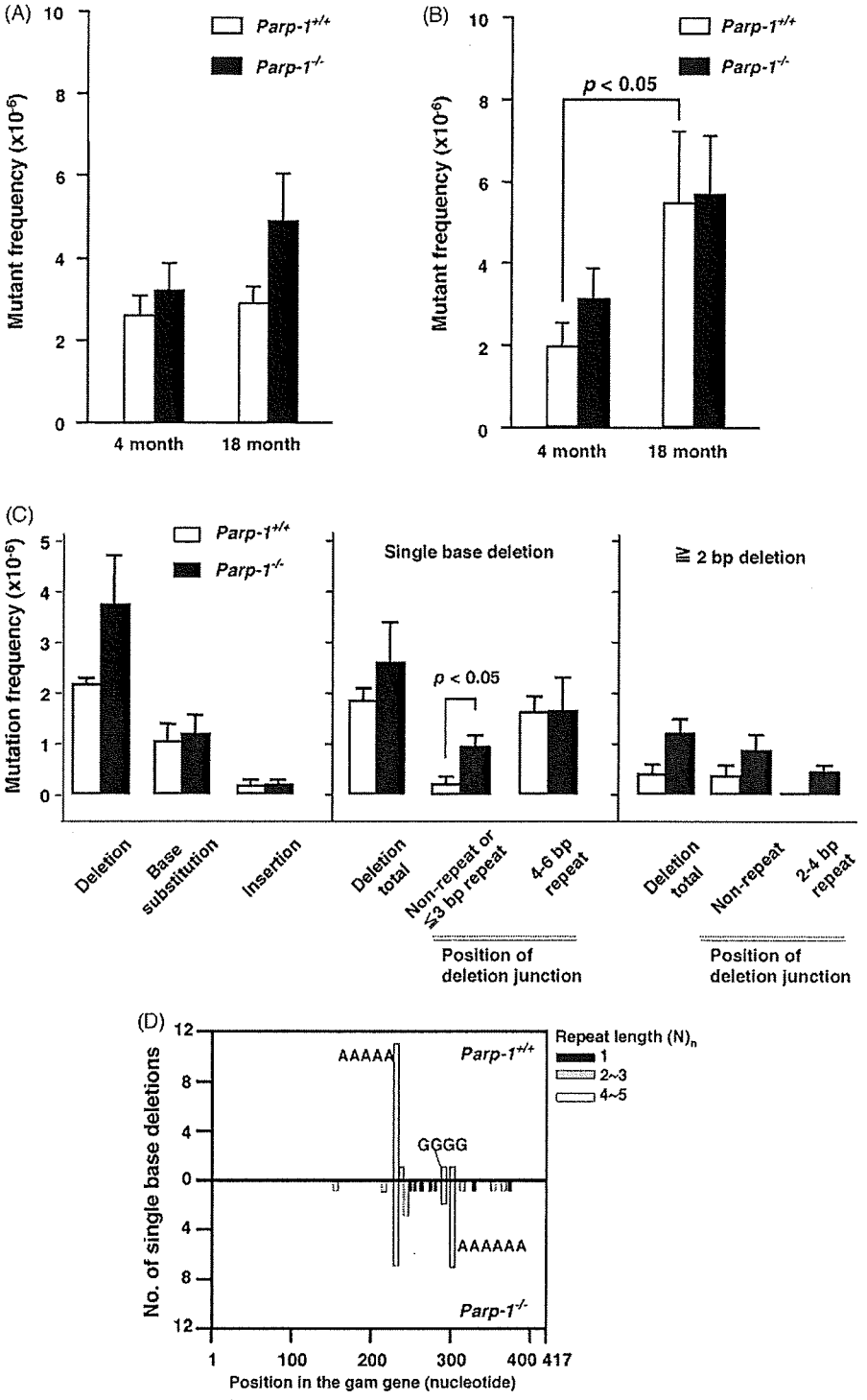

Fig. 1. Spontaneous mutant frequencies of the *red/gam* and *gpt* genes in the liver of *Parp-1*$^{-/-}$ and *Parp-1*$^{+/+}$ mice at 4 and 18 months of age. (A) Spontaneous mutant frequencies of the *red/gam* genes in the livers. (B) Spontaneous mutant frequencies in the *gpt* genes of the livers. Error 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 $2–6 \times 10^{-6}$ and $1–5 \times 10^{-6}$, 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.

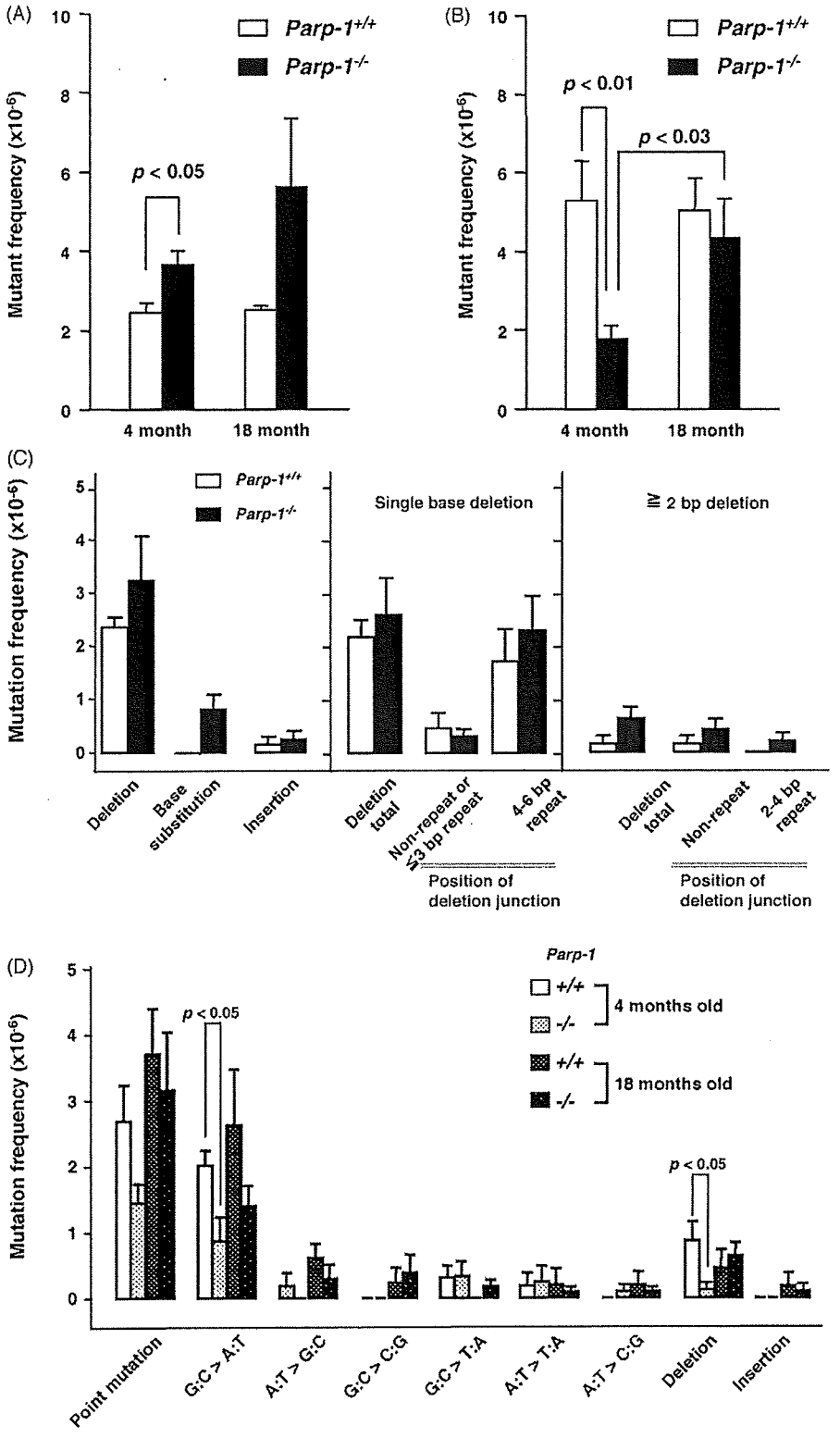

Fig. 2. Spontaneous mutant frequencies of the *red/gam* and *gpt* genes in the brain of *Parp-1*$^{-/-}$ and *Parp-1*$^{+/+}$ mice at 4 and 18 months of age. (A) Spontaneous mutant frequencies of the *red/gam* genes. (B) Spontaneous mutant frequencies in the *gpt* genes. Error bars represent standard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

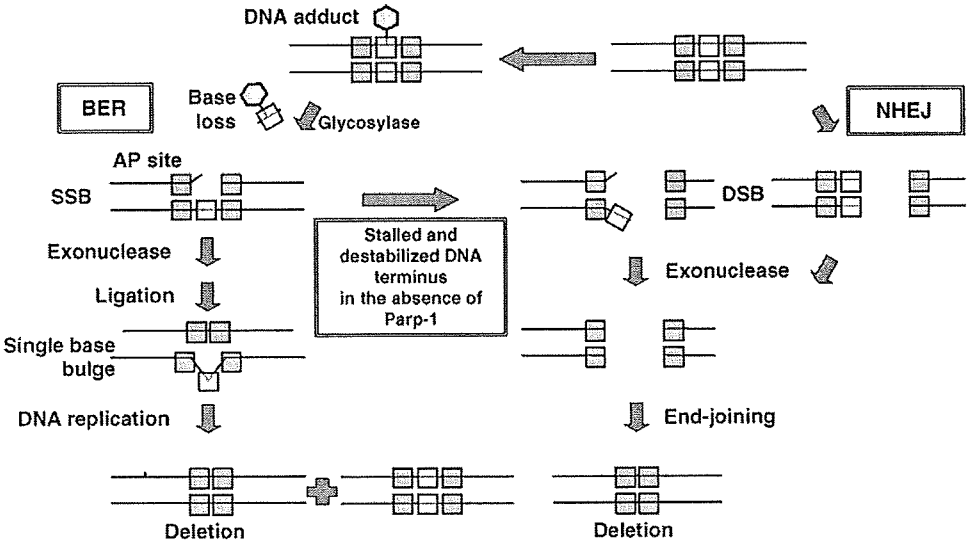

Fig. 3. A model for augmented development of deletion mutation through imprecise BER or NHEJ process in the absence of Parp-1. During BER, after single strand breaks are introduced following damaged base removal, the DNA terminus may be destabilized in the absence of Parp-1. Base loss could occur by 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.

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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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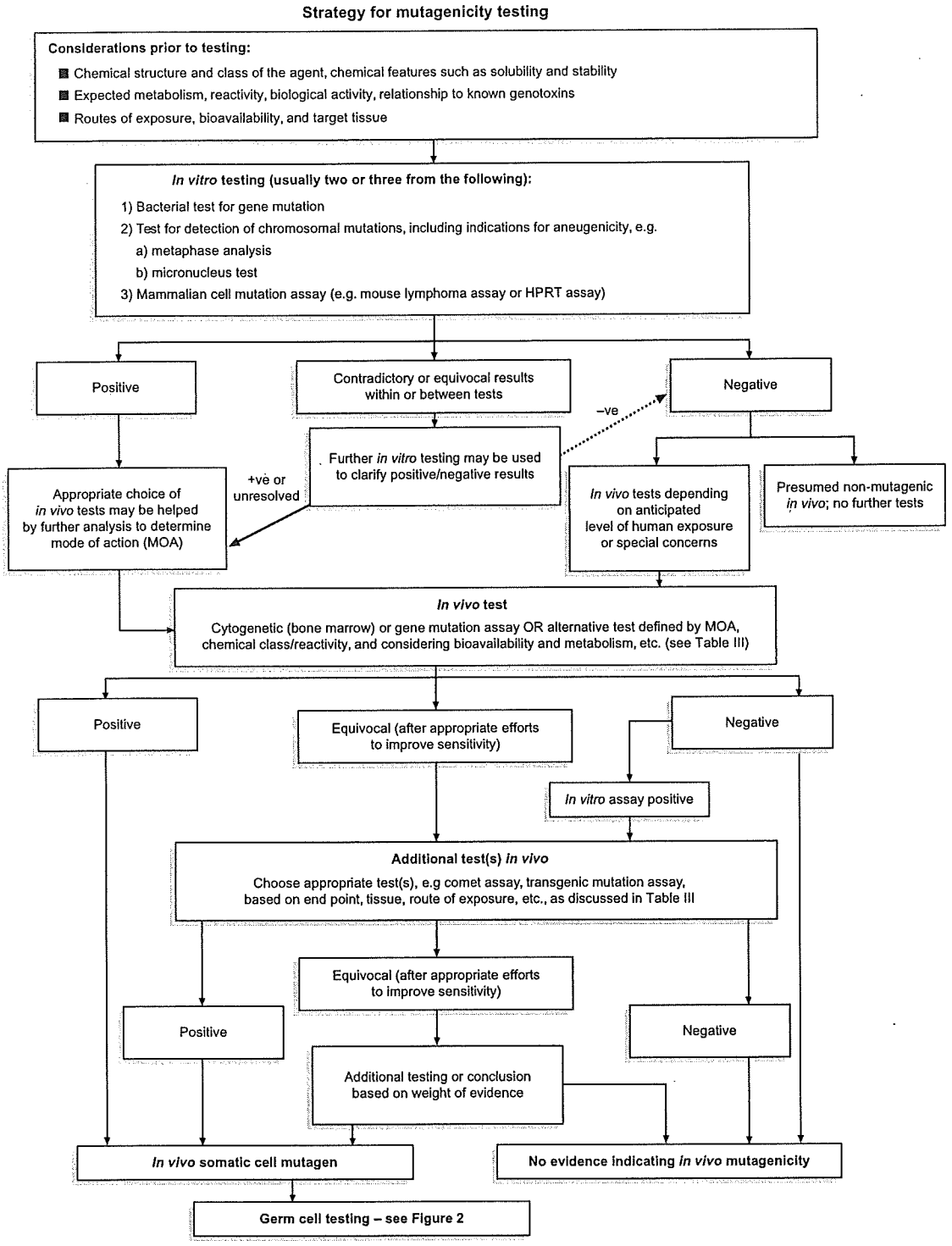

Fig. 1. Strategy for mutagenicity testing.

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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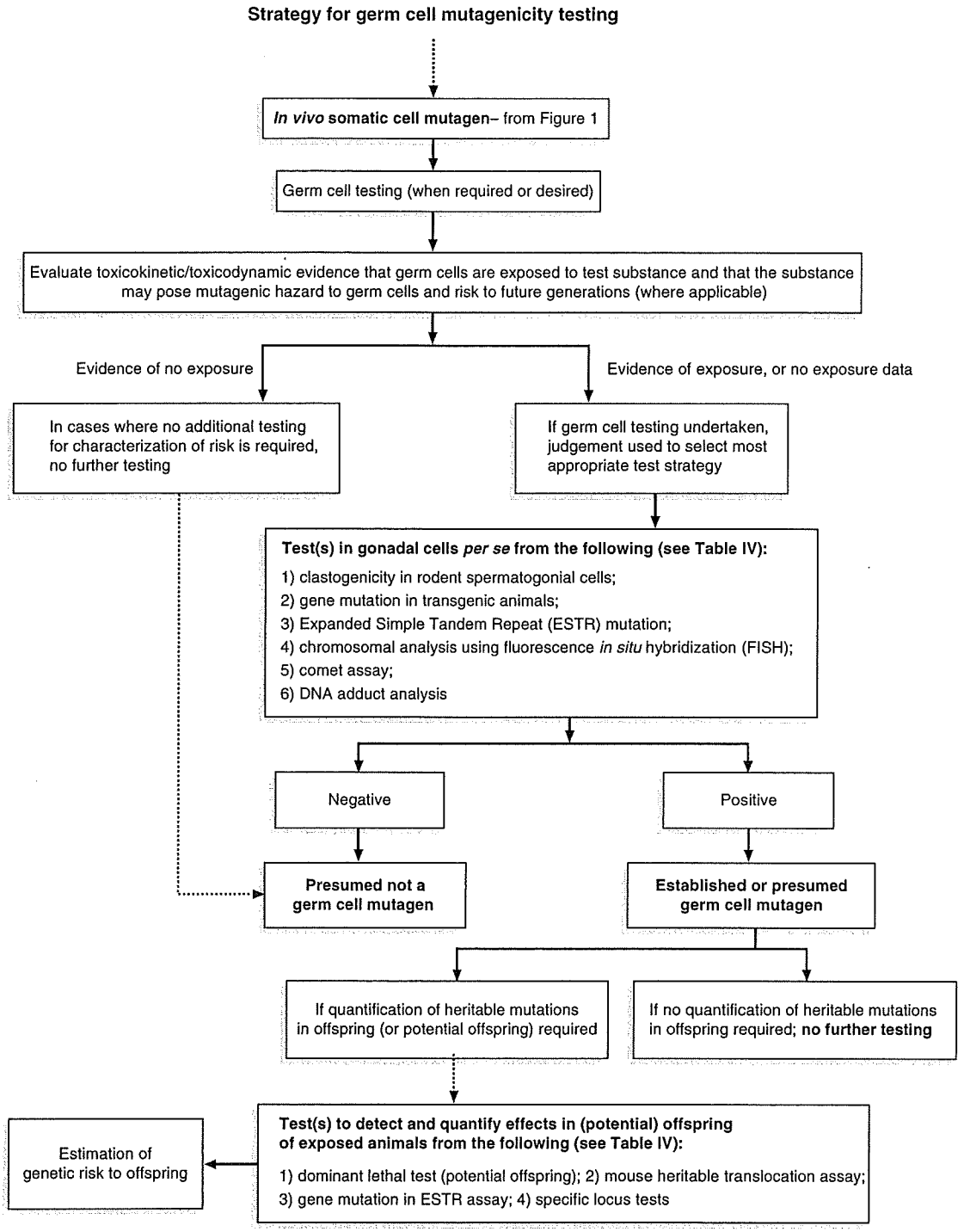

Fig. 2. Strategy in germ cell mutagenicity testing.

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