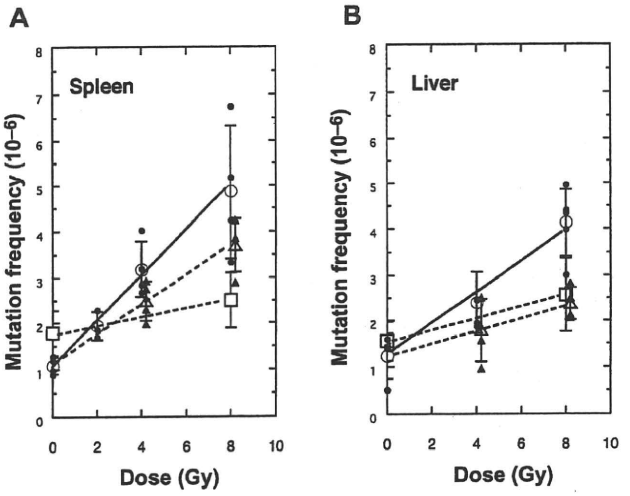

FIG. 1. Radiation-induced mutations at different dose rates. *Gpt* delta × SWR F_1 mice were irradiated with γ rays at a rate of 920 mGy/min, 1 mGy/min and 12.5 μGy/min. Mutation frequencies in the spleen (panel A) and liver (panel B) were determined in the *red-gam* genes. The small closed symbols indicate mutant frequencies for each mouse and the large open symbols indicate the means ± SD. Circles with solid lines indicate the dose response at 920 mGy/min, triangles with dotted lines 1 mGy/min, and squares with dotted lines 12.5 μGy/min.

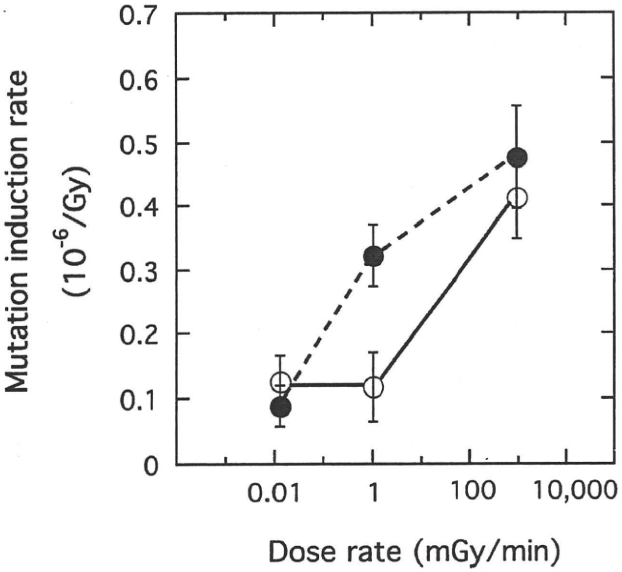

FIG. 2. Dose-rate dependence of the radiation-induced mutation rate in the *red-gam* genes. The slopes of the lines in Fig. 1 were analyzed by linear regression and plotted as a function of the dose rate. The open circles with solid lines indicate the liver, and the closed circles with dotted lines indicate the spleen. The vertical lines indicate standard errors of the slopes. The mutation induction rates in spleen and liver at 1 mGy/min are statistically significantly different ($P = 0.0037$).

primers surrounding one terminal of the inserted sequence at its original location. The positions of the primers were at 4476–4496 (21 mer) and 6638–6658 (21 mer) in lambda EG10.

Statistical Analysis

The statistical analyses were performed using StatView 5.0 software (http://www/hulinks.co.jp/software/statview). Mutant frequencies were analyzed with the *t* test. The dose–response curves of mutation induction were examined by regression analyses. The variations in the dose responses under different dose rates as well as between the spleen and liver were analyzed by ANCOVA. Occurrences of different types of mutations were analyzed with the contingency test. In all cases, a *P* value of less than 0.05 was considered to be a statistically significant difference.

RESULTS

Mutations Induced by Radiation Delivered at Different Dose Rates

Mutation frequencies observed after irradiation with different doses of high- (920 mGy/min), medium- (1 mGy/min) and low-dose-rate (12.5 μGy/min) γ rays in the spleens and livers of *gpt* delta mice are shown in Fig. 1. The mutant frequencies for each individual mouse is shown in Supplementary Tables S1 and S2. In control nonirradiated mice, slight increases in mutation frequency were observed between 2 and 18 months of age in the two tissues. The increase in the spleen was from $(1.083 \pm 0.180) \times 10^{-6}$ (4 mice) to $(1.783 \pm 0.492) \times 10^{-6}$ (9 mice), and the difference was statistically significant with a *P* value of 0.0033. The slight increase in the liver was not statistically significant ($P = 0.243$). After 2 to 8 Gy of high- or medium-dose-rate radiation, a linear increase in the mutant frequency was observed in the two tissues. The linear regression analyses of the results showed the relationships between mutant frequency ($\times 10^{-6}$) and dose (in Gy) as follows: mutant frequency $1.11 + (0.477 \pm 0.081)D$, $r = 0.871$ for high-dose-rate spleen, $1.14 + (0.323 \pm 0.048)D$, $r = 0.922$ for medium-dose-rate spleen, $0.743 + (0.413 \pm 0.064)D$, $r = 0.917$ for high-dose-rate liver, and $1.364 + (0.118 \pm 0.053)D$, $r = 0.643$ for medium-dose-rate liver. The mutant frequencies after irradiation with the low dose rate (12.5 μGy/min) were $(2.496 \pm 0.610) \times 10^{-6}$ (10 mice) in spleen and $(2.560 \pm 0.808) \times 10^{-6}$ (10 mice) in liver, whereas the levels in age-matched control mice were $(1.783 \pm 0.492) \times 10^{-6}$ (9 mice) in spleen and $(1.553 \pm 0.424) \times 10^{-6}$ (8 mice) in liver. The differences between the 18-month-old nonirradiated control mice and the irradiated mice were statistically significant in both spleen ($P = 0.0128$) and liver ($P = 0.0043$). The linear regression analyses of these data showed the following relationships: mutant frequency $= 1.78 + (0.089 \pm 0.031)D$, $r = 0.572$ for spleen and $1.554 + (0.126 \pm 0.040)D$, $r = 0.622$ for liver.

The slopes of the dose–response regression lines were plotted as a function of the dose rate in Fig. 2. The mutation induction rate in spleen was different between the high and low dose rate ($P = 0.0002$) and between the medium and low dose rate ($P = 0.0370$) but not between the high and medium dose rate ($P = 0.14$). In the liver,

the mutation induction rate with high-dose-rate radiation was different from medium- and low-dose-rate radiation at statistically significant levels, $P = 0.0024$ and 0.0035, respectively. The mutation induction rates at 1 mGy/min and 12.5 μGy/min were similar. When the mutation induction rates in spleen and liver were compared, a statistically significant difference was found for medium dose rate ($P = 0.0037$) but not for high or low dose rate.

Molecular Nature of Radiation-Induced Mutations

In an attempt to determine whether specific types of mutations were induced by high- and low-dose-rate irradiation, 26 to 38 mutant clones were collected randomly from three mice from each experimental group and the DNA was sequenced. Some mutants showed identical alterations to other mutants isolated from the same DNA sample. These mutants were eliminated from counting because they were likely to have represented mutated DNA produced through replication of DNA and may not have reflected original mutations. The total number of original mutants sequenced was 236. All of the mutant clones showed deletion mutations in the *gam* gene, except for two clones that showed base substitutions in the *gam* gene and deletions or tandem mutations in the *red* gene.

The original mutants were classified into six groups: 1-bp deletion, 2–1,000-bp deletion, more than 1,000-bp deletion, insertions, multiple mutations, and complex mutations. A multiple mutation was defined as two or more mutations in separate positions in the *red-gam* gene or in its neighboring region. Complex mutations included all kinds of mutations that were not included in the former groups. They could be explained by the simultaneous occurrence of a deletion and insertion in one location. The percentages of each type of mutation found in irradiated and nonirradiated mice are shown in Fig. 3. In nonirradiated mice, in both tissues, the 1-bp deletions were dominant followed by the deletion of more than 1,000 bp. This pattern was similar even as the mice aged (up to 18 months). In irradiated mice, the fraction of 2-bp to 1-kbp deletions was elevated regardless of tissue or mode of irradiation. The elevation in mutations after high-dose-rate irradiation with 4 Gy in spleen and 8 Gy in liver and after low-dose-rate irradiation in the spleen with a total dose of 8 Gy were statistically significant at the level of $P = 0.0232$, 0.000745 and 0.0128, respectively. The elevation in the spleen after a high-dose-rate 8-Gy irradiation and in the liver after a low-dose-rate irradiation were not statistically significant, with P values of 0.054 and 0.11, respectively. These results suggest that deletion mutations of 2 to 1,000 bp are induced by radiation. No significant difference in the mutation spectra after high- or low-dose-rate irradiation were found.

Other types of mutations such as insertions and multiple and complex mutations were not frequent. The exact sequence changes found in the multiple and complex mutations are listed in Table 2. All 11 complex mutations could be explained by the simultaneous occurrence of a deletion and an insertion in one place. Eleven out of 14 multiple mutations showed two mutations in separate positions. Some of these included complex mutations and tandem mutations. The remaining three multiple mutations were more complicated. Clone 18L6-10 from the liver of a chronically irradiated mouse showed two base substitutions and one base deletion in nearby locations (Table 2). These mutations could also be explained by a 5-bp deletion of 25,100–25,104 and a simultaneous insertion of AG. In that case, this clone should be classified as a complex type mutation. The clone 18L2-1 found in the liver of an old nonirradiated mouse showed five base substitutions and one large deletion of –7,664 bp. Three of the five base substitutions were located at 29, 20 and 13 bp from the left end terminal of the deletion. The other two base substitutions were located at 238 and 828 bp from the right end of deletion. All five base substitutions were G:C to A:T transitions. The third multiple mutation was one found in the spleen of a high-dose-rate-irradiated mouse, 2S4-5, exposed to 8 Gy. This showed two base substitutions at 36,025 and 38,876 together with a complex mutation at a distant site. The complex mutation was composed of an 8,936-bp deletion (24,136 to 33,071) and a 6,139-bp insertion in the same location. Most of the inserted sequence, 6,136 bp out of 6,139 bp, showed homology to another part of lambda EG10 (12 to 6,147) in an inverted direction. At the left end junction of the deletion and insertion, an extra insertion of ACA was observed. These alterations are illustrated in Fig. 4. The presence of the original sequence of 12 to 6,147 bp was confirmed at the correct position in the mutant clone with PCR by using primers located at the upstream and downstream sides of 6,147. Thus the inserted sequence must have been duplicated from the original sequence rather than translocated.

DISCUSSION

The present study shows that the mutagenic effects of radiation in somatic tissues depend on both the dose rate and the tissue. In the liver, the efficiency of mutation induction was reduced from 0.41×10^{-6}/Gy to 0.12×10^{-6}/Gy when the dose rate was reduced from 920 mGy/min to 1 mGy/min, but it remained at a similar level when the dose rate was reduced further to 12.5 μGy/min (0.13×10^{-6}/Gy). The efficiency at high dose rates was similar to that observed previously with 0.77 Gy/min of X rays using *gpt* delta mice (*18*). The dose-rate dependence resembled the previous observations made in spermatogonia (*3, 4*), splenic T cells (*1*), and intestinal

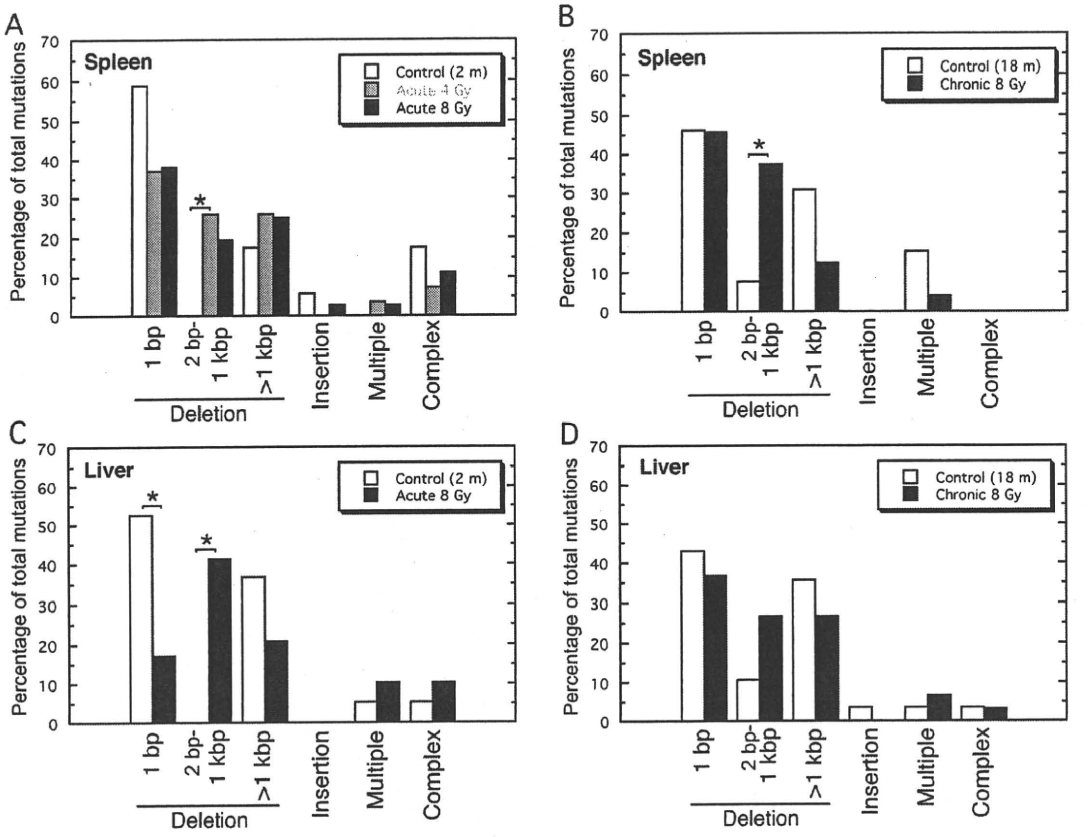

FIG. 3. Frequencies of different types of mutations in control and irradiated mice. Deletion mutations were classified into three groups according to the size of deleted fragments: 1 bp, 2 bp–1 kbp and more than 1 kbp. The incidence of each type of mutation among the non-redundant mutations was calculated. Open bars indicate unirradiated age-matched control mice while the gray and black bars show 4-Gy-irradiated and 8-Gy-irradiated mice, respectively. Panel A: Spleen with or without high-dose-rate irradiation with 4 Gy or 8 Gy. Panel B: Spleen with or without low-dose-rate irradiation with 8 Gy. Panel C: Liver with or without high-dose-rate irradiation with 8 Gy. Panel D: Liver with or without low-dose-rate irradiation with 8 Gy. The percentages of 2- bp–1-kbp deletions were elevated after irradiation. Asterisks show statistically significant differences between control and irradiated mice.

stem cells (*2*). In spleen, on the other hand, the efficiency decreased steadily from 0.48×10^{-6}/Gy to 0.089×10^{-6}/Gy as the dose rate was reduced from 920 mGy/min to 12.5 μGy/min. The efficiency was higher in spleen than in liver when the dose rate was 1 mGy/min but was similar at 920 mGy/min and 12.5 μGy/min (Fig. 2).

The reduction of the mutation induction rate observed when the dose rate decreased from 1 mGy/min to 12.5 μGy/min in spleen does not agree with a previous study on splenic T lymphocytes in which *Hprt* mutation rates showed a dose-rate effect between 500 mGy/min and 0.69 mGy/min but no further reduction at 99 μGy/min (*1*). This discrepancy might be explained by an underestimation of the *Hprt* mutation frequency, especially with low-dose-rate exposures, because the *Hprt* mutant frequency is reported to decline with time (*1*). For low-dose-rate irradiation, the mutations induced at early stages might be eliminated if the assays were performed at long times after the beginning of the irradiation.

In the present study, the dose–response curves were estimated to be linear for the three dose rates examined. Linearity was also observed for mutagenic effects in germ-line cells (*4*). On the other hand, many other genetic effects, including chromosome abnormalities, had linear-quadratic dose responses (*22*). Thus the dose response of mutagenic effects could also be linear-quadratic rather than linear. Because the data obtained in our study were limited, it is difficult to determine whether the dose–response relationship for the mutagenic effects were linear or linear-quadratic (Fig. 1, Supplementary Tables S1, S2).

The low dose rate we adopted was 12.5 μGy/min, and the irradiation continued for 483 days. Under these conditions, many of the mutations observed would reflect mutations accumulated in stem cells, especially in spleen, which is a cell-proliferating tissue. The high and medium dose rates, on the other hand, were 920 mGy/min and 1 mGy/min and continued for 8.7 min and 5.56 days to reach to total dose of 8 Gy, respectively. The

TABLE 2
Unusual Mutations

Tissue	Age (months)	Dose (Gy)[a]	Mutant number	Position	Change[b]	Class[c]
Spleen	2	4	2S1-6	24,987–24,997	GCCA···CTGA → GC(G)GA (–11+1)	C
			2S2-7	25,054–25,055	GGCGTT → GG(T)TT (–2+1)	C
			2S3-1	25,102	GGCCT → GGGCT	M
				25,108	CCTTTTTCC → CCTTTTCC (–1)	
		8	2S4-5	24,136–33,071	GCCA···TTAC → GC(AC···GA)GG (–8,936+6139)	C,M
				36,025	AACCA → AATCA	
				38,876	GATAA → GAATT	
			2S6-2	24,981–24,983	TGTTCTG → TG(GG)TG (–3+2)	C
			2S6-6	24,513–25,284	GCGA···AGGG → GC(AA)GG (–772+2)	C
			2S6-8	25,016–25,017	ATCAAA → AT(T)AA (–2+1)	C
	18	0	18S1-12	23,942	CAGC → CA(CA···CA)GC (+17)	M
				24,730–26,052	TCTT···ACAC → TCAC (–1,323)	
			18S2-12	20,075	AGAT → AG(TC···AG)AT (+28)	M
				20,345–26,215	ACCA···TTGG → ACGG (–5,870)	
			18S3-1	21,208	AGAAA → AGGAA	M
				21,581–25,835	TGGG···CAGT → TG(T)GT (–4,255+1)	C
			18S3-6	24,511–25,737	GCAG···GCTT → GCTT (–1,227)	M
				25,762	GCGCG → GCACG	
		8(crh)	18S5-12	23,700–24,828	CGAG···AGTG → CGTG (–3,979)	M
				25,075	ATAGC → ATCGC	
Liver	2	0	2L2-1	24,943–24,944	CCTGGT → CC(A)GT (–2+1)	C
			2L3-8	25,049	CCCGT → CCGT (–1)	M
				32,876–39,221	TCCC···TCTG → TCTG (–6,297)	
		8	2L4-9	25,055–25,057	GCGTTGC → GC(TG)GC (–3+2)	C
			2L4-12	24,948–24,949	TGGTGA → TGAAGA	T,M
				24,955–24,967	CAAT···CATG → CATG (–13)	
			2L6-3	25,019	AAACG → AACG (–1)	M
				26,083	TGGTT → TGTTT	
			2L6-5	24,983	TTCTG → TTTG (–1)	M
				25,147	GGGCG → GGTCG	
			2L6-10	23,514–25,232	TGAA···TAGC → TG(CT)GC (–1,719+2)	C
	18	0	18L1-4	24,902–25,002	GTGC···ACTC → GT(AA)TC (–101+2)	C
			18L2-1	23,894	CAGGC → CAAGC	M
				23,902	TGCTT → TGTTT	
				23,908	AGCGT → AGTGT	
				23,925–31,588	CCAC···TGTA → CCTA(–7,664)	
				31,825	CGGCG → CGACG	
				32,414	TGCAG → TGTAG	
	18	8(chr)	18L6-2	24,760–24,761	TTGGCA → TTTTCA	T,M
			18L6-6	25,017	TCAAA → TCTAA	
			18L6-10	25,056–25,820	CGTT···TGGT → CG(A)GT (–765+1)	C
				25,100	CAGGC → CAAGC	M
				25,103	GCCTT → GCGTT	
				25,108	CCTTTTTCC → CCTTTTCC (–1)	

[a] (chr) indicates chronic irradiation at a dose rate of 12.5 μGy/min for 483 days.
[b] Altered sequences are underlined. Bases shown in parentheses indicate inserted bases. The numbers in parentheses show the size of deletions indicated by a minus sign, and the size of insertions indicated with a plus sign.
[c] Unusual mutation classes are C for complex, M for multiple, and T for tandem.

mutation frequency was assayed 1 week after the end of irradiation. The mutations observed under these conditions would represent mostly those in the progenitor cells and terminally differentiated cells rather than stem cells. Thus the differences in the mutation induction rates observed for medium- and low-dose-rate-irradiated spleen (Fig. 2) might reflect the different radiosensitivities of stem cells and progenitor/differentiated cells. However, this concept does not appear to be applicable to the liver, because the mutation induction rates were similar for medium- and low-dose-rate irradiation (Fig. 2) and because the cell proliferation rate in liver is very slow.

The dose-rate effect evaluated by the mutation induction rate for 920 mGy/min divided by that for 12.5 μGy/min was 5.4 for spleen (0.477/0.089) and 3.3 for liver (0.413/0.126) (Fig. 2). These values corresponded roughly to the dose-rate effects for tumor induction in many mouse tissues examined in the past, which varied from 2 to 11 depending on tissue, strain of mouse,

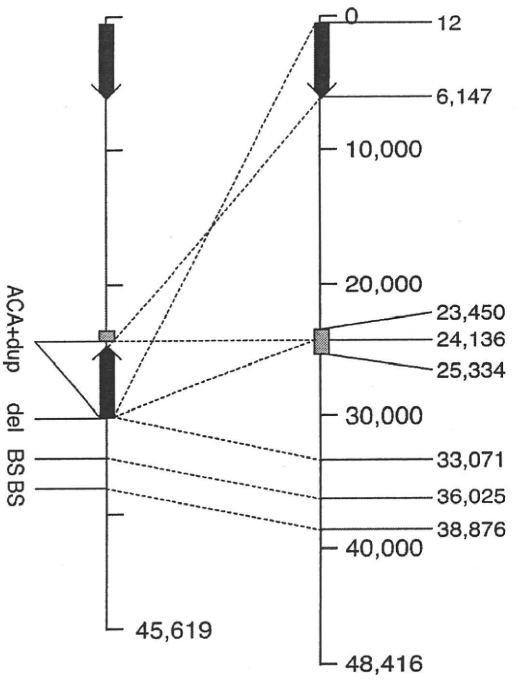

FIG. 4. Schematic illustration of the lambda EG10 genome showing the intricate mutations found in the liver of an aged control mouse. The right vertical line indicates the lambda EG10 genome, and the numbers show the positions in base pairs from the cos site. The left vertical line shows mutated lambda EG10. Gray squares in the middle show the *red-gam* genes before and after the mutation event. Bold black arrows indicate the sequence of lambda EG10 located between 12 and 6,147. In the mutant DNA, five alterations were found: two base substitutions at bp 36,025 and 38,876, deletion of base pairs from 24,136 to 33,071, duplication and insertion of 12 to 6,147 bp at 24,136; and insertion of an ACA sequence at the upper terminal of the duplicated fragment.

sex, etc. (*23*). Although the dose-rate effects specific for spleen and liver tumor induction in the mouse strain we used are not available, the similarity of the dose-rate effects in mutation induction and tumor induction may support the idea that mutation induction is closely related to radiation carcinogenesis.

Analysis of the molecular nature of mutations using DNA sequencing revealed that the predominant type of mutation observed was a deletion of 1 to 8,112 bp in both nonirradiated and irradiated mice. Among these deletions, the frequency of medium-size deletions of 2 to 1,000 bp was elevated in irradiated tissues (Fig. 3), indicating that they were induced by radiation. Similar results have been observed previously in the spleen (*17*). The fraction of large deletion of more than 1 kbp showed similar levels in spleen before and after acute irradiation with 4 and 8 Gy (Fig. 3A), whereas the fraction decreased after low-dose-rate irradiation (Fig. 3B), although the difference was not statistically significant ($P = 0.0697$). This may suggest that the occurrence of large deletions is suppressed after low-dose-rate irradiation compared to high-dose-rate irradiation. A similar phenomenon was reported in cultured cells (*7*). A slight reduction of large deletions was also observed in liver (Fig. 3C). In this tissue, however, the trend of reduction after irradiation was observed after high-dose-rate irradiation but not after low-dose-rate irradiation (Fig. 3D). This may suggest a tissue difference in radiation-induced mutation between spleen and liver.

Studies in yeast have revealed three kinds of DNA repair systems that generate deletion-type mutations, non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), and single-strand annealing (SSA), which function in the repair of DNA double-strand breaks (*24, 25*). One of the major characteristics of these repair systems is the difference in size of the homologous sequences that are used in the ligation through annealing of the separated DNA ends generated by a double-strand break. They were 1 to 4 nucleotides in NHEJ, 5 to 20 nucleotides in MMEJ, and more than 20 nucleotides in SSA (*24*). Similar phenomena were observed in chicken and mammalian cells (*25, 26*). Thus, in the work described here, homologous sequences near the termini of the deletion mutations were examined and grouped into three classes depending on the size of the homologous sequences at the break points 0, 1–4 and 5 or more. The 0-bp homology class was considered as a separate group because this kind of deletion was observed previously to be elevated by radiation (*14–17*). The maximum size of the homologies observed in the present experiment was 12 nucleotides; homologies of more than 20 nucleotides, which correspond to SSA, were not found. The percentages of each kind of mutation among the total number of deletion mutations are shown in Fig. 5. Deletions in the multiple and complex mutations were not included. The percentage of zero homology was elevated in spleen after 8 Gy high-dose-rate irradiation (Fig. 5A) ($P = 0.0443$). The increase was accompanied by a decrease in 5 or more nucleotide homologies. This may suggest that the contribution of MMEJ is small during repair of radiation-induced DNA double-strand breaks, at least in the spleen. An increase of deletions without homologous terminal sequences was also observed in the liver after 8 Gy acute irradiation (Fig. 5C), although the difference was not statistically significant ($P = 0.0964$). The frequencies of deletions with 1–4-bp homology did not change much among the samples. When all of the mutations found in control and irradiated mice were summed up, the frequency of deletions containing 5 or more bases of terminal homology appeared to be higher in the spleen than in the liver. These events comprised 30 out of 114 deletion mutations in the spleen and only 10 out of 92 deletion mutations in the liver ($P = 0.00533$). Thus it is likely that the contribution of MMEJ is higher in the spleen than in the liver. This is in agreement with the fact that MMEJ works more efficiently in the S and

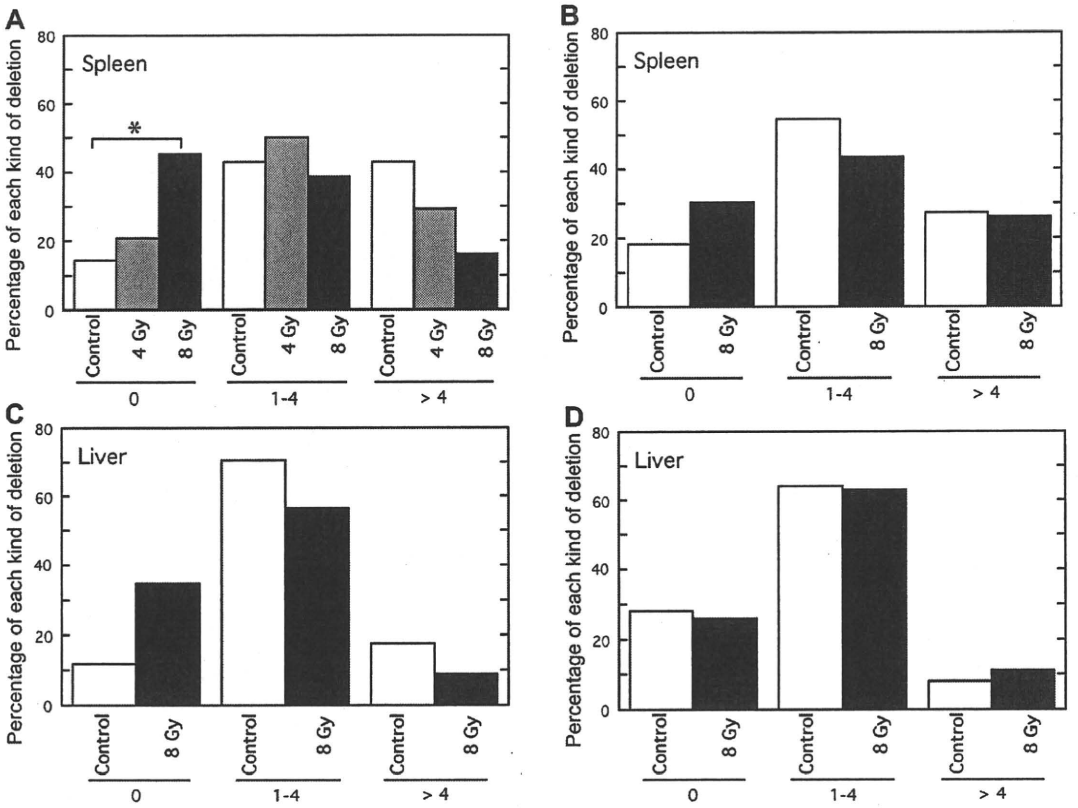

FIG. 5. Comparisons of the frequencies of deletion mutations showing repeated sequences of different sizes at the junctions of the deletions. The deletions were classified into three groups: no homology at the junction, 1 to 4-bp-long homology, and 5-bp-long or more homology. The frequency of each group of mutations was calculated for spleen (panel A) and liver (panel C) in high-dose-rate-irradiated mice and in the spleen (panel B) and liver (panel D) of low-dose-rate-irradiated mice. Statistically significant differences are indicated by an asterisk.

G_2 phases of the cell cycle (*24, 25*), because the spleen is a tissue in which many cells are dividing and are therefore in S and G_2 phase, whereas the liver contains almost exclusively slowly dividing cells in the G_0 or G_1 phase.

Among the 236 non-redundant mutations found in the present study, 14 were multiple mutations. This frequency of 5.9×10^{-2} (14/236) was much higher than the expected value for the coincidental occurrence of two or more single mutations, because the expected frequency of a single mutation was of the order of 10^{-6}. The mutation frequency observed was 1 to 5×10^{-6} (Fig. 1), and most of these (222 out of 236) were single mutations. This high frequency of multiple mutations was observed in many other systems (*27*), including normal tissues of the Big-Blue mouse (*28*) and the epithelial tissues in the intestines of aged Muta™ mice (*29*). A possible mechanism that could explain the production of these multiple mutations could be the involvement of some trans-lesional DNA polymerases, which replicate DNA with an extremely high error rate and can result in two or more mutations within a DNA stretch only several kbp long (*30–33*).

To understand how these multiple mutations were generated, it would be helpful to scrutinize their molecular nature. Among the 14 multiple mutations, 13 showed changes in DNA length including deletions, insertions and complex-type mutations. One exception was a base substitution in the *gam* gene and a tandem base substitution in the *red* gene (18L6-2). Frequent associations of deletion mutations and other types of mutations in nearby positions along a single DNA strand have been reported in two cases: a preferential occurrence of base substitution mutations in the *Ki-ras* proto-oncogene allele containing 37 base deletions in the second intron (*34*) and a high rate of single nucleotide mutations in the areas neighboring deletion and insertion mutations in evolutionary changes (*35*). Although the molecular mechanisms of these phenomena have not been elucidated, a possible association with error-prone trans-lesional DNA polymerases in MMEJ is one possibility (*25*).

The mutant clone 18L2-1 found in the liver of an aged control mouse (Table 2) had one deletion of –7,664 bp together with 5 base substitutions in the neighboring area. All of the 5 base substitutions were G:C to A:T transition mutations. This could be induced by an imbalance in the pool size of deoxyribonucleotide triphosphates. In cultured cells, an increase of the dTTP pool size has been shown to result in the induction of

G:C to A:T transition mutations (*36, 37*). The clustering of the base substitutions in the nearby regions of the deletion mutation may suggest that the pool size effect becomes more effective when DNA polymerization takes place together with a deletion mutation. The deletion mutation found in this clone did not have any homologous sequences at the terminals, suggesting that it was not generated by MMEJ or SSA.

In conclusion, the mutagenic effects of radiation in spleen and liver were shown to be dependent on the dose rate. The detail information about the mutation induction rates as well as the molecular characteristics of mutations revealed some differences between the two tissues. Further studies on the other tissues would be important to understand dose-rate effects at the organismal level.

SUPPLEMENTARY INFORMATION

Tables S1 and S2. Mutant frequencies in spleen and liver, respectively, of each individual mouse. http://dx.doi.org/10.1667/RR1932.1.S1

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Strategies in case of positive *in vivo* results in genotoxicity testing☆

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ABSTRACT

At the 2009 International Workshop on Genotoxicity Testing in Basel, an expert group gathered to provide guidance on suitable follow-up tests to describe risk when basic *in vivo* genotoxicity tests have yielded positive results. The working group agreed that non-linear dose-response curves occur *in vivo* with at least some DNA-reactive agents. Quantitative risk assessment in such cases requires the use of (1) adequate data, *i.e.*, the use of all available data for the selection of reliable *in vivo* models to be used for quantitative risk assessment, (2) appropriate mathematical models and statistical analysis for characterizing the dose-response relationships and allowing the use of quantitative and dose-response information in the interpretation of results, (3) mode of action (MOA) information for the evaluation and analysis of risk, and (4) reliable assessments of the internal dose across species for deriving acceptable margins of exposure and risk levels. Hence, the elucidation of MOA and understanding of the mechanism underlying the dose-response curve are important components of risk assessment. The group agreed on the need for (i) the development of *in vivo* assays, especially multi-endpoint, multi-species assays, with emphasis on those applicable to humans, and (ii) consensus about the most appropriate mathematical models and statistical analyses for defining non-linear dose-responses and exposure levels associated with acceptable risk.

1. Introduction

The International Workshops on Genotoxicity Testing (IWGT), in addition to their historical focus on the refinement of genetic toxicology test protocols, have established working groups to recommend appropriate strategies for the use and interpretation of genetic toxicology tests and assessment of the risk of genotoxic exposures. Müller et al. [1] describe the objectives of this IWGT effort, identify areas of focus for the IWGT strategy working groups, and provide initial recommendations for hazard assessment. An IWGT working group has previously provided recommendations on follow-up testing in case of *in vitro* positive results in genotoxicity assays, defined criteria for developing a weight-of-evidence

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decision on marginal and non-reproducible results, and has provided a general decision tree for implementing a testing strategy [2]. This general strategy has been extended by a recent working group of the Health and Environmental Sciences Institute, a part of the International Life Sciences Institute (HESI/ILSI), with a focus on interpretation and follow-up testing of positive results *in vitro* [3].

At the August 17–19, 2009, meeting of the IWGT in Basel, Switzerland, a working group was charged with development of recommendations for appropriate follow-up actions when testing results clearly demonstrate genotoxic effects *in vivo*. The objectives of this working group were to develop consensus and to provide recommendations on the following specific topics:

(1) The use of *in vitro* and *in vivo* results in the interpretation and design of *in vivo* assays,
(2) The use of appropriate *in vivo* models for risk assessment,
(3) The evaluation and impact of mode of action (MOA) information, and
(4) Quantitative aspects of the interpretation and use of dose-response information.

This report summarizes the outcome of that working group meeting, including points on which consensus was achieved and those for which further consideration and discussion are needed.

2. Topic 1: Use of *in vitro* and *in vivo* results for risk assessment

In vivo tests are generally considered the most appropriate for quantitative risk estimations. However, they have certain limitations, which are associated both with the current lack of simple assays for relevant endpoints in certain tissues (and the consequent need to interpret surrogate information) and with the difficulty of obtaining mechanistic information in the context of often complex *in vivo* interactions. Thus it was agreed that *in vivo* assays should not be considered in isolation, but that information generated in *in vitro* assays is useful for interpreting the *in vivo* results, defining mode(s) of action, and offering guidance for any additional testing that may be necessary. *In vitro* studies can often be designed to address mechanistic questions that can aid extrapolation to humans. The working group affirmed that when *in vivo* testing is conducted as a follow-up to positive *in vitro* results, the endpoint(s) studied *in vivo* needs to be either the same as that affected *in vitro* or a surrogate demonstrated to be appropriate for predicting the affected endpoint [2] [3]. It was noted that most test systems in genetic toxicology are not specific to a single endpoint and that most DNA-damaging agents affect multiple endpoints, but many chemicals exhibit a predominance of certain types of damage (*e.g.*, point mutations *vs.* chromosomal breaks and rearrangements). Hence, the characteristics of the assays involved (both *in vitro* and *in vivo*) must be taken into account, with recognition of the spectrum of endpoints likely to be affected. Moreover, the selection of tissue(s) *in vivo* should consider pharmaco-/toxicokinetic and pharmaco-/toxicodynamic information about the test material, considering the relevant route(s) of administration. Furthermore, all pertinent toxicological information, including the identification of target organs in sub-acute and sub-chronic studies, should be considered in the design of follow-up *in vivo* genotoxicity studies.

Since absorption, distribution, metabolism, and elimination (ADME) of a compound are integral parts of *in vivo* assays, and ADME extrapolation across species is central to risk assessment, it was acknowledged that the results of the *in vivo* genotoxicity assays should generally have more weight than the *in vitro* assays in genotoxicity risk assessment. Moreover, *in vivo* test selection and design, including selection of tissues for analysis, should be based on appropriate information about anticipated human exposures and account for any known interspecies differences. As *in vivo* testing in genetic toxicology is usually conducted in rodents (mice and rats), well-known limitations exist when extrapolating results from such experiments to the human situation. This is in contrast to other areas of general toxicity testing (*e.g.*, for pharmaceuticals, food additives, and agricultural chemicals), in which extrapolation for humans is normally based on testing in both rodent and non-rodent species. Nonetheless, the working group agreed that if one or more *in vitro* tests are positive, and no measurable genotoxic effects are detected in appropriate *in vivo* endpoints in adequately exposed tissues in relevant animal species, the risk of *in vivo* genotoxic effects can be considered to be negligible. This requires that the follow-up testing *in vivo* is conducted at appropriate doses (*i.e.*, investigated tissues were exposed to sufficient levels of the test material) and that the experimental design is appropriate to show that the *in vitro* effects are not manifested *in vivo*.

The working group also agreed that when genotoxicity is identified in an animal model then an appropriate evaluation of risk in relation to anticipated human exposure should be conducted: *i.e.*, a quantitative risk assessment for the genetic effect should be conducted. The following sections briefly consider the appropriate *in vivo* models that can be used for risk assessment, and then discuss follow-up strategies that can be applied in order to characterize the genotoxic risk for humans.

3. Topic 2: Selection of appropriate *in vivo* models for risk assessment

There was limited discussion of the use of appropriate *in vivo* models for risk assessment. Selection of endpoints and species was not discussed extensively, but there was consensus that transgenic animal models in which neutral reporter genes are used to monitor mutation are acceptable surrogates for naturally occurring genes for assessment of *in vivo* mutagenic risk. The models include transgenic rodent assays with *lacI*, *lacZ* (phage and plasmid), *cII* and *gpt* delta target genes [4]. It appears that such non-transcribed transgenic constructs are useful genetic mutation markers as they are "neutral" and therefore mutations can accumulate during exposure, unlike transcribed genes that may be subject to selective pressure. Thus, the working group agreed that data from transgenic animals with recoverable neutral reporter genes were of comparable quality and predictability for carcinogenicity compared with other standard mutagenicity tests based on endogenous active genes, and that they fill an important need in current regulatory practices (*e.g.*, *in vivo* follow-up testing).

Promising assays include the new *Pig-a* assay [5–12], flow cytometric micronucleus assays [13–27], and *gpt* delta rat and mouse models [28–33]. In particular, the *Pig-a* assay, based on the loss of the glycosylphosphatidylinositol (GPI) membrane anchor of the cell membrane, shows great promise as a high throughput method which, when fully validated, should facilitate the acquisition of data necessary to define *in vivo* dose-response and kinetics relationships that are critical to risk assessment. This assay could easily be coupled with the analysis of micronuclei in peripheral blood. A major advantage of the *Pig-a* and micronucleus assays is that they are conducted using peripheral blood and are therefore relatively non-invasive and can be conducted in any species (including human) [34,35] as part of general toxicity.

The main disadvantage of the *Pig-a* and erythrocyte micronucleus assays is that at present they evaluate only damage in hematopoietic cells, and so are currently not amenable to many target tissues of mutagenesis and carcinogenesis (liver, GI-tract, lung, kidney). Therefore, other assays such as the comet assay or transgenic mutation assays (especially the *gpt* delta model, which

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is able to detect both base substitutions and gene deletions) would be needed for the evaluation of *in vivo* genotoxicity in other target organs in which mutations play a significant etiological role in disease. There was consensus that there is a need to continue the development of *in vivo* assays, especially multi-endpoint, multi-species assays, with emphasis on those applicable to samples from human origin. In the spirit of the 3Rs (the Replacement, Reduction and Refinement of the use of experimental animals) in toxicology, integration of *in vivo* genotoxicity assays into 28-day repeat dose toxicity assays or short-term carcinogenicity assays may be an important future direction [4]. Integration into toxicology studies also facilitates comparison of genotoxic responses with other toxicity endpoints and with pharmacokinetic and metabolism information.

4. Topic 3: Quantitative aspects of the interpretation and use of dose-response information

As already reported in the literature [36–39], the working group considered the hypothesis that agents documented to induce genetic damage *via* interaction with non-DNA targets may exhibit a non-linear dose-response relationship with a "threshold" dose below which DNA damage is not expected to occur. For agents that act *via* such non-linear mechanisms, the No Observed Genotoxicity Effect Level (NOGEL) is generally considered an appropriate metric to which additional safety margins may be applied in determining an acceptable safe exposure limit [40]. In such cases, the risk assessment methods applied would be the same as those used for any other toxicological endpoint. For example, in the case of impurities in pharmaceutical agents, the calculation of a permissible daily exposure (PDE) starting with the no observed effect level (NOEL) (or lowest observed effect level (LOEL)) and using five different 'uncertainty factors' has been suggested [40]. The magnitude of the uncertainty factor depends on the degree of certainty for the respective extrapolations from the test systems to the human exposure situation. In the case of pharmaceuticals, an acceptable margin of exposure (MOE) also depends on the benefit of treatment to the exposed patient or population.

The main focus of the working group discussion was the use of quantitative dose-response information to assess the risk of genetic damage due to human exposure to DNA-reactive compounds. In the case of carcinogenicity, the current default assumption is that genotoxic carcinogens that interact with DNA will generally show linear non-threshold dose-responses. However, it has recently been demonstrated that some genotoxic carcinogens that interact with DNA show non-linear dose-response curves with apparent thresholds, *i.e.*, practical thresholds [41–51]. In this paper "threshold" is used to describe a dose below which the incidence of the measured genotoxic effects cannot be distinguished from the background and its associated confidence interval.

Among the non-linear dose-response examples a key case is the recent incident in which the pharmaceutical Viracept was contaminated with ethyl methane sulfonate (EMS), which led to intensive study of the genotoxicity exposure-response relationship for the well-studied genotoxic agent EMS. For this reason, this was selected by the working group as a case study for the quantitative evaluation of the dose-response curves. It was demonstrated in this case that assessment of exposure and response information could be used to define an exposure level, accepted by the European regulatory authority, below which intensive follow-up studies were not considered essential since there is no significant human safety concern. This information, reported at the meeting by Elmar Gocke and Lutz Müller, has now been published ([52,53]; all details in [51]) and is summarized briefly in Appendix A. In their analysis the authors reported that in the case of EMS:

(1) DNA adducts produced by EMS can be repaired error-free,
(2) The existence of several dose groups without any effect below the threshold and their comparison against a large cohort of vehicle controls allows the estimation of a threshold dose and its associated confidence interval,
(3) Assessment of exposure to free EMS in several species appears to be a reasonable basis for human exposure modelling,
(4) It appears that conventional cross-species exposure scaling methods (as used in other areas of toxicology), together with safety margin calculations to balance uncertainties about the exact threshold dose in other species (or other tissues or different age or disease conditions), can be used for risk management for this genotoxic carcinogen.

The direct nature of the genetic damage induced by EMS, which does not involve any major metabolic steps, makes cross-species scaling and risk assessment less complicated than in many other cases, in which metabolic activation or detoxification processes have to be taken into account.

After the Basel meeting, a cancer study using very large numbers of trout exposed to dibenzo[a,l]pyrene was published [54]. The size of this study allowed the determination of statistically significant increases of 1 cancer in 1000 animals. The sensitivity and hence statistical accuracy is more that two orders of magnitude higher than in a usual rodent cancer study with lifetime administration of the test substance.

It was shown that linear extrapolation of the dose-response in the low dose range overestimated the actual cancer risk, and appropriate modelling of the sublinear dose-response curve indicated that the virtual safe dose (VSD; 1 induced cancer in 1,000,000 individuals) was about 1000 fold higher than predicted by linear extrapolation. Notably, the dose-response of the induction of the bulky DNA adducts was close to linear, indicating that at low doses an error-free removal is apparently operative even for the bulky dibenzo[a,l]pyrene adducts. Alternatively, error-free bypass DNA synthesis across the lesion may occur, thereby suppressing the resultant mutations.

Based on the above data and other results recently reported in the literature (*e.g.*, see [41–45] [48] [50] [54–60]) the working group agreed that non-linear response curves and operational thresholds occur *in vivo* with at least some DNA-reactive agents. In other words, some agents will exhibit a "practical threshold": *i.e.*, a dose below which exposure does not add appreciably to existing background rates of DNA damage. Much more data are needed from studies with carefully determined dose-response curves to determine if generalizations across agents are possible. At present, each case requires appropriate data and careful statistical scrutiny. Possible mechanisms/modes of action underlying non-linear dose-response relationships should also be investigated. Consensus is needed on appropriate mathematical models and statistical analyses for characterizing these dose-response relationships and risk levels, and for deriving acceptable margins of exposure. While DNA primary damage can be used for exposure assessment (*i.e.*, as a biomarker of exposure), stable mutations (which are a biomarker of effect) should be given much more weight for risk assessment.

There was consensus that dose and exposure metrics must be justified for each situation of interest, that cross-species extrapolation should consider the same factors that are important for other toxicity endpoints, including relative metabolism, PK differences, surface area scaling, and internal dose, in addition to DNA repair and translesion DNA synthesis differences and relative apoptosis efficiency. Exposure metrics may include the traditional measures of plasma and tissue exposure (C_{max}/AUC).

The working group supported the approach suggested by Lutz and Lutz regarding the analysis of dose-response data for a continuous response variable with background to determine if a threshold

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of response is present. This approach, recently published [61], involves definition of the background frequency and variability, followed by a statistical analysis of the data to check whether a fit by a "hockey stick" model is significantly better than a linear regression. If the hockey stick model applies, then the next step consists of a linear regression analysis for the data below the best estimate of the break point in the dose-response curve, estimating the slope of the upper limit of a confidence interval of the linear regression, and calculating the response at the threshold dose. In the context of EMS in Viracept, a 5% error level was proposed [62,63] and a 95% confidence interval was given for the estimate of the calculated threshold dose.

Conventions for unacceptable increases above the existing spontaneous levels need to be established within the scientific community, with consideration of the irreversible nature of mutation induction. The question of a theoretically-expected linear dose-related increase below the threshold dose could be addressed by linear regression of the data below the break point and estimation of an upper limit of the slope. The biological relevance of this slope can then be discussed against the normal variation of background measures in the controls [61]. Other approaches to analysis of thresholds (*e.g.*, [64]) should also be considered, and consensus is still needed about the most appropriate mathematical models and statistical analyses for defining threshold response and exposure levels associated with acceptable risk.

The working group also considered whether genotoxicity data can be used to derive acceptable MOEs, in a manner that is often applied to non-cancer risk factors (*e.g.*, [65]) and sometimes to risk from genotoxic carcinogens [66,67]. To this end, *in vivo* data can be modeled to estimate benchmark dose (BMD_x levels (*i.e.*, dose associated with a defined increase, *x*, of genotoxic damage above background) that could be compared with an estimated human exposure level, as proposed by the European Food Safety Authority (EFSA) for genotoxic carcinogens. As an example an MOE >10,000 relative to the carcinogenic BMD_{10} has been identified as a "low concern" for genotoxic carcinogens by EFSA (2005) [67]. In other words, the threshold of toxicological concern (TTC) levels can be defined for *in vivo* genotoxicants based on benchmark dose level and MOE determinations. Hence, one could determine a permitted daily exposure level with appropriate safety margins for genotoxic carcinogens [53]. For this MOE approach to be applied to *in vivo* genotoxicity data it will be necessary to define the relevant endpoint(s) to be considered and the biologically meaningful increase over background upon which the benchmark dose and safety margins would be chosen. For example, there would be a need for consensus on whether the NOGEL, a particular benchmark dose based on initial response, or other parameter was an appropriate reference exposure parameter for the genotoxic endpoint and also how that exposure metric related to the estimated cancer risk, or other endpoint of concern, for genotoxic agents. Moreover, it can be anticipated that the NOGEL will vary depending on the genetic effect induced and test method applied, and consensus on the selection of relevant endpoints and tests is needed.

The working group felt these approaches should be explored further, but was not able to define the necessary processes at the time of the meeting. The value of such approaches would be that mutagenicity dose-response curves can be determined with far greater precision than carcinogenicity dose-response curves, and so the acceptable margin to avoid genotoxic effects (which might lead to carcinogenicity or other adverse effects) could be determined with much better precision than the acceptable margin to avoid carcinogenicity.

In addition to application of quantitative dose-response information, secondary factors that may modify dose-response relationships were also considered [68–70]. Examples are cell proliferation state, modification or interspecies differences in repair and bypass DNA synthesis capacity or in levels of electrophilic "traps" such as thiols. It was recognized that these factors may be tissue specific and that such factors must be considered when applying quantitative methods to analyse dose-response information.

In summary, there was consensus that quantitative approaches to the assessment of the health risk of exposures to genotoxic agents are necessary when the potential for genotoxic damage that could lead to heritable changes is identified *in vivo*. IWGT will continue to develop recommendations for their implementation.

5. Topic 4: Evaluation and impact of mode of action (MOA) information

Elucidation of MOA of individual compounds is an important component of risk assessment. The better the information about MOA and dose-response relationships, the more certain is the interpretation of dose-response relationships and the determination of an acceptable exposure level in humans. When performing MOA analysis and extrapolating to humans, all available relevant data should be used—not only genotoxicity data.

One example presented was a drug candidate with positive *in vitro* findings that were due to species-specific metabolism that do not occur in humans (Appendix A). Results obtained with the chelating agent nitrilotriacetic acid (NTA) were presented as an example of a rodent nephrocarcinogen with an *in vivo* positive result due to an indirect mechanism of action (Appendix A). When carcinogenicity data are available, genotoxicity should be examined in the target organs for chemical carcinogenesis, using the same species and strains, when possible. Mechanisms underlying the shape of the dose-response curve should be investigated as thoroughly as is feasible both *in vitro* and *in vivo*.

Many chemicals are both mutagens and carcinogens. When conducting an MOA assessment for the induction of the tumors, it is important to consider whether the chemical is actually a mutagenic carcinogen. It should be noted that mutagens should not automatically be assumed to be mutagenic carcinogens. This determination depends on a comprehensive evaluation using a MOA framework and the assessment of key events [71,72].

A strategy for using *in vivo* mutation data to inform cancer MOA was presented. The strategy uses transgenic rodents to evaluate whether a carcinogen can induce mutation in the tumor target tissue. A modified Hill Criteria analysis [73,74] is used to determine whether the induced mutation response is consistent with a mutagenic MOA. This requires an assessment of temporality and dose-response concordance between the mutation dose-response and the tumor dose-response. A case study using riddelliine and dichloroacetic acid (DCA) was presented. Both of these chemicals are mutagens and liver carcinogens. Riddelliine induces mutations in the liver after only a few weeks exposure, while DCA induces mutations in the liver after 60 weeks exposure. A benchmark dose analysis of the mutation and cancer data dose-response curves indicates dose-response concordance for riddelliine but not for DCA. Taken together the temporality analysis and the dose-response concordance analysis for these two chemicals indicate that riddelliine is likely a mutagenic carcinogen, but DCA likely has a different mode of action. The details of this approach are published [75].

It was suggested that future *in vivo* mutation studies to inform MOA should be designed based on the cancer study. Species, dose route, and dose levels should be selected based on the cancer study and should include enough doses, particularly at the lower end of the dose-response curve, to provide an adequate assessment of the dose-response. The design should include chronic exposure and interim sacrifices to provide a dose-response curve at multiple time points. The timing of the interim sacrifices should be based on any

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known preneoplastic lesions that occur prior to tumor development. It is possible that the treatment may need to be extended to up to a year, as was the case in the DCA example. Experiments can be designed to evaluate possible MOAs in addition to the induction of mutation.

The extent to which *in vivo* mutagenicity can be associated with adverse effects other than cancer, and the importance of risk assessment of genotoxicity, *per se*, was discussed. In addition to germline mutations that result in well-recognized human diseases, a number of human diseases are caused by *de novo* somatic mutations [76]. More recently, Borlak and co-workers have shown that both somatic and germline mutations result in cardiac septation defects [77–79]. Accordingly, it was affirmed that cancer is not the only adverse health outcome associated with genetic damage, and, therefore *in vivo* genotoxicity should be considered an adverse effect whatever the evidence of carcinogenicity. Data were also presented suggesting that negative carcinogenicity data may not always provide assurance of the lack of genotoxicity *in vivo* in other species or with different exposures. The Maillard reaction product 4-hydroxy-2,5-dimethylfuran-3(2H)-one, negative in a rat carcinogenicity and positive in mutagenicity studies *in vivo* in mouse somatic and germ cells, was discussed as an example [80].

These examples illustrate the need for expert evaluation of all available data to determine the appropriate follow-up investigation that may be necessary for *in vivo* and/or *in vitro* positive genotoxicity data, even when negative carcinogenicity data are available. It was agreed that further review and discussion is warranted before any specific recommendations can be provided on this topic.

6. Conclusions

In conclusion, appropriate models for risk assessment of *in vivo* genotoxicants have been discussed in an IWGT group, and the working group agreed on the following points:

(1) When *in vivo* testing is conducted as a follow-up to positive *in vitro* results, an appropriate experimental design should be used to determine if the *in vitro* effects are manifested *in vivo*, *i.e.*, in adequately exposed tissues in relevant animal species using the same endpoint as that affected *in vitro* or a surrogate demonstrated to be appropriate for predicting the affected endpoint.

(2) Transgenic animal models with recoverable neutral reporter genes are useful for assessing mutagenic activity in different tissues, and are of comparable quality and predictivity for assessment of *in vivo* mutagenic risk as compared to endogenous genes. They therefore fill an important need in current regulatory practice (*e.g.*, *in vivo* follow-up testing). The *Pig*-a assay, flow cytometric micronucleus assays, and *gpt* delta rat and mouse models are promising assays; *Pig-a* and micronucleus assays because they are conducted using peripheral blood and can be conducted in any species, and *gpt* delta model because it is able to detect both point mutations and gene deletions. The comet assay and transgenic mutation assays remain the principal assays allowing the evaluation of *in vivo* genotoxicity in any target organ. There is a need to continue the development of *in vivo* assays, especially multi-endpoint, multi-species assays, with emphasis on those applicable to samples from human origin. Integration of *in vivo* genotoxicity assays into general toxicity assays, such as 28-day repeat dose toxicity assays, is worth considering in the light of its advantages in efficiency, provision of comparative toxicology, pharmacokinetic and metabolic information, and the spirit of the "3Rs" in regulatory toxicology.

(3) Non-linear response curves may occur *in vivo* with non-DNA-reactive and at least some DNA-reactive agents. More data are needed to determine if generalizations across types of agents are possible. Each case requires appropriate data, justified doses and exposure metrics, and careful statistical scrutiny. Consensus is needed on appropriate mathematical models and statistical analyses for characterizing these dose-response relationships and risk levels, and for deriving acceptable margins of exposure. Among the possible approaches are (a) the use of mathematical models and statistical analyses (*e.g.*, "hockey stick" model) to define the background frequency and its variability, and to analyse the dose-response curves, (b) the estimation of the break point in the dose-response curve, *e.g.*, NOGEL with additional safety margins, or BMD to which may be applied an acceptable safe margin of exposure (MOE). In some instances, human exposure levels thought to pose negligible safety concerns can be defined for *in vivo* genotoxicants.

(4) Genotoxic effects *in vivo* generally have more weight than *in vitro* effects in genotoxicity risk assessment, and the absence of measurable *in vivo* effects in target tissues with adequate exposure and metabolic activity indicates that the risk of *in vivo* genotoxic effects can be considered to be negligible in relation to the anticipated human exposure. While DNA primary damage can be used for exposure assessment, stable mutations, *i.e.*, biomarkers of effect, should be given much more weight for risk assessment.

(5) All pertinent toxicological information should be considered in the design of follow-up *in vivo* genotoxicity studies. Elucidation of MOA of individual compounds is an important component of risk assessment, *i.e.*, mechanisms underlying the shape of the dose-response, MOA framework and the assessment of key events, temporality and dose-response concordance between the mutation dose-response, and the tumor dose-response when carcinogenicity data are available.

Conflict of interest

None.

Appendix A. Case examples presented and discussed

Roche Viracept® case

Roche's protease inhibitor nelfinavir mesylate (Viracept®) produced between March 2007 and June 2007 was found to contain elevated levels of EMS, a known mutagen (alkylating agent), leading to a global recall of the drug. EMS levels present in the contaminated drug were predicted not to exceed a dose of ~2.75 mg/day (~0.055 mg/kg/day for a 50 kg patient) based on the daily dose of 2500 mg Viracept/day. As existing toxicology data on EMS did not permit an adequate patient risk assessment, a comprehensive animal toxicology evaluation of EMS was conducted. The general toxicity of EMS was investigated in rats exposed for 28 days. Two studies that assessed DNA damage were performed in mice: chromosomal damage was assessed using a micronucleus assay and gene mutations were detected using the Muta™ Mouse transgenic model. In addition, experiments designed to extrapolate animal exposure to humans were undertaken. A general toxicity study showed that the toxicity of EMS occurred only at doses ≥60 mg/kg/day, which is far above the doses received by patients. Studies for chromosomal damage and *lacZ* mutants in mice (in bone marrow and gastrointestinal tract) demonstrated a clear threshold effect with EMS, with no measurable effect at and below 25 mg/kg/day, under 4-week continuous dosing conditions. In the same experiment, a threshold in liver was determined to exist

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Table 1
Threshold analysis (hockey-stick model).

Study	Organ	No observed effect level (mg/kg)	Threshold dose (mg/kg)	95% Confidence interval of threshold dose (mg/kg)
Micronucleus test	Bone marrow	80	89.81	56.67–118.25
Muta™mouse	Bone marrow	25	35.45	21.46–45.73
Muta™mouse	Liver	50	51.31	25.67–99.10
Muta™mouse	GI-tract	25	24.51	12.97–38.51

Table 2
Slope analysis for the low dose range.

Study	Organ	Slope at low dose region	95% Confidence interval of slope
Micronucleus test	Bone marrow	−0.10	−0.20 to −0.001
Muta™mouse	Bone marrow	−0.19	−1.19 to 0.81
Muta™mouse	Liver	−0.10	−0.69 to 0.48
Muta™mouse	GI-tract	0.48	−0.96 to 1.92

(The slope is given as number of micronucleated polychromatic erythrocytes out of 4000PCE/mg/kg, and mutant frequency per million cells/mg/kg, for *in vivo* micronucleus test and gene mutation assay in Muta™Mouse, respectively).

at 50 mg/kg/day. A detailed statistical analysis using the approach developed by [61] estimated the 95% statistical confidence intervals for the threshold dose and the slopes below the threshold for the investigated endpoints and organs [62,63]. The confidence in this analysis reflecting a threshold is strengthened by the fact that four dose levels for each organ measured yielded no discernable mutation difference from the control and that three independent control groups were used for the experiment (Tables 1 and 2).

Exposure analysis (C_{max}) in mice, rats and monkeys demonstrated that ~370-fold higher levels of EMS than that ingested by patients are needed to saturate known, highly conserved, error-free, mammalian DNA repair mechanisms for alkylation. Yet, as the half-life of EMS was higher in rats than in mice, and higher in non-human primates than in rats, the calculations of its AUC (area under the exposure-time curve) at the threshold dose of 25 mg/kg/day yielded an AUC-based safety factor of ~28 (*vs.* the C_{max}-based factor of ~370) [81]. Because all mutagenic DNA alkylations seem to be repairable at daily doses up to 25 mg/kg EMS, it can be argued that the C_{max} (which is largely half-life independent) is the main factor for risk assessment in this "EMS in Viracept" case.

In summary, the animal studies suggested that patients who took nelfinavir mesylate (Viracept) with elevated levels of EMS are not at increased risk for carcinogenicity, mutagenicity, or teratogenicity, since mutations are prerequisites for these events. As exposure biomarkers such as adducts on globin or DNA, do generally follow linear dose-response relationships in the case of EMS, these data clearly show that such biomarkers cannot be used for risk assessment or risk management processes in this case but that risk assessment should be based on "fixed" mutational events. Although non-linear behaviour of mutations *in vivo* has been demonstrated previously, these data give the first reliable experimental basis for comprehensive risk management in a low dose exposure scenario.

In vitro effects due to species-specific metabolism

An example of irrelevant *in vivo* positive findings due to an interspecies difference in metabolic capacity was presented. A drug candidate was positive in a comet assay performed on rat stomach and negative in rat liver. This compound is known to be hydroxylated in the stomach and then glucuroconjugated by UDP-glucuronosyltransferase (UDPGT) 1A8. This UDPGT 1A8 isoform is not expressed in the rat gastrointestinal tract [82] while it is highly expressed in human gastric mucosa [83]. *In vitro* assays on isolated mucosa were performed and demonstrated that (1) the glucuroconjugated metabolite was observed in human gastric mucosa but not in the rat, (2) the hydroxylated metabolite was present in gastric rat mucosa but not in the human, and (3) the hydroxylated metabolite was a direct genotoxic compound in the Ames assay and in the *in vitro* micronucleus assay. It was concluded that the parent compound is positive in the comet assay in rat gastric mucosa but most probably not in human, and that there was no genotoxic concern in human associated with this compound.

Rodent nephrocarcinogen with an indirect mechanism of action

Nitrilotriacetic acid (NTA) induced marked increases in DNA damage after a single oral treatment at high doses in the *in vivo* rodent comet assay on kidney cells at both short (3–6 h) and long-term (22–26 h) sampling times. NTA demonstrated no mutagenic activity in the Ames test but was positive in the *in vitro* micronucleus assay on L5178Y mouse lymphoma cells without and with metabolic activation by aroclor 1254-induced liver or kidney rat S9-mix. An assay on CTLL2/Bcl2 cells coupled to the apoptosis measurement with and without metabolic activation demonstrated a positive response and confirmed the absence of interference of apoptosis. The direct mutagenic activity of NTA was confirmed in the mouse lymphoma *tk*+/− gene mutation assay and in the chromosomal aberrations test on human lymphocytes. However, tested in combination with an excess of Ca^{2+}, NTA gave negative results on L5178Y mouse lymphoma cells, in the *in vitro* comet and in the micronucleus assays, while Ca^{2+} only partly abolished the formation of DNA strand breaks on rat primary kidney cells. The higher sensitivity of renal cells to Ca^{2+} variations could explain the positive response observed *in vivo*. The carcinogenicity of NTA could be a consequence of the intracellular variations of Ca^{2+}, leading to a local and indirect genotoxic mechanism. This suggests that in the case of NTA, a threshold dose may exist beyond which kidney tumor-generating events will be displayed [84].

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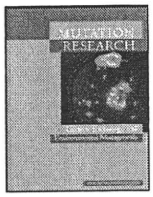

Preface

Nucleotide pool damage and its biological consequences

It is our pleasure and honor to publish a special issue of Mutation Research "Nucleotide Pool Damage and Its Biological Consequences". This is extended presentation of a session of "Health Effects of Nucleotide Pool Damage" in the 10th International Conference on Environmental Mutagens (10th ICEM) in Florence in Italy in August 2009. The session was organized by us (Bignami and Nohmi), and six out of 11 authors who contribute to this special issue were invited speakers in the session. After ICEM, we invited another five persons to join the group to publish this special issue and all kindly accepted the invitation. We are very pleased that this special issue is published within a relatively short period of time.

Needless to say, nucleotide pools as well as DNA are important substrates for DNA polymerases. Accurate DNA synthesis requires well balanced dNTP pools and the imbalance leads to mutations and cell death. In addition, excess oxidation of nucleotide pools in aerobic metabolism or inflammation results in a variety of cellular abnormalities including genome instability. The representative example of oxidation of dNTPs is the formation of 8-oxo-dGTP in nucleus and mitochondria, which may induce mutations, cellular senescence, neurological diseases and cancer. To combat the detrimental effects of oxidized dNTPs, cells evolve nucleotide pool sanitizing enzymes such as MTH1. However, some of the oxidized dNTPs escape from the defense systems and eventually incorporated into DNA by polymerases. In this issue, 11 authors discuss biological and health consequences of nucleotide pool damage from various viewpoints. The collected papers may be beneficial to people in pharmaceutical industries as well because modified dNTPs are an important class of pharmaceuticals. It is our hope that this special issue will contribute to wider recognition of the importance of nucleotide pool damage in health sciences.

Finally we acknowledge Dr. David Kirkland, an editor of special issue of Mutation Research, who encouraged us to publish this special issue.

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Minireview

DNA polymerases involved in the incorporation of oxidized nucleotides into DNA: Their efficiency and template base preference

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ABSTRACT

Genetic information must be duplicated with precision and accurately passed on to daughter cells and later generations. In order to achieve this goal, DNA polymerases (Pols) have to faithfully execute DNA synthesis during chromosome replication and repair. However, the conditions under which Pols synthesize DNA are not always optimal; the template DNA can be damaged by various endogenous and exogenous genotoxic agents including reactive oxygen species (ROS), and ROS oxidize dNTPs in the nucleotide pool from which Pols elongate DNA strands. Both damaged DNA and oxidized dNTPs interfere with faithful DNA synthesis by Pols, inducing various cellular abnormalities, such as mutations, cancer, neurological diseases, and cellular senescence. In this review, we focus on the process by which Pols incorporate oxidized dNTPs into DNA and compare the properties of Pols: efficiency, i.e., k_{cat}/K_m, k_{pol}/K_d or V_{max}/K_m, and template base preference for the incorporation of 8-oxo-dGTP, an oxidized form of dGTP. In general, Pols involved in chromosome replication, the A- and B-family Pols, are resistant to the incorporation of 8-oxo-dGTP, whereas Pols involved in repair and/or translesion synthesis, the X- and Y-family Pols, incorporate nucleotides in a relatively efficient manner and tend to incorporate it opposite template dA rather than template dC, though there are several exceptions. We discuss the molecular mechanisms by which Pols exhibit different template base preferences for the incorporation of 8-oxo-dGTP and how Pols are involved in the induction of mutations via the incorporation of oxidized nucleotides under oxidative stress.

Contents

1. Introduction

Chromosome DNA is continuously exposed to various endogenous and exogenous genotoxic agents. Among these agents, oxidation is one of the most common threats to genetic stability [1,2]. Each human cell is estimated to metabolize approximately 10^{12} molecules of oxygen per day, and approximately 1% of oxygen

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metabolism results in the production of reactive oxygen species (ROS) via one electron reduction [3,4]. These reactive molecules include superoxide, hydrogen peroxide, hydroxyl radicals, and singlet oxygen, with hydroxyl radicals thought to be the most predominant reactive species [5]. ROS are also generated in cells when they are exposed to radiation and chemical carcinogens. To counteract the oxidative stress induced by ROS, cells have evolved multiple defense mechanisms. Enzymes, such as catalase or superoxide dismutase, detoxify ROS and low-molecular-weight scavengers, such as glutathione, reduce the toxicity of ROS [6]. Nevertheless, some ROS molecules escape from the defense systems and inevitably damage the bio-molecules. Thus, ROS have been implicated in the etiology of human degenerative diseases, aging, and cancer [7,8].

Although ROS generate a variety of modified bases in DNA, 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-dG) is the best characterized oxidized base in chemistry and biology [9–11] and is used as a biomarker of DNA oxidation. Approximately 10^3 8-oxo-dG molecules are generated in normal human cells per day [2,12]. 8-oxo-dG pairs with cytosine in the *anti* conformation but assume the *syn* conformation when pairing with adenine [13–15]. In fact, both dATP and dCTP are inserted opposite template 8-oxo-dG during DNA synthesis [16]. The latter pairing can result in G:C to T:A transversion [17]. To prevent the mutagenesis caused by the lesion, human cells possess multiple repair mechanisms [18], including DNA glycosylases in the base excision repair pathway, such as 8-oxoguanine glycosylase (OGG1) and MutY glycosylase homologue (MUTYH), which excise 8-oxo-G when paired with cytosine and adenine opposite 8-oxo-dG, respectively [19]. The Cockayne syndrome proteins CSA and CSB in transcription-coupled nucleotide excision repair also involve the exclusion of 8-oxo-dG from DNA [20–22]. Despite the presence of repair mechanisms, 8-oxo-dG accumulates in senescent cells and the brain cortex of aged humans, which may cause various cellular abnormalities [23–26].

In addition to the direct oxidation of deoxyguanosine (dG) in DNA, 8-oxo-dG can be generated by the incorporation of oxidized dGTP (8-oxo-dGTP) into DNA by DNA polymerases (Pols). 8-oxo-dGTP can be incorporated into the template strand opposite deoxycytidine (dC) or deoxyadenosine (dA) and the latter may cause A:T to C:G transversions [27]. In fact, *Escherichia coli mutT* mutants, which lack the ability to hydrolyze 8-oxo-dGTP to its mono-phosphate form, exhibit more than 1000 times higher frequencies of spontaneous A:T to C:G transversions than controls [28]. Mice lacking MTH1, a mammalian homologue of MutT, display enhanced tumor formation in the lung, liver, and stomach [29]. In human cells, suppression of MTH1 expression induces cellular senescence [30]. In contrast, the over-expression of hMTH1 reduces total cellular 8-oxo-dG levels in human cells and transgenic mice [31,32]. Over-expression also suppresses genome instability in cells with defective mismatch repair (MMR) mechanisms and causes ameliorated neuropathological and behavioral symptoms resembling Huntington's disease in mice. Thus, the oxidized dNTP pool is recognized as a source of spontaneous mutagenesis, carcinogenesis, cellular senescence, and neurological disease.

In human cells, MTH1 hydrolyzes 8-oxo-dGTP and other oxidized dNTPs, such as 2-hydroxy-dATP (2-OH-dATP) and 8-oxo-dATP, to the mono-phosphate forms in the nucleotide pool [33]. In addition to MTH1, cells possess MTH2, which hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP [34], and Nudix type 5 (NUDT5) protein, which hydrolyzes 8-oxo-dGDP to the mono-phosphate [35]. MMR prevents the mutations caused by the incorporation of 8-oxo-dGTP [31]. 8-oxo-dG incorporated during replication can become a target of MMR machinery, which removes the incorporated 8-oxo-dG from DNA and initiates DNA re-synthesis.

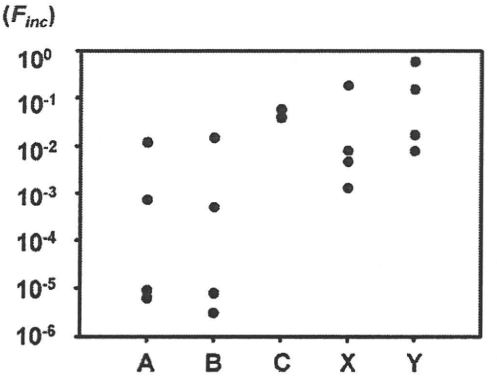

Fig. 1. Efficiency of the incorporation of 8-oxo-dGTP into DNA by family A, B, C, X, and Y Pols. F_{inc} indicates the ratio of the efficiency of incorporating 8-oxo-dGTP opposite a favorable template base versus that of incorporating normal dNTPs opposite the same template base. F_{inc} was calculated from the kinetic parameters in the references (see Table 1). Pols include hPolγ, *E. coli* Pol I Klenow fragment (exo⁻), and T7 Pol for family A; hPolα, bovine Polδ, *E. coli* Pol II, and φ29 Pol for family B; *E. coli* Pol III* and α subunit of Pol III for family C; hPolβ, hPolλ, and African swine fever virus Pol X for family X; and hPolη, Polι, and Polκ for family Y.

Measurements of intracellular levels of 8-oxo-dGTP in nucleotide pool are a challenging issue. Initial attempt to measure the level of 8-oxo-dGTP in *E. coli* was failed [36]. Recent measurements with improved sensitive methods indicate that the level of 8-oxo-dGTP in mitochondria is similar to that of normal dTTP, which is 1–10% of the level of normal dGTP [37]. It remains to be seen the levels of 8-oxo-dGTP and other oxidized dNTPs in nucleus in oxidative stressed and non-stressed human cells.

To exert adverse effects, oxidized dNTPs must be incorporated into DNA by Pols. In culture medium, 8-oxo-dG is readily incorporated into the genomic DNA of human cells upon phosphorylation [38]. As shown below, however, various Pols have distinct properties in terms of their efficiency in incorporating 8-oxo-dGTP into DNA. In this review, we use the term "efficiency" to mean k_{cat}/K_m or V_{max}/K_m in steady-state kinetic analyses and k_{pol}/K_d in pre-steady-state kinetic analyses. Some Pols incorporate 8-oxo-dGTP into DNA very efficiently, whereas others do so poorly. So far, we have surveyed the maximum difference of F_{inc} (i.e. the ratio of the efficiency for incorporating 8-oxo-dGTP versus that of incorporating normal dNTP) among various Pols to be 10^5 (Fig. 1, Table 1). For example, human Polη incorporates 8-oxo-dGTP opposite template dA with 20–60% efficiency compared to normal dTTP incorporation [39,40], whereas *E. coli* Pol II exo⁻ incorporates it opposite template dC with 0.0003% efficiency compared to normal dGTP incorporation [41]. In addition, the preference of the Pols for template bases is distinct (Table 2); Polη incorporates 8-oxo-dGTP opposite template dA almost exclusively [40], and *E. coli* Pol II exo⁻ incorporates it opposite template dC more favorably (the ratio for incorporation opposite template dC:dA is 22:1) [41].

In this review, we compare the relative efficiency and template base preferences of Pols for incorporating 8-oxo-dGTP. Because Pols are categorized into six families (A, B, C, D, X, and Y) based on their primary structures [42], we review the properties of Pols family by family. The A, B, or C families of Pols are involved in chromosome replication with high fidelity and high processivity, and the X and Y families are responsible for DNA repair synthesis and translesion synthesis (TLS). Pols family D is restricted to Archaea, and their properties for 8-oxo-dGTP incorporation have not been reported. Therefore, we omit the D-family Pols from this review. We also discuss mechanisms underlying the distinct properties of some Pols and the biological consequences of the incorporation of 8-oxo-dGTP.

Table 1
Relative efficiencies of DNA polymerases for incorporating 8-oxo-dGTP compared to normal dNTP.

Family	DNA polymerase	F_{inc}	Reference
A	KF (exo$^-$)	1.2×10^{-2}	[49]
	KF (exo$^-$)	7.2×10^{-4}	[47]
	T7 Pol	9.1×10^{-6}	[41]
	KF (exo$^-$)	6.4×10^{-6}	[41]
B	Polα (calf thymus)	1.5×10^{-2}	[49]
	φ29 Pol	5.0×10^{-4}	[56]
	Polα (calf thymus)	7.9×10^{-6}	[57]
	Pol II (exo$^-$)	3.1×10^{-6}	[41]
C	Pol III*	5.6×10^{-2}	Yamada et al., unpublished data
	α Subunit of Pol III	3.9×10^{-2}	[28]
X	hPolβ	1.8×10^{-1}	[68]
	hPolβ	8.0×10^{-3}	[69]
	hPolλ	4.7×10^{-3}	[69]
	ASFV Pol X	1.3×10^{-3}	[70]
Y	hPolη	5.9×10^{-1}	[40]
	hPolη	1.5×10^{-1}	[39]
	hPolι	1.7×10^{-2}	[40]
	hPolκ	7.9×10^{-3}	[39]

F_{inc} was calculated as the ratio of the efficiency of incorporating 8-oxo-dGTP opposite the favorite template base versus incorporating normal dNTPs opposite the same template base. The "efficiency" was defined as k_{cat}/K_m or V_{max}/K_m in steady-state kinetic analyses [28,39–41,47,49,56,57,68] or k_{pol}/K_d in pre-steady-state kinetic analyses [69,70].

2. Efficiency and template base preferences of Pols

2.1. A-family Pols

A-family Pols are defined as homologues of *E. coli* DNA Pol I, which was the first Pol to be described and is involved in the synthesis of Okazaki fragments during lagging strand synthesis and in DNA repair [43,44]. Representative members of this family are Polγ and Polθ in humans, and T7 Pol in phages. In general, Pols in this family inefficiently incorporate 8-oxo-dGTP into DNA compared to the incorporation of normal dNTPs (Fig. 1, Table 1). Polγ, which functions solely in mitochondrial DNA replication and has 3′ to 5′ exonuclease activity [45], incorporates 8-oxo-dGTP opposite template dA with a F_{inc} of approximately 10^{-4} [46]. This enzyme favorably incorporates 8-oxo-dGTP opposite template dA compared to template dC at a ratio of 13:1 (Table 2). The resulting 8-oxo-dG:dA pair is extended, rather than excised, by the exonuclease activity [46]. T7 Pol, which is involved in the replication of T7 phage, also poorly incorporates 8-oxo-dGTP with a F_{inc} of approximately 10^{-4} and preferably incorporates it opposite template dA compared to dC [41]. In contrast, the *E. coli* Pol I Klenow fragment deficient in exonuclease activity (KF exo$^-$) incorporates 8-oxo-dGTP opposite dA comparable to dC [41,47,48]. It incorporates 8-oxo-dGTP inefficiently ($F_{inc} = 10^{-5}$–10^{-6}). 8-oxo-dGTP may be more efficiently incorporated into template run sequences, such as CCCCCC, by KF(exo$^-$) and B-family Polα [49]. KF exo$^-$ also incorporates 5-hydroxydeoxycytidine tri-phosphate and 5-hydroxydeoxyuridine tri-phosphate into DNA more efficiently than 8-oxo-dGTP [47]. The degree of excision of 8-oxo-dGTP opposite template dA is enzyme-dependent: Polγ and KF excise it poorly while T4 Pol (B-family Pol) excises it efficiently [46,50]. The A-family Pols exhibit a broad range of template base preferences for the incorporation of 8-oxo-dGTP into DNA.

2.2. B-family Pols

B-family Pols are homologues of *E. coli* Pol II. This enzyme is encoded by *polB*, which is induced by DNA damage under the control of SOS regulation [51]. Pol II has both DNA Pol activity and 3′ to 5′ exonuclease activity in a single polypeptide [52]. Although Pol II is not involved in chromosome replication, the mammalian B-family members are responsible for the replication of the genome [42]. In humans, the B-family Pols, i.e., Polα, Polδ, and Polε, are involved in replication and DNA repair [53,54], and another B-family member, Polζ, plays a role in TLS with Y-family Pols [55]. Bacteriophage φ29 Pol is a protein-primed DNA-dependent replicase belonging to the B family. Similar to the A-family Pols, the B-family Pols poorly incorporate 8-oxo-dGTP into DNA. φ29 Pol incorporates 8-oxo-dGTP 2000-fold less efficiently than unmodified dGTP (Fig. 1, Table 1) [56]. The enzyme has a preference for pairing 8-oxo-dGTP with template dC (Table 2), and φ29 Pol extends the correct 8-oxo-dG:dC pair preferentially, with an efficiency similar to that of the

Table 2
Favorite template base for incorporation of 8-oxo-dGTP by various Pols.

Favorite template	DNA polymerase	Family	dA/dC[a]	Reference
dA » dC	hPolι	Y	dA only	[40]
	Dbh	Y	dA only	[59]
	Dpo4	Y	dA only	[59]
	hPolη	Y	660:1, 180:1	[39,40]
dA > dC	hPolλ	X	35:1	[69]
	T7 Pol	A	31:1	[41]
	hPolβ	X	24:1, 11:1	[68,69]
	Pol III *	C	20:1	Yamada et al., unpublished data
	hPolγ	A	13:1	[46]
	hPolκ	Y	11:1	[39]
	Pol IV	Y	ND	[48]
dA ≈ dC	KF (exo$^-$)	A	1.6:1, 0.44:1	[47,41]
	α Subunit of Pol III	C	1.3:1	[28]
	HIV-1 RT	RT	0.5:1	[41]
	ASFV Pol X	X	0.5:1	[70]
	φ29 Pol	B	0.33:1	[56]
	DNA Pol B1 (*Sso*)	B	ND	[59]
dA < dC	Bovine Polδ + PCNA	B	0.032:1	[84]
	Pol II (exo$^-$)	B	0.045:1	[41]
	hPolα	B	ND[b]	Katafuchi et al., unpublished data

[a] The ratio of dA/dC was determined as the ratio of the efficiency of incorporation 8-oxo-dGTP opposite template dA compared to that of incorporating it opposite template dC. The efficiency was defined as k_{cat}/K_m or V_{max}/K_m in steady-state kinetic analyses [28,39–41,47,48,56,59,68,84] or k_{pol}/K_d in pre-steady-state kinetic analyses [46,69,70].
[b] ND: the ratio was not determined. RT stands for reverse transcriptase.