TABLE IV. DNA Sequence Analysis of *gpt* Mutations Obtained From the Lungs of DE, DEP-Treated, and Control Mice

Type of mutation	Nucleotide	Sequence change		Amino acid change	Number				
					Control (DE)	DE	Control (DEP and DEP extract)	DEP	DEP extract
Base substitution									
Transition									
G:C → A:T	3	atG → atA		Met → Ile				1	
	26	tGg → tAg		Trp → Stop		2[a]			
	27	tgG → tgA		Trp → Stop	1				
	64	Cga → Tga	CpG	Arg → Stop	6[c]	8[b]		4[b]	7[d]
	82	Caa → Taa		Gln → Stop		1			1
	87	tgG → tgA		Trp → Stop	1	1		1	1
	92	gGc → gAc		Gly → Asp		1			
	110	cGt → cAt	CpG	Arg → His	5[c]	14[d]	3[a]	16[c]	15[f]
	113	gGc → gAc		Gly → Asp		1			
	115	Ggt → Agt	CpG	Gly → Ser	1	6[d]	1	4[b]	3[b]
	116	gGt → gAt		Gly → Asp	1	1	2		2[a]
	128	gGt → gAt		Gly → Asp		1			
	145	Gaa → Aaa		Glu → Lys	1			1	1
	176	tGt → tAt		Cys → Tyr		1			
	185	aGc → aAc		Ser → Asn		3[b]			
	262	Gat → Aat		Asp → Asn	1			1	
	274	Gat → Aat		Asp → Asn	1			2[a]	1
	281	gGt → gAt		Gly → Asp		1			
	287	aCt → aTt		Thr → Ile	1				
	290	gCg → gTg	CpG	Ala → Val	1				
	346	Ccg → Tcg		Pro → Ser					1
	350	gCt → gTt		Ala → Val				1	1
	391	Caa → Taa		Gln → Stop		2[a]		1	1
	401	tGg → tAg		Trp → Stop		6[b]	1	3[b]	
	402	tgG → tgA		Trp → Stop		4[b]			
	406	Gaa → Aaa		Glu → Lys		6[a]			1
	409	Cag → Tag		Gln → Stop				1	
	416	tGg → tAg		Trp → Stop				1	
	417	tgG → tgA		Trp → Stop				7[a]	
	418	Gat → Aat		Asp → Asn	2[a]	10[c]		5[b]	2
	451	Ggt → Agt	CpG	Gly → Ser				1	
A:T → G:C	17	aTc → aCc		Ile → Thr		1			
	41	aTc → aCc		Ile → Thr			1		
	56	cTc → cCc		Leu → Pro	1	2[a]			3[a]
	146	gAa → gGa		Glu → Gly					1
	181	Tcc → Ccc		Ser → Pro					1
	269	cTg → cCg		Leu → Pro	1				1
	400	Tgg → Cgg		Trp → Arg	1				
	415	Tgg → Cgg		Trp → Arg					1
	419	gAt → gGt		Asp → Gly	1			1	
Transversion									
G:C → T:A	3	atG → atT		Met → Ile				1	
	7	Gaa → Taa	CpG	Glu → Stop	1			2	2[a]
	15	taC → taA		Tyr → Stop					1
	26	tGg → tTg		Trp → Leu				1	
	59	gCa → gAa		Ala → Glu		1			
	79	Gaa → Taa		Glu → Stop					1
	107	aGc → aTc		Ser → Ile	1				
	115	Ggt → Tgt	CpG	Gly → Cys	1				
	116	gGt → gTt		Gly → Val		1		1	
	140	gCg → gAg	CpG	Ala → Glu		1	1	1	
	143	cGt → cTt	CpG	Arg → Leu					2[a]
	145	Gaa → Taa		Glu → Stop			1	1	1
	176	tGt → tTt		Cys → Phe				2[a]	2[a]
	182	tCc → tAc		Ser → Tyr				1	
	185	aGc → aTc		Ser → Ile		1			
	189	taC → taA	CpG	Tyr → Stop	3			1	

TABLE IV. *Continued*

Type of mutation	Nucleotide	Sequence change		Amino acid change	Number				
					Control (DE)	DE	Control (DEP and DEP extract)	DEP	DEP extract
	190	Gat → Tat	CpG	Asp → Tyr				1	
	205	Cgc → Agc	CpG	Arg → Ser				1	
	208	Gag → Tag	CpG	Glu → Stop				1	
	220	Ctg → Atg		Leu → Met					1
	230	gCa → gAa		Ala → Glu				1	
	244	Gaa → Taa	CpG	Glu → Stop				1	
	262	Gat → Tat		Asp → Tyr				1	
	268	Ctg → Atg		Leu → Met				1	
	274	Gat → Tat		Asp → Tyr	1				2[a]
	287	aCt → aAt		Thr → Asn	1				1
	304	Gaa → Taa		Glu → Stop		2[a]		1	
	313	Cca → Aca		Pro → Thr				1	
	319	Gcg → Tcg		Ala → Ser		1			
	320	gCg → gAg	CpG	Ala → Glu		1	1	1	1
	346	Ccg → Acg		Pro → Thr					1
	389	cCg → cAg	CpG	Pro → Gln					1
	401	tGg → tTg		Trp → Leu	1	2[a]		1	1
	402	tgG → tgT		Trp → Cys		3[a]		1	
	406	Gaa → Taa		Glu → Stop	7[d]	3[a]		4[b]	4[c]
	409	Cag → Aag		Gln → Lys		2	1	1	
	411	caG → caT		Gln → His				1	
	412	Ccg → Acg		Pro → Thr					1
	413	cCg → cAg	CpG	Pro → Gln	1				
	418	Gat → Tat		Asp → Tyr		3[a]		6[a]	1
G:C → C:G	6	agC → agG	CpG	Ser → Arg		2		1	
	46	Gca → Cca		Ala → Pro		1			
	112	Ggc → Cgc		Gly → Arg					1
	115	Ggt → Cgt	CpG	Gly → Arg		2[a]			1
	125	cCg → cGg	CpG	Pro → Arg					1
	127	Ggt → Cgt		Gly → Arg	1			1	
	130	Gcg → Ccg		Ala → Pro				1	
	139	Gcg → Ccg		Ala → Pro		1			
	145	Gaa → Caa		Glu → Gln					1
	185	aGc → aCc		Ser → Thr					2
	190	Gat → Cat	CpG	Asp → His					1
	262	Gat → Cat		Asp → His				1	
	280	Ggt → Cgt	CpG	Gly → Arg					1
	281	gGt → gCt		Gly → Ala				1	
	290	gCg → gGg	CpG	Ala → Gly			1		
	295	Gcg → Ccg		Ala → Pro					1
	340	Gca → Cca	CpG	Ala → Pro		1			1
	402	tgG → tgC		Trp → Cys				1	
	413	cCg → cGg	CpG	Pro → Arg					1
	442	Cca → Gca		Pro → Ala			1		
	443	cCa → cGa		Pro → Arg				1	1
A:T → T:A	8	gAa → gTa		Glu → Val				1	
	10	Aaa → Taa		Lys → Stop				1	
	11	aAa → aTa		Lys → Ile				1	
	12	aaA → aaT		Lys → Asn					1
	83	cAa → cTa		Gln → Leu				1	
	134	tTa → tAa		Leu → Stop		1			
	146	gAa → gTa		Glu → Val		1			
	164	gTc → gAc		Val → Asp				1	
	187	Tac → Aac		Tyr → Asn			1		
	214	Aaa → Taa		Lys → Stop				1	
	260	aTt → aAt		Ile → Asn	1				
	311	tAt → tTt		Tyr → Phe					1
	365	gTt → gAt		Val → Asp	1				
	419	gAt → gTt		Asp → Val		4			

TABLE IV. *Continued*

Type of mutation	Nucleotide	Sequence change	Amino acid change	Number				
				Control (DE)	DE	Control (DEP and DEP extract)	DEP	DEP extract
A:T → C:G	10	Aaa → Caa	Lys → Gln					1
	17	aTc → aGc	Ile → Ser				1	
	56	cTc → cGc	Leu → Arg				4	
	106	Agc → Cgc	Ser → Arg				1	1
	188	tAc → tCc	Tyr → Ser				1	
	312	taT → taG	Tyr → Stop					1
	345	aaA → aaC	Lys → Asn					1
	419	gAt → gCt	Asp → Ala				1	
Deletion								
−1 base	8–12	gAAAAAt → gAAAAt		4[b]				1
	32	aTg → ag					1	
	34–35	gTTg → gTg			1	1		
	37	gCa → ga			2[a]			
	83–84	cAAt → cAt						1
	97	tAt → tt						1
	115–116	cGGt → cGt			1			
	137	cTg → cg			1			
	170–171	aCCg → aCg			1			
	176	tGt → tt		1				
	201–202	aCCa → aCa		1				
	237	gCg → gg					1	1
	272	gTg → gg			1			
	315–318	cAAAAg → cAAAg					1	
	321	cGc → cc			1			
	352–353	tGGt → tGt			1			
	369	aTg → ag					1	
	407–408	gAAc → gAc						1
	412–413	gCCg → gCg			1			
	416–418	tGGGa → tGGa		2[a]			1	2[a]
	426	gCg → gg			1			
	431	gTa → ga						1
	442–443	gCCa → gCa		1	1			
	444–445	cAAt → cAt					2[a]	1
	449–450	tCCg → tCg						1
	451–452	cGGt → cGt					5[d]	1
>2	97–111	tATTGCCGTAAGCCGTg → tg						1
	149–165	cTGGGTATTCGTCATGTCg → cg						1
	158–170	cGTCATGTCGATACc → cc					1	
	170–171	aCCg → ag			1			
	177–196	gTATTTCCAGCTACGATCACGa → ga			1			
	226–227	aCGc → ac			1			
	250–255	cTTCATCg → cg					1	
	252–254	tCATc → tc			1			
	262–273	tGATGACCTGGTGg → tg			1			
	292–320	gGTTGCGATTCGTGAAAT-GTATCCAAAAGCg → gg					1	
	375–377	aTGTt → at					1	
Insertion	8–12	gAAAAAt → gAAAAAAt			1			
	35	tg → tCg					1	
	83–84	cAAt → cAAAt					1	
	120–121	tGGt → tGGGt						2
	269	cTg → cTTg			1			
	301	cg → cGTGAAATg					1	
	312	tc → tGc					1	
Other	46–47	tGCa → tAGa			1			
	401–402	tGGa → tAAa						1

[a]Mutations found in 2 different mice.
[b]Mutations found in 3 different mice.
[c]Mutations found in 4 different mice.
[d]Mutations found in 5 different mice.
[e]Mutations found in 7 different mice.
[f]Mutations found in 8 different mice.

TABLE V. Classification of *gpt* Mutations From the Lungs of Control, DEP-, and DEP Extract-Treated Mice

Type of mutation in *gpt*	Control		DEP All		DEP extract All		DEP (mg) 0.125		DEP (mg) 0.25		DEP (mg) 0.5		DEP extract (mg) 0.05		DEP extract (mg) 0.1		DEP extract (mg) 0.2	
	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%	Number	%
Base substitution																		
Transition																		
G:C → A:T (CpG site)	7 (4)	44	50 (25)	39	37 (25)	37	18 (13)	46	20 (11)	43	12 (1)	29	10 (6)	30	12 (9)	35	15 (10)	44
A:T → G:C	1	6	1	1	7	7	0	0	1	2	0	0	3	9	2	6	2	6
Transversion																		
G:C → T:A	4	25	35	28	23	23	9	23	10	21	16	39	7	21	8	24	8	24
G:C → C:G	2	13	7	6	12	12	4	10	1	2	2	5	8	24	1	3	3	9
A:T → T:A	1	6	6	5	2	2	2	5	2	4	2	5	0	0	2	6	0	0
A:T → C:G	0	0	8	6	4	4	1	3	3	6	4	10	0	0	2	6	2	6
Deletion																		
−1	1	6	12	9	11	11	3	8	6	13	3	7	2	6	5	15	4	12
>2	0	0	4	3	2	2	1	3	3	6	0	0	1	3	1	3	0	0
Insertion	0	0	4	3	2	2	1	3	1	2	2	5	2	6	0	0	0	0
Other	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	3	0	0
Total	*16*	*100*	*127*	*100*	*101*	*100*	*39*	*100*	*47*	*100*	*41*	*100*	*33*	*100*	*34*	*100*	*34*	*100*

reported by Sun et al. [1984], Bond et al. [1986], and Yu et al. [1991] demonstrated that in the lungs the concentration of organic compounds released from DEP reaches a steady state at an early phase of exposure. Alternatively, DE-induced mutagenesis may occur via a mechanism that is easily saturated, such as DNA adduct formation, generation of reactive oxygen species, or inflammation. Further studies are required to examine whether mutagenicity is increased in DNA repair system-deficient mice by DE treatment.

The IARC classification categorizes DE as a Group 2A substance and DEP contains various potent mutagenic PAHs and nitro-PAHs such as B[*a*]P and DNPs. To assess the health risks of DE, we attempted to identify components responsible for mutagenicity in the lungs of *gpt* delta mice. As mentioned above, intratracheal instillation of DEP or DEP extract increased the MF linearly, depending on the dosage (Table II). Inhalation of DE and instillation of DEP and DEP extract induced mutations at hotspots in *gpt* (mutation loci identified from three or more mice at nucleotide numbers 64, 110, 115, 185, 401, 402, and 418 (DE inhalation); 64, 110, 115, 401, and 418 (DEP); and 64, 110, and 115 (DEP extract); Table IV). Analysis of sequences adjacent to the mutated guanine bases (Fig. 1) identified the sequences GGA, TGA, CGG, and CGT as major targets for mutation. These results indicate that DE, DEP, and DEP extract targeted the same nucleotide sequences on *gpt*, producing DNA adducts. According to the Environmental Health Criteria 171 [International Programme on Chemical Safety, 1996], the carbon black in DEP and DEP extract contributes to the carcinogenicity of DE. Our study demonstrates that DEP extract exerts potent in vivo mutagenicity in the lung and that the mutation hotspots and target sequences are similar for mice that are treated with inhalation of DE or instillation of DEP and DEP extract. These observations suggest that compounds in the DEP extract induce mutations in the lung.

We compared the mutation spectra caused by the different treatments and observed that following inhalation of DE, G:C → A:T transitions were the predominant mutation in *gpt* delta mice (Table III). This result confirms previous observations in the lungs of Big Blue® rats [Sato et al., 2000]. In the lungs of DEP and DEP extract-instilled mice, G:C → A:T transitions were also the major base substitutions but G:C → T:A transversions were induced at a higher frequency than by inhalation of DE (Table V). We expected that G:C → T:A transversions would be the predominant base substitution induced by DE, since B[*a*]P is considered to be a major mutagenic component in DE, and such transversions are a common B[*a*]P-induced mutation in the mouse lung [Hashimoto et al., 2005]. As G:C → T:A transversions were induced by DEP- and DEP extract-instillation, it is likely that B[*a*]P contributes partly to DE-induced mutagenesis. However, to understand the mechanism underlying DE-induced carcinogenesis, the mutagen responsible for G:C → A:T transitions must be identified.

A number of mutagenic compounds have been identified in DEP using a Salmonella assay [Jeffrey et al., 1990; Tokiwa et al., 1993], and Salmeen et al. [1984] suggested that mono- and dinitro-PAHs such as 1,3-, 1,6- and 1,8-DNP may account for between 30 and 40% of the mutagenic activity. We have shown that intratracheal instillation of 1,6-DNP into the lungs of *gpt* delta mice [Hashimoto et al., 2005, 2006] induced mainly G:C → A:T transitions but not G:C → T:A transversions. In addition, the mutation

hotspots induced by DE inhalation were similar to those induced by 1,6-DNP [Hashimoto et al., 2006] but not by B[*a*]P [Hashimoto et al., 2005]. These results suggest that 1,6-DNP and related compounds are likely to be among the main contributors to mutagenesis induced by DE inhalation. Further studies are required to identify all the major mutagens in DE and to understand the mechanisms of mutagenesis induced by DE inhalation.

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Differential effects of low- and high-dose X-rays on *N*-ethyl-*N*-nitrosourea-induced mutagenesis in thymocytes of B6C3F1 *gpt*-delta mice

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Abstract

Carcinogenesis in humans is thought to result from exposure to numerous environmental factors. Little is known, however, about how these different factors work in combination to cause cancer. Because thymic lymphoma is a good model of research for combined exposure, we examined the occurrence of mutations in thymic DNA following exposure of B6C3F1 *gpt*-delta mice to both ionizing radiation and *N*-ethyl-*N*-nitrosourea (ENU). Mice were exposed weekly to whole body X-irradiation (0.2 or 1.0 Gy), ENU (200 ppm) in the drinking water, or X-irradiation followed by ENU treatment. Thereafter, genomic DNA was prepared from the thymus and the number and types of mutations in the reporter transgene *gpt* was determined. ENU exposure alone increased mutant frequency by 10-fold compared to untreated controls and over 80% of mutants had expanded clonally. X-irradiation alone, at either low or high dose, unexpectedly, reduced mutant frequency. Combined exposure to 0.2 Gy X-rays with ENU dramatically decreased mutant frequency, specifically G:C to A:T and A:T to T:A mutations, compared to ENU treatment alone. In contrast, 1.0 Gy X-rays enhanced mutant frequency by about 30-fold and appeared to accelerate clonal expansion of mutated cells. In conclusion, repeated irradiation with 0.2 Gy X-rays not only reduced background mutation levels, but also suppressed ENU-induced mutations and clonal expansion. In contrast, 1.0 Gy irradiation in combination with ENU accelerated clonal expansion of mutated cells. These results indicate that the mode of the combined mutagenic effect is dose dependent.

Keywords: Combined genotoxic effect; *gpt*-delta mouse; *N*-Ethyl-*N*-nitrosourea; Radiation; Clonal expansion

1. Introduction

Humans are continuously and simultaneously exposed to numerous environmental mutagens. It is important, therefore, to determine the dose–effect relationship of combined exposure when assessing human health risks. Mutagenic DNA damage arises from interaction of DNA with a myriad of endogenous and exogenous agents [1]. Ionizing radiation induces DNA strand breaks and base modifications, directly or indirectly via reactive oxygen species. Such DNA damages result in base substitutions, insertions and deletions. Thus, the recent increase in the utilization of, and hence chance for exposure to, medical ionizing radiation has raised an aspect of social concern. Alkylating agents also modify DNA, thereby inducing base substitutions that lead to point mutations [2,3]. These agents are found in plants, food, cigarette smoke, fuel combustion products, and commonly used industrial solvents. In addition, ionizing radiation and some alkylating agents are used for cancer chemotherapy. *N*-Ethyl-*N*-nitrosourea (ENU) is a potent alkylating mutagen and carcinogen that induces G:C to A:T transitions,

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A:T to G:C transitions and A:T to T:A transversions *in vivo* [4–8]. Since ionizing radiation and alkylating agents are widely distributed and utilized, understanding their toxicity and the cellular response mechanism(s) is important. Occasionally, we are exposed to these agents simultaneously in the environment or for therapeutic reasons. However, currently available data are not sufficient to delineate the mode and the mechanism of combined action of these agents.

The combined effect of radiation and alkylating agents has been examined in animal tumor models, including thymic lymphomas, brain tumors, mammary tumors and colon tumors [9–12]. Thymic lymphoma (TL) is an excellent model for human T-cell acute lymphoblastic leukemia and has been used for the study of radiation carcinogenesis. Concomitant exposure to butylnitrosourea and X-rays for 12 weeks accelerates the development of thymic lymphoma in BDF1 mice, in a manner dependent on the dose of radiation [9,13]. Irradiation 2–5 weeks before methylnitrosourea exposure accelerates thymic lymphoma development [13]. Irradiation for 5 days, but not 30 days, before administrating ENU treatment enhances lymphomagenesis [9].

DNA mutations play a central role in carcinogenesis. The frequency and type of mutations that result from combined treatment may shed light on the molecular mechanism(s) underlying the carcinogenic effects of combined exposure to alkylating agents and radiation. In order to delineate such mechanisms, we have examined the occurrence of mutations in thymic cells of B6C3F1 (*gpt*+/−) mice after combined exposure to X-rays and ENU. Repeated exposure to high-dose X-rays (1.0 Gy) followed by ENU increased the frequency of mutants and facilitated clonal expansion of mutated cells. However, unexpectedly, when low-dose X-rays (0.2 Gy) preceded ENU, mutant frequency was reduced primarily due to a decrease in G:C to A:T and A:T to T:A mutations. This is the first report, to our knowledge, that provides a new evidence on the mode and mechanism of combined exposure, which clearly differs between low and high dose of radiation.

2. Materials and methods

2.1. Mice

Male C3H/He mice were purchased from Charles River Laboratories (Kanagawa, Japan). Female *gpt*-delta C57BL/6J mice carrying approximately 80 copies of λEG10 DNA in haploid genome were obtained from Japan SLC (Shizuoka, Japan) [14]. Mice were housed five to a cage in a room maintained at 23 ± 2 °C and 50 ± 10% humidity, with a 12 h dark–light cycle. All mice were fed standard laboratory diet MB-1 (Funabashi Farm Co., Ltd., Chiba, Japan) and water *ad libitum*.

2.2. Chemicals

ENU (CAS No. 759-73-9) was purchased from Nakarai Tesque (Kyoto, Japan). Reagents for M9 buffer preparation were purchased from Nakarai Tesque and Wako Pure Chemical Industries (Osaka, Japan). Dimethyl sulfoxide and 6-thioguanine (6-TG) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Chloramphenicol (Cm) was purchased from Wako Pure Chemical Industries. Bacto yeast extract, Bacto tryptone and Bacto agar were obtained from Difco (Franklin Lakes, NJ, USA).

2.3. Irradiation

X-ray irradiation was performed using a Pantak HF-320 machine (PANTAK Ltd., East Haven, CT, USA) at 200 kV, 20 mA, and a dose rate of 0.7 Gy/min.

2.4. Combined X-ray exposure and ENU treatment

Mice (4–5 weeks old) were exposed weekly to 0.2 or 1.0 Gy X-rays for 4 consecutive weeks, because this X-ray exposure method is the most leukemogenic [15]. ENU was dissolved in reverse osmosis water (Elix UV10, Millipore, Bedford, MA) at pH 6.0 to prepare 200 ppm (1.17 mM) solution. To avoid degradation, ENU solution was administered in drinking water in brown bottle immediately after preparation. Freshly prepared ENU solution was exchanged on every Monday, Wednesday, Friday and Saturday during treatment. The amount of ENU solution up-taken was approximately 3 ml per mouse per day. For combined exposures, X-ray irradiation at 0.2 or 1.0 Gy for 4 consecutive weeks was followed by 4 weeks of ENU treatment (Fig. 1). Four weeks after the end of ENU treatment, mice were sacrificed and thymuses were collected and frozen immediately in liquid nitrogen, then stored at −80 °C until use. These animal experiments were conducted in compliance with guidelines for animal experiments of the National Institute of Radiological Sciences for the care and use of laboratory animals.

2.5. Genomic DNA extraction and in vitro packaging

The thymuses (20 mg) were homogenized in a Dounce homogenizer (Kontes, Vineland, NJ, USA) and genomic DNA was isolated using the RecoverEase DNA isolation kit (Stratagene, La Jolla, CA, USA). Transpack Packaging Extract (Stratagene, La Jolla, CA, USA) was used to rescue λEG10 phages from genomic DNA (10 μl) isolated from the thymus.

2.6. gpt mutation assay

The *gpt* mutagenesis assay was performed on five mice per group according to methods previously described [16]. Briefly, λEG10 phage was incubated with *E. coli* YG6020 at 37 °C for 20 min. After incubation, *E. coli* was incubated at 37 °C with vigorous agitation for 30 min. *E. coli* was mixed with 0.6% molten soft agar with or without 6-TG and the entire contents poured onto the M9 + Cm + 6-TG or M9 + Cm plates and incubated at 37 °C for 3 or 2 days. After incubation, colonies were counted and on M9 + Cm + 6-TG plates were subject to colony PCR for *gpt* gene. At least three independent experiments were performed per mouse.

Mutant frequency was calculated by dividing the number of colonies growing on M9 + Cm + 6-TG plates by the number of colonies growing on M9 + Cm plates.

Recurrent mutations derived from the same tissue of a single animal could be the result of clonal expansion that occurred early after mutagen treatment. When multiple identical mutations were recovered from an individual mouse, the

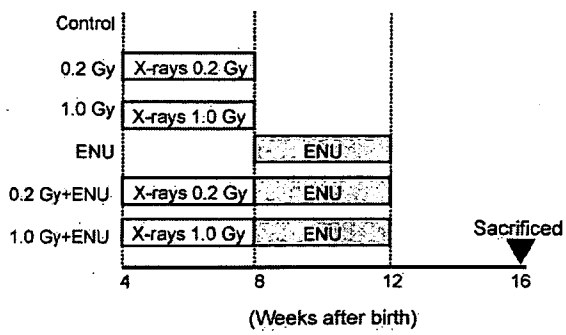

Fig. 1. Experimental design for *gpt* mutation analysis of thymic DNA from mice treated with X-ray irradiation, ENU or a combination of the two. Mice were exposed to X-rays weekly. ENU was administered at a concentration of 200 ppm in drinking water.

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data were corrected for any clonal expressions by counting just the mutation as defined by independent mutation [17,18]. *Mutation frequency* was calculated as the ratio of calculated total independent mutants to the total number of colonies growing on M9 + Cm plates. Mutational common hot spots were defined as sites where the same mutation was observed in three or more mice from the same group. Clonality was calculated as the ratio of clonal (total − independent) mutations to the total mutations [19,20].

2.7. PCR and DNA sequencing analysis of gpt

A 739-bp DNA fragment containing *gpt* was amplified by PCR using two primers (primer 1 (forward): 5′-TACCACTTTATCCCGCGTCAGG-3′, primer 2 (reverse): 5′-ACAGGGTTTCGCTCAGGTTTGC-3′). The reaction mixture contained 5 pmol of each primer and 200 μM of each dNTP. PCR amplification was carried out using Taq DNA polymerase (Takara Bio, Shiga, Japan) with a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The reaction was started by incubation at 96 °C for 180 s, followed by 29 cycles of 30 s at 94 °C, 30 s at 58 °C, 60 s at 72 °C and an additional 180 s incubation at 72 °C after the final cycle.

PCR products were purified using Exo-SAP It (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). DNA sequencing of *gpt* was performed using Big Dye Terminator v3.1 (Applied Biosystems) on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) with sequencing primer (5′-ATCTCTATAATCTCGCGCAACC-3′) according to the manufacturer's instructions. Oligonucleotide primers were obtained from Hokkaido System Science (Sapporo, Japan).

2.8. Statistical analysis

Mutant frequency is presented as mean ± S.D. Statistical significance was evaluated with the Student's *t*-test and Fisher's exact test using Graphpad Prism software (Graphpad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Thymus weight after treatment with ENU and X-rays

First, we measured body and thymus weight after combined exposure to X-rays and ENU (Table 1). The thymus weight of mice exposed to 0.2 or 1.0 Gy X-rays, ENU alone, or 0.2 Gy X-rays followed by ENU did not differ from that of non-irradiated controls. Thymus from mice exposed to 1.0 Gy X-rays followed by ENU weighed slightly greater than control thymus (68 ± 22 mg vs. 43 ± 9 mg; $P < 0.05$), suggestive of an onset of leukemogenesis. The body weight of all treated mice, except the 0.2 Gy followed by ENU mice, did not differ significantly from control.

3.2. Thymus gpt mutant frequency analysis

The *gpt* reporter transgene was used to analyze the occurrence of mutations in the thymus. The frequency of *gpt* mutants in each control and treated thymus is shown in Table 1 and Fig. 2. It is evident that ENU increased mutant frequency by 10-fold relative to untreated controls. Surprisingly, the mutant frequency in mice exposed to 0.2 or 1.0 Gy X-rays alone was significantly reduced compared to the control ($P < 0.05$). The mutant frequency in mice exposed to 0.2 Gy X-rays in combination with ENU was also, unexpectedly, reduced compared to ENU treatment alone, almost to the level of the untreated controls. In contrast, exposure

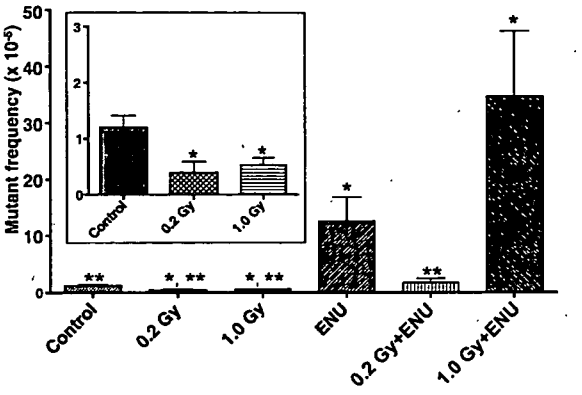

Fig. 2. Mutant frequency analysis of *gpt*⁻ recovered from thymus DNA from control, irradiated (0.2 or 1.0 Gy), ENU-treated, and irradiated/ENU-treated mice. The inset shows an expanded scale for mutant frequency for the first three conditions. $^{*}P < 0.05$, significantly different from control. $^{**}P < 0.05$, significantly different from ENU. Bars represent mean ± S.D.

to 1.0 Gy X-rays in combination with ENU increased the mutant frequency by 3-fold compared to ENU treatment alone. In mouse #30 of ENU group, mutant frequency was not different from that in control group (0.91×10^{-5} vs. 1.20×10^{-5}), but the weight of thymus was significantly increased compared with control (Table 1). These results suggested that the thymocyte with no *gpt* mutation expanded in this mouse.

3.3. Characteristics of the gpt mutants

The class- and site-distribution of mutations are shown in Tables 2 and 3, respectively.

In control group, G:C to A:T mutation was predominantly detected, especially at both non-CpG and CpG sites (0.24×10^{-5} and 0.30×10^{-5}, respectively). In 0.2 and 1.0 Gy groups, however, G:C to A:T mutation at CpG was reduced by 6-fold compared to control, respectively (0.2 Gy, 0.05×10^{-5}; 1.0 Gy, 0.05×10^{-5}). In ENU group, G:C to A:T mutation was generated at non-CpG sites (8.26×10^{-5}) especially at positions 86 and 409. These sites might be hot spot for mutation by ENU, because four out of five mice commonly had these mutations. In addition, mutants with G:C to T:A and A:T to T:A in ENU group were also increased at the rate of 1.47×10^{-5} and 2.36×10^{-5}, respectively.

In 0.2 Gy followed by ENU group, however, the mutant frequency of G:C to A:T and A:T to T:A was significantly reduced compared to the ENU-treated group ($P < 0.001$). Particularly, mutants with G:C to A:T transitions decreased by 37-fold (ENU, 8.26×10^{-5}; 0.2 Gy + ENU, 0.24×10^{-5}).

In three mice of 1.0 Gy followed by ENU group, more than 1000 mutants were detected per thymus (#26, 27 and 29); 201, 168 and 135 mutants were analyzed for each mouse, respectively (Table 3). The class- and site-distribution of mutations differed between mice and jackpot mutation was shown. In mouse #26, 143 of 201 mutations were G:C to A:T transitions at position 87. Almost all of the mutations (164 of 168) detected in mouse #27 were A:T to C:G transversions at position 106 (Table 3). Ninety-eight of 135 mutations in mouse #29 were A:T to G:C transition

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Table 1
Mutant and mutation frequency in thymus DNA from control, irradiated, ENU-treated, and combined treated mice

Treatment	Mouse ID	Body weight (g)	Average ± S.D. (mg)	Thymus weight (mg)	Average ± S.D. (mg)	Number of Cm colonies ($\times 10^5$)	Number of mutants	Mutant frequency ($\times 10^{-5}$)	Average ± S.D. ($\times 10^{-5}$)	Number of mutants sequenced	Number of independent mutants	Independent mutants (%)	Mutation frequency ($\times 10^{-5}$)	Average ± S.D. ($\times 10^{-5}$)
Control	1	25.7		45		18.3	32	1.75		28	22	79	1.38	
	2	25.8		39		12.7	16	1.26		14	13	93	1.17	
	3	24.6	26.5 ± 1.7	57	43 ± 9	16.2	21	1.30	1.2 ± 0.48	15	10	67	0.86	1.00 ± 0.43
	4	28.6		38		13.4	17	1.27		9	9	100	1.27	
	5	28.1		36		35.8	15	0.42		14	10	71	0.30	
	6	27.0		48		5.3	6	1.14		4	4	100	1.14	
	7	28.4		49		31.5	1	0.03		0	0	N.D.	N.D.	
0.2 Gy	8	24.6	26.3 ± 1.6	39	46 ± 6	28.3	4	0.14	0.39 ± 0.46[a]	1	1	100	0.14	0.59 ± 0.51[a]
	9	27.1		53		6.9	1	0.14		0	0	N.D.	N.D.	
	10	24.7		56		6.0	3	0.50		3	3	100	0.50	
	11	25.2		45		18.3	15	0.82		12	10	83	0.68	
	12	25.6		48		23.4	8	0.34		8	7	88	0.30	
1.0 Gy	13	24.6	25.0 ± 0.5	45	46 ± 1	47.8	12	0.25	0.53 ± 0.29[a]	9	9	100	0.25	0.49 ± 0.27
	14	24.5		45		15.0	13	0.87		10	10	100	0.87	
	15	25.5		47		8.5	3	0.35		2	2	100	0.35	
	16	24.5		35		17.7	110	6.21		110	15	14	0.85	
	17	26.7		43		2.3	66	28.21		58	12	21	5.84	
ENU	18	24.6	25.1 ± 1.0	36	41 ± 5	20.6	281	13.62	12.56 ± 9.67[a]	274	25	9	1.24	2.01 ± 2.15
	19	25.2		45		53.4	616	11.54		110	9	8	0.94	
	20	24.4		45		13.3	43	3.23		38	14	37	1.19	
	21	24.0		39		8.0	6	0.75		6	6	100	0.75	
	22	24.9		41		28.8	31	1.08		31	19	61	0.66	
0.2 Gy + ENU	23	24.6	24.4 ± 0.4[a]	35	40 ± 4	7.4	11	1.48	1.74 ± 1.76	10	9	90	1.34	1.12 ± 0.79
	24	24.8		47		5.0	24	4.82		22	11	50	2.41	
	25	24.0		38		31.3	18	0.57		17	13	76	0.44	
	26	24.4		64		18.5	1028	55.72		201	7	3	1.94	
	27	25.4		43		20.9	1001	47.94		168	5	3	1.43	
1.0 Gy + ENU	28	24.6	24.8 ± 0.9	100	68 ± 22[a]	5.7	75	13.09	34.74 ± 25.90[a]	59	8	14	1.77	1.61 ± 0.50
	29	25.9		54		27.5	1539	56.07		135	5	4	2.08	
	30	23.8		77		15.4	14	0.91		11	10	91	0.83	

N.D.: not determined.
[a] $n = 3$.
[a] $P < 0.05$, statistically significant difference vs. control.

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Table 2
Classification and mutant frequency (M.F.) and mutation frequency (m.f.) of *gpt* mutations recovered from thymus DNA from control, irradiated, ENU-treated, and combined treated mice

	Control			0.2 Gy			1.0 Gy			ENU			0.2 Gy+ENU			1.0 Gy+ENU		
	No.	M.F. ($\times 10^{-5}$)	No.	m.f. ($\times 10^{-5}$)	No.	M.F. ($\times 10^{-5}$)	No.	m.f. ($\times 10^{-5}$)[a]	No.	m.f. ($\times 10^{-5}$)	No.	M.F. ($\times 10^{-5}$)	No.	m.f. ($\times 10^{-5}$)	No.	M.F. ($\times 10^{-5}$)	No.	m.f. ($\times 10^{-5}$)	No.	M.F. ($\times 10^{-5}$)	No.	m.f. ($\times 10^{-5}$)
G:C to A:T (at non-CpG)	16	0.24	15	0.23	5	0.24	8	0.37	7	0.09	388	8.26	20	0.54	12	0.24	11	0.21	149	9.02	6	0.28
G:C to A:T (at CpG)[b]	20	0.30	11	0.17	1	0.05	4	0.07	3	0.04	1	0.02	1	0.03	7	0.14	5	0.10	5	0.30	4	0.18
G:C to T:A	16	0.24	15	0.23	0	<0.05	0	<0.07	12	0.16	12	0.15	69	1.47	15	0.30	10	0.19	20	1.21	4	0.18
G:C to C:G	3	0.05	3	0.05	0	<0.05	0	<0.07	1	0.01	1	0.01	2	0.04	2	0.05	0	<0.02	1	0.06	1	0.05
Total G:C[c]	55	0.83	44	0.69	6	0.29	6	0.44	25	0.32	23	0.30	460	9.79	38	1.02	34	0.69	26	0.50	175	10.59	15	0.69
A:T to G:C	11	0.17	7	0.11	0	<0.05	0	<0.07	7	0.09	6	0.08	4	0.09	4	0.11	17	0.34	8	0.15	103	6.23	6	0.28
A:T to T:A	0	<0.02	0	<0.02	1	0.05	1	0.07	1	0.01	1	0.01	111	2.36	24	0.64	31	0.63	20	0.39	129	7.81	10	0.46
A:T to C:G	1	0.02	1	0.02	1	0.05	1	0.07	1	0.01	1	0.01	13	0.28	7	0.19	2	0.04	2	0.04	164	9.93	1	0.05
Total A:T[c]	12	0.18	8	0.13	2	0.10	2	0.15	9	0.12	8	0.10	128	2.72	35	0.94	50	1.01	30	0.58	396	23.97	17	0.78
1 bp deletion	6	0.09	6	0.09	0	<0.05	0	<0.07	6	0.08	6	0.08	2	0.04	2	0.05	1	0.02	1	0.02	2	0.12	2	0.09
1 bp insertion	2	0.03	2	0.03	0	<0.05	0	<0.07	1	0.01	1	0.01	0	<0.01	0	<0.02	1	0.02	1	0.02	1	0.06	1	0.05
>2 bp frameshifts	5	0.08	4	0.06	0	<0.05	0	<0.07	0	<0.01	0	<0.01	0	<0.02	0	<0.02	0	<0.02	0	<0.02	0	<0.06	0	<0.05
Total frameshifts	13	0.20	12	0.19	0	<0.05	0	<0.07	7	0.09	7	0.09	2	0.04	2	0.05	2	0.04	2	0.04	3	0.18	3	0.14
Total	80	1.20	64	1.00	8	0.39	8	0.59	41	0.53	38	0.49	590	12.56	75	2.01	86	1.74	58	1.12	574	34.74	35	1.61

No.: number of mutation(s). M.F.: specific mutant frequency was calculated by multiplying the mutant frequency by the ratio of the number of mutations in each class among the total number of *gpt* mutants. m.f.: specific mutation frequency was calculated by multiplying the mutation frequency by the ratio of independent mutation in each class to the total number of independent *gpt* mutant.

[a] $n = 3$.

[b] Number of occurrences of the G:C to A:T mutation at the 5′-CpG-3′ site.

[c] Total number of mutations occurring at G:C or A:T base pairs.

at position 419. These results indicate that the increased frequency of mutation in this group was caused by clonal expansion of cells possessing a unique mutation. None of the mutation sites were identical to those observed as hot spots in mice treated with ENU alone.

3.4. Mutation frequency

Unlike mutant frequency, difference in overall mutation frequency after ENU was calculated to be small among groups. However, A:T to T:A mutation frequency in ENU group, either combined or not combined with 1.0 Gy X-rays, was increased compared with control or X-ray irradiation groups (Table 2), while mutation at G:C sites remained unchanged statistically. The total mutation frequency by ENU group reduced from 2.01×10^{-5} to 1.12×10^{-5} when combined with 0.2 Gy, although statistically not significant. In contrast, repeated 1.0 Gy did not alter the overall frequency of ENU-induced mutation (1.61×10^{-5}).

3.5. Clonality of gpt mutations

The mutant frequency was significantly larger than mutation frequency in the groups treated with ENU alone, and with 1.0 Gy combined with ENU (Table 1). This means that mutation occurrence in ENU and 1.0 Gy combined with ENU groups were mainly caused by clonal expansion. We established the approximate level of clonality for each group. Clonality was less than 20% in the control group, which was the same as that observed in *lacI* transgenic mice [19]. Clonalities in 0.2 Gy X-ray and 1.0 Gy X-ray groups were also similar to that in the control group. It increased dramatically to over 80% in the ENU-treated group (Fig. 3). When 0.2 Gy X-ray exposure was combined with ENU, clonality was drastically reduced compared with ENU alone (24.6% vs. 82.2%, $P < 0.001$). In contrast, 1.0 Gy X-rays, except mouse #30, facilitated clonal expansion of mutated cells, as shown by the high percentage of subpopulations with specific mutations and the shift from an oligoclonal to monoclonal population (Table 3).

4. Discussion

In this study we investigated the combined effect of ENU and X-rays on the occurrence of mutations in the reporter transgene *gpt* in mouse thymus DNA. Repeated irradiation with 0.2 Gy X-rays not only reduced background mutation levels, but also suppressed ENU-induced mutations and clonal expansion. In contrast, 1.0 Gy irradiation in combination with ENU accelerated clonal expansion of mutated cells.

Reduction of the background mutation frequency by repeated dosing with 0.2 Gy X-rays or 1.0 Gy X-rays was unexpected. A decrease in G:C site mutations, including G:C to A:T at the CpG site, primarily accounted for this effect. The *gpt* in *gpt*-delta transgenic mice may be methylated at the CpG site [21]. Mammalian cell, in general, is heavily methylated at C-5 of cytosine residues at CpG dinucleotides. When 5-methylcytosine was deaminated, it converts to thymine, which results in G:C

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Table 3
Mutational spectrum of *gpt* gene mutations recovered from thymus DNA from non-, X-ray-, ENU- and combined treated mice

Base positions[a]	CpG site	Control		0.2 Gy		1.0 Gy		ENU		0.2 Gy + ENU		1.0 Gy + ENU	
		No. of mutant	No. of independent mutation (mouse ID)	No. of mutant	No. of independent mutation (mouse ID)[b]	No. of mutant	No. of independent mutation (mouse ID)	No. of mutant	No. of independent mutation (mouse ID)	No. of mutant	No. of independent mutation (mouse ID)	No. of mutant	No. of independent mutation (mouse ID)
G:C to A:T													
3		2	2 (1, 4)					194	4 (16,17,18,19)				
7	CpG					1	1 (14)	1	1 (18)			143	1 (26)
37		1	1 (5)							1	1 (22)	1	1 (26)
58	CpG												
64	CpG	12	4 (1,2,3, 5)	1	1 (6)	2	1 (11)			3	2 (21,25)	1	1 (28)
86													
87													
92				2	2 (6,8)								
107		2	2 (1, 2)							1	1 (22)	1	1 (26)
109	CpG	2	1 (5)										
110	CpG	1	1 (3)										
113				1	1 (10)			2	2 (19,20)	3	2 (21,22)	2	1 (26)
115	CpG	3	3 (2,3,4)			1	1 (12)	1	1 (17)	1	1 (22)	1	1 (27)
116								19	2 (16,18)				
128		1	1 (1)					83	2 (19,20)				
176						1	1 (11)	2	2 (17,19)				
189								1	1 (19)				
262						1	1 (12)						
274		2	2 (1,2)	1	1 (10)			1	1 (19)	1	1 (21)	2	1 (30)
280	CpG	1	1 (2)										
281		1	1 (1)			1	1 (15)					1	1 (30)
287								1	1 (16)				
301	CpG											1	1 (28)
391										1	1 (22)		
401				1	1 (6)	1	1 (13)			1	1 (22)		
402										2	2 (21,23)	1	1 (26)
406		3	2 (1,2)							1	1 (22)		
409								84	4 (16,17,18,19)			1	1 (26)
413	CpG	1	1 (2)										
417		1	1 (2)			1	1 (13)						
418		3	3 (1,2,5)			3	2 (11,12)						
										5	4 (22,23,24,25)		
Total at non-CpG		16	15	5	5	8	7	388[c]	20	12	11	149[c]	6
Total at CpG		20	11	1	1	4	3	1[c]	1	7	5	5[c]	4
G:C to T:A													
3												1	1 (30)
7	CpG					1	1 (11)	32	2 (16,18)				
15								1	1 (18)				
19	CpG												
26						1	1 (12)			1	1 (24)		
37						1	1 (13)						

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79										1	1(27)
109	CpG	1	1 (4)								
110	CpG	1	1 (4)								
113						1	1 (16)				
115	CpG	1	1 (1)	1	1 (13)			1	1 (24)		
116		1	1 (3)	1	1 (15)	1	1 (16)				
143	CpG	1	1 (3)					1	1 (22)		
176				1	1 (14)						
185		1	1 (2)								
189								1	1 (23)		
190	CpG							1	1 (22)		
208	CpG					1	1 (18)	9	4 (22,23,24,25)	1	1(28)
232		1	1 (1)								
244	CpG	1	1 (5)	1	1 (13)	8	2 (18,19)				
265						3	2 (16,18)				
304		2	2 (1,2)								
401		1	1 (5)			19	3 (16,17,18)				
402		3	2 (1,5)	3	3 (11,13,14)						
406		1	1 (1)	1	1 (14)	1	1 (17)				
409		1	1 (1)			2	1 (17)	1	1 (24)	17	1(29)
418				1	1 (13)						
Total		16	15	12	12	69*	15	15	10	20*	4
G:C to C:G											
7	CpG					1	1 (17)			1	1(30)
115	CpG	1	1 (1)								
125	CpG					1	1 (18)				
289		1	1 (1)								
402				1	1 (15)						
436	CpG	1	1 (4)								
Total		3	3	1	1	2	2			1	1
A:T to G:C											
2		2	2 (4,5)							1	1(30)
8								1	1 (25)	1	1(29)
25		4	3 (1,2,4)								
56				1	1 (14)						
119								9	2 (22,25)		
149								1	1 (24)		
175				1	1 (11)						
181						1	1 (17)				
187						1	1 (17)			1	1(30)
188								1	1 (22)		
191								1	1 (21)	1	1(30)
269		2	1 (5)	1	1 (14)			1	1 (25)		
275										1	1(27)
277								3	1 (22)		
343				1	1 (14)						
419		3	1 (1)	3	2 (11,14)	1	1 (17)			98	1(29)
457						1	1 (18)				
Total		11	7	7	6	4	4	17	8	103*	6
A:T to T:A											
2						2	2 (18,19)	1	1 (25)		
12				1	1 (13)						
35								1	1 (25)	18	1(29)
52						5	2 (16,18)			1	1(27)
56						1	1 (18)				
81								1	1 (22)		
88						14	1 (19)	8	2 (23,24)	43	2(26, 28)
95								1	1 (25)		
134						16	4 (16,17,18,19)				

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Table 3 (*Continued*)

Base positions[a]	CpG site	Control		0.2 Gy		1.0 Gy		ENU		0.2 Gy + ENU		1.0 Gy + ENU	
		No. of mutant	No. of independent mutation (mouse ID)	No. of mutant	No. of independent mutation (mouse ID)[b]	No. of mutant	No. of independent mutation (mouse ID)	No. of mutant	No. of independent mutation (mouse ID)	No. of mutant	No. of independent mutation (mouse ID)	No. of mutant	No. of independent mutation (mouse ID)
164				1	1(6)			3	3 (16,18,19)	2	2 (24,25)		
173								1	1 (18)				
177										1	1 (23)		
214										1	1 (24)		
223												1	1(30)
254												1	1(28)
257								2	1 (16)			1	1(28)
260										1	1 (25)		
263								8	2 (18,19)	7	3 (22,23,24)	63	2(26, 28)
312								1	1 (19)	2	1 (22)		
329								1	1 (18)			1	1(30)
345								56	4 (16,17,18,19)				
375										1	1 (22)		
400										2	2 (23,24)		
407										1	1 (23)		
420								1	1 (19)	1	1 (22)		
Total				1	1	1	1	111*	24*	31*	20*	129*	10*
A:T to C:G													
1								8	4 (16,17,18,19)				
9								1	1 (19)				
65				1	1(10)								
106								2	1 (19)			164	1(27)
218		1 (1)	1										
312										1	1 (25)		
331										1	1 (25)		
345						1	1 (14)	2	1 (18)				
Total		1	1	1	1	1	1	13	7	2	2	164*	1

No. of mutants: total number of mutations detected, include clonal mutations. Total number was shown the total number of mutations. No. of independent mutations: total number was shown the total number of independent mutations. The data were corrected for any clonal expansions by counting only one mutation when multiple identical mutations were recovered form an individual mouse. ID of mice with a mutation was shown in parentheses on the right-hand side of the total number of independent mutations.

[a] Position in the *gpt* coding sequence when A of start codon is labeled as 1.

[b] $n = 3$.

* $P < 0.05$ vs. control.

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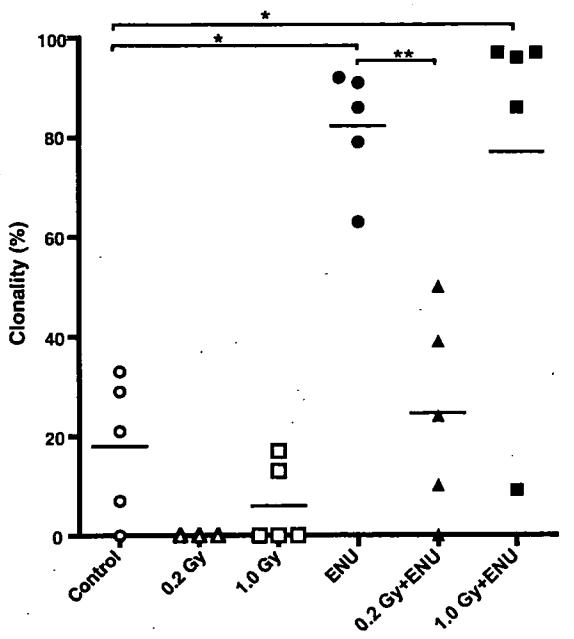

Fig. 3. Mutation clonality was enhanced by ENU as well as by 1.0 Gy X-rays in combination with ENU, but was suppressed by repeated irradiation with 0.2 Gy X-rays before ENU treatment. $^{*}P < 0.05$, significantly different from control. $^{**}P < 0.05$, significantly different from ENU. Bars represent means.

to A:T mutation after DNA replication if G:T mismatch is not repaired [22]. Repeated irradiation with 0.5 Gy X-rays leads to a decrease in DNA methylation via loss of histone H4-Lys20 trimethylation in thymus tissue [23], which could account for the reduced G:C to A:T mutation occurrence at CpG sites. Low-dose radiation (within 0.2 Gy) reduces thymic DNA fragmentation and apoptosis relative to non-irradiated background levels [24], suggesting that activation of DNA repair mechanisms may also contribute to the reduced frequency of mutations.

We show here that repeated 0.2 Gy irradiation also dramatically reduces ENU-induced mutations and the number of hot spot sites, primarily by decreasing the number of G:C to A:T transitions and G:C to T:A and A:T to T:A transversions. ENU produces ethylated base damage such as O^6-ethylguanine, O^4-ethylthymine and O^2-ethylthymine, which induces G:C to A:T transition, A:T to G:C transition and A:T to T:A transversion, respectively [4–8]. Base damage can be removed by multiple DNA repair systems. O^6-Methylguanine-DNA methyltransferase (*Mgmt*) recognizes and directly binds O^6-ethylguanine and removes the ethyl residue, thereby preventing G:C to A:T transition [25–28]. It is shown that transient expression of *Mgmt* is stimulated by X-irradiation both *in vitro* and *in vivo* within 3–48 h [29–31]. Importantly, the inductive effect of X-irradiation on O^6-alkylguanine-DNA alkyltransferase activity corresponds with a reduced incidence of rat CNS tumors after X-irradiation combination with ENU [10]. We have also examined if the expression of *Mgmt* mRNA increased after repeated X-irradiation of 0.2 Gy followed by ENU treatment. It turned out that the induction of *Mgmt* was negligible in irradiated thymus: fold increase was just 1.1 after the last irradiation. Subsequent ENU treatment increased *Mgmt* expression as a function of time. The 2-fold induction of *Mgmt* in thymus with 0.2 Gy followed by ENU, however, was smaller than 3.9-fold induction in thymus with ENU alone. Therefore, decrease in mutation frequency by 0.2 Gy could not be explained by expression level of *Mgmt*. Mechanism(s) other than *Mgmt* induction might be involved. Nucleotide excision repair can also repair alkylating base damage [32–34]. Chinese hamster ovary cells lacking *XPD/ERCC2* are highly susceptible to ENU-induced *Hprt* mutations [32]. DNA microarray studies indicate that *XPC* is up-regulated in human blood lymphocytes after 0.2 Gy X-ray exposure [35]. Taken together, it is required to determine activation or inactivation of other DNA repair pathways that reduce or enhance the overall rate of ENU-induced mutations.

We noticed that ENU mutations were predominantly induced at A:T site. It is reported that O^6-ethylguanine was repaired fast by *Mgmt* and nucleotide excision repair, while O^4-ethylthymine and O^2-ethylthymine were persistent lesions and the repair of these lesions occurred only at a very slow rate [25,32,36]. *In vivo* mutagenicity assays in mouse T lymphocytes have shown that predominant mutations induced by ENU are A:T to T:A [37].

Cells with hot spot point mutations expanded oligoclonally after ENU treatment. Clonal thymic lymphomas selected from oligoclonal preleukemic cells have been reported in virus-induced or radiation-induced T-cell lymphomagenesis [38,39]. Interestingly, the treatment with 0.2 Gy X-rays followed by ENU reduced oligoclonality, whereas 1.0 Gy X-rays accelerated ENU-induced clonal expansion. It is reported that high-dose whole body irradiation at 1.0 Gy induced p53 dependent transcription of *Noxa*, *Killer/DR5*, *Fas*, *Pidd* and *Perp* genes in thymus, whereas irradiation at 0.2 Gy did not induce these gene expressions [40]. Low-dose irradiation likely again activates multiple repair pathways and cell cycle control. In contrast, high-dose radiation may select specific clones with a growth advantage.

Point mutations of K-*ras*, *p53* and *Ikaros* occur frequently in ENU-induced T-cell lymphomas [41,42]. These mutations may enhance responsiveness of cells to growth factor induction of proliferation or resistance to apoptosis, thereby improving survival and increasing clonal expansion. When myeloma cells are cultured on normal bone marrow stromal cells, or in the presence of IL-6, activation of the K-*ras* oncogene provides a growth advantage over cells lacking activated K-*ras* [43]. The progression from low grade to high-grade brain tumors is associated with clonal expansion of cells that have acquired a *p53* mutation that endows the cells with a selective growth advantage [44]. T-cells with reduced or dominant-negative Ikaros activity, which may result from either a lack of or a point mutation in the zinc finger responsible for DNA binding, exhibit a greater proliferative response to IL-2 [45,46]. Irradiation of thymic epithelial cells enhances IL-7 production, and thymocytes at preleukemic stage proliferate more vigorously in response to IL-7 [47,48]. Taken together, these results suggest that high-dose radiation provides a thymic microenvironment ripe for the occurrence of prelymphoma cells, which harbor growth-advantageous mutations following ENU treatment.

In conclusion, low-dose X-rays (0.2 Gy) reduce not only the frequency of spontaneously occurring but also ENU-induced

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mutations, suggestive of an adaptive response. Low-dose X-rays also reduce the clonal expansion of cells following ENU treatment, whereas 1.0 Gy X-rays accelerate cell expansion. Thus, low- and high-dose radiations play two different roles in lymphomagenesis when combined with ENU exposure.

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Involvement of Y-Family DNA Polymerases in Mutagenesis Caused by Oxidized Nucleotides in *Escherichia coli*

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***Escherichia coli* DNA polymerase IV incorporated 2-hydroxy-dATP opposite template guanine or thymine and 8-hydroxy-dGTP exclusively opposite adenine in vitro. Mutator phenotypes in *sodIfur* strains were substantially diminished by deletion of *dinB* and/or *umuDC*. DNA polymerases IV and V may be involved in mutagenesis caused by incorporation of the oxidized deoxynucleoside triphosphates.**

Excess oxidation is a major threat to the genomic integrity of most living organisms. Reactive oxygen species oxidize deoxynucleoside triphosphates (dNTPs), as well as DNA, and some of the oxidized dNTPs have been shown to be mutagenic when they are incorporated in DNA. 8-Oxo-7,8-dihydro-2′-deoxyguaniosine 5′-triphosphate (8-OH-dGTP) leads to A · T-to-C · G transversions when it is incorporated opposite adenine (A) in the template (5, 14). To counteract the mutagenic 8-OH-dGTP, *Escherichia coli* has a sanitizing enzyme, MutT, that hydrolyzes 8-OH-dGTP (20). When the *mutT* gene is inactivated, the frequency of mutation of A · T to C · G increases more than a thousandfold compared with the wild-type frequency (35). In the case of 2-oxo-1,2-dihydro-2′-deoxyadenosine 5′-triphosphate (2-OH-dATP), G · C-to-T · A transversions occur when it is incorporated opposite guanine (G) in the template (14, 16). Another sanitizing enzyme, Orf135, degrades 2-OH-dATP in *E. coli*, and G · C-to-T · A mutations occur in an *orf135*-deficient strain more frequently than in the wild-type strain (15, 17).

The members of the Y family of DNA polymerases (DNA Pols) are involved in error-free and error-prone translesion synthesis (TLS) of damaged template DNA in various species, including humans (13, 26). Recently, involvement of Y-family DNA polymerases in the incorporation of damaged dNTPs was suggested by in vitro experiments performed with purified DNA Pols (28). The archaeal Y-family DNA Pols from *Sulfolobus* sp. and the human DNA Pols exclusively incorporate 8-OH-dGTP opposite A in the template DNA and incorporate 2-OH-dATP opposite G and thymine (T). Thus, it would be interesting to examine the in vivo roles of Y-family DNA Pols in the incorporation of mutagenic dNTPs into DNA. *Escherichia coli* strain QC1736 seems to be an appropriate background to investigate the roles of Y-family DNA Pols (DNA Pol IV and Pol V encoded by *dinB* and *umuDC*, respectively) in the mutagenesis caused by oxidized nucleotides. Iron metabolism is deregulated in this strain due to the lack of the Fur protein, a negative regulator of iron uptake (29). This strain also lacks both superoxide dismutases (SodA and SodB), which cata-

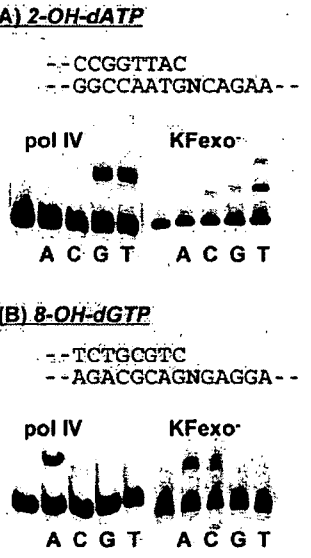

FIG. 1. Incorporation of oxidized nucleotides by DNA polymerases. The incorporation of 2-OH-dATP (A) and 8-OH-dGTP (B) into DNA by DNA Pol IV and KF exo⁻ of *E. coli* was assayed as described previously (28). Cy3-conjugated primer, annealed to the template at a 1:1 ratio (0.1 μM), was incubated with DNA Pol IV (0.1 μM) or KF exo⁻ (0.02 U), and then 50 μM 2-OH-dATP (A) or 50 μM 8-OH-dGTP (B) was added. No other dNTPs were added to the reaction mixtures. All the reactions were carried out at room temperature for 30 min. The reaction products were analyzed on 15% denatured polyacrylamide gels, and the bands were visualized using a Molecular Imager FX Pro system (Bio-Rad, Richmond, CA). The oligonucleotide sequences of the primer and template were 5′-Cy3-CGCGCGAAGAC CGGTTAC-3′ and 5′-GAAGGGATCCTTAAGACNGTAACCGGTCTT CGCGCG-3′, respectively, for 2-OH-dATP and 5′-Cy3-CGGAGCTCGGT CGGCGTCTGCGTC and 5′-AGCCGCAGGAGNGACGCAGACGCC GACCGAGCTCCG-3′, respectively, for 8-OH-dGTP (N = A, C, G, or T). Parts of the sequences of the primer and template are shown. The unlabeled lanes on the left indicate the positions of Cy3-labeled primers without extension.

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TABLE 1. Strains used in this study[a]

Strain	Characteristics	P1 transduction or conjugation	Reference or source
CC101	Derivative of strain P90C [*araA*(*lac proB*)$_{XIII}$] carrying F′ *lacIZ- proB*$^+$; *lacZ* has a mutation (GAG to TAG) at codon 461		7
CC104	Derivative of strain P90C [*araA*(*lac proB*)$_{XIII}$] carrying F′ *lacIZ- proB*$^+$; *lacZ* has a mutation (GAG to GCG) at codon 461		7
AR30	Δ*dinB61*::*ble sulA211*		4
DE2302	*thr-1 ara-14 leuB6* Δ(*gpt proA*)*62 lacY1 tsx-33 supE44 galK2 hisG4 rpsL31 xyl-5 mtl-1 arg3 thi-1 uvrA6* Δ(*umuDC*)*595*::*cat fadR615*::Tn*10 purB58*		34
EC8	*thr-1 ara-14 leuB6* Δ(*gpt-proA*)*62 lacY1 tsx-33 supE44 galK2 hisG4 rpsL31 xyl-5 mtl-1 argE3 thi-1 uvrA6* Δ(*umuDC*)*596*::*ermGT fadR*$^+$ *purB*$^+$		11
KY1056sFtet101	AB1157 derivative; harboring F′ derived from CC101, which has Tn*10* in it for selection of F′		K. Yamamoto
KY1056sFtet104	AB1157 derivative; harboring F′ derived from CC104, which has Tn*10* in it for selection of F′		K. Yamamoto
YG6125A	AB1157 derivative; harboring F′ derived from CC101, which has Tn*10* in it for selection of F′ and Δ*dinB*::*kan*		This study
YG6125B	AB1157 derivative; harboring F′ derived from CC104, which has Tn*10* in it for the selection of F′ and Δ*dinB*::*kan*		This study
QC1736	Δ(*argF-lac*)*U169 rpsL* Δ*sodA3 sodB*::MudPR *fur*::*kan*; Cm^r Km^r		29
YG6177	Like QC1736 but Δ*dinB61*::*ble*; Cm^r Km^r Zc^r	AR30 (P1) → QC1736	This study
YG6180	Like QC1736 but Δ*umuDC*(*596*)::*ermGT*; Cm^r Km^r	DE2302/EC8 (P1) → QC1736	This study
YG6124	Like QC1736 Δ*dinB61*::*ble*; Δ*umuDC*(*596*)::*ermGT*; Cm^r Km^r Zc^r	DE2302/EC8 (P1) → YG6177	This study
YG6175[b]	Like QC1736 but harboring F′ from CC101; Cm^r Km^r Tc^r	KY1056sFtet101 → QC1736	This study
YG6176[b]	Like QC1736 but harboring F′ from CC104; Cm^r Km^r Tc^r	KY1056sFtet104 → QC1736	This study
YG6178[b]	Like QC1736 but Δ*dinB61*::*ble* and harboring F′ from CC101; Cm^r Km^r Tc^r Zc^r	YG6125A → YG6177	This study
YG6179[b]	Like QC1736 but Δ*dinB61*::*ble* and harboring F′ from CC104; Cm^r Km^r Tc^r Zc^r	YG6125B → YG6177	This study
YG6181[b]	Like QC1736 but Δ*umuDC*(*596*)::*ermGT* and harboring F′ derived from CC101; Cm^r Km^r Tc^r	KY1056sFtet101 → YG6180	This study
YG6182[b]	Like QC1736 but Δ*umuDC*(*596*)::*ermGT* and harboring F′ from CC104; Cm^r Km^r Tc^r	KY1056sFtet104 → YG6180	This study
YG6126[b]	Like QC1736 but Δ*dinB61*::*ble* and Δ*umuDC*(*596*)::*ermGT* and harboring F′ from CC101; Cm^r Km^r Tc^r Zc^r	YG6125A → YG6124	This study
YG6127[b]	Like QC1736 but Δ*dinB61*::*ble* and Δ*umuDC*(*596*)::*ermGT* and harboring F′ from CC104; Cm^r Km^r Tc^r Zc^r	YG6125B → YG6124	This study

[a] The deletion strains for *dinB* encoding DNA Pol IV were constructed by P1 transduction as indicated. The *umuDC* deletion encoding DNA Pol V was introduced into QC1736 and YG6177 by two-step P1 transduction (11). (P1) indicates that P1vir phage lysate was prepared in the strain. F′ with a mutation for specific detection of changes from G · C to T · A or from A · T to C · G was separately introduced by conjugation as indicated. The arrows indicate the directions of transfer for P1 transduction and conjugation. Chloramphenicol, kanamycin, tetracycline, and zeocin were used at concentrations of 10 μg/ml, 25 μg/ml, 10 μg/ml, and 50 μg/ml, respectively. Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Zc^r, zeocin resistance.

[b] Strain used for the LacZ reversion assay.

lyze the breakdown of the superoxide anion. Thus, both iron overload and superoxide stress occur in strain QC1736, which leads to high rates of spontaneous mutation from A · T to C · G and from G · C to T · A (24). The hot spots and sequence contexts of A · T-to-C · G mutations are almost identical to those in a *mutT* strain (25). In contrast, the hotspots of G · C-to-T · A mutations are very different from those in *mutM mutY* strains, in which 8-OH-G in DNA acts as a major mutagenic lesion. Expression of the cDNA of the human counterpart of *E. coli* MutT, MTH1, which hydrolyzes both 8-OH-dGTP and 2-OH-dATP (12), suppresses the mutator phenotype of the strain. Thus, it has been concluded that the targets contributing to the oxidative mutagenesis in the *sodIfur* mutant are oxidized dNTPs, such as 8-OH-dGTP and 2-OH-dATP, rather than DNA (25).

We first examined the specificity with which the purified native form of DNA Pol IV (31) incorporates 2-OH-dATP and 8-OH-dGTP in vitro. DNA Pol IV predominantly incorporated 2-OH-dATP opposite template G and T, and the frequency of incorporation opposite G was almost equal the frequency of incorporation opposite T (Fig. 1 A). In contrast, Klenow fragment exo$^-$ (KF exo$^-$) (New England Biolabs, Massachusetts) predominantly incorporated 2-OH-dATP opposite T. DNA Pol IV almost exclusively incorporated 8-OH-dGTP opposite A, and KF exo$^-$ incorporated 8-OH-dGTP opposite A and cytosine (C) (Fig. 1 B). These in vitro results suggest possible involvement of Y-family DNA polymerases in oxidative mutagenesis through misincorporation of the oxidized dNTPs during DNA synthesis in *E. coli*.

To examine the in vivo roles of Y-family DNA Pols, mutation frequencies were compared for *sodIfur* strains with and without Pol IV and Pol V (Table 1). Both A · T-to-C · G and G · C-to-T · A transversion frequencies were reduced by 80 to 90% by deletion of either *dinB* or *umuDC* or both in the *sodIfur* strains (Table 2). Interestingly, the double mutants (Δ*dinB* Δ*umuDC*) exhibited levels of mutation frequency similar to those of single mutants (Δ*dinB* or Δ*umuDC*). These results suggest that the base substitutions by erroneous incorporation

TABLE 2. Mutation frequencies for the *sodAB fur* strains with and without DNA Pol IV and DNA Pol V[a]

Expt	Mutation frequency (10^{-6})							
	Pol IV$^+$/Pol V$^-$ (YG6176)	Pol IV$^-$/Pol V$^+$ (YG6179)	Pol IV$^+$/Pol V$^-$ (YG6182)	Pol IV$^-$/Pol V$^-$ (YG6127)	Pol IV$^+$/Pol V$^+$ (YG6175)	Pol IV$^-$/Pol V$^+$ (YG6178)	Pol IV$^+$/Pol V$^-$ (YG6181)	Pol IV$^-$/Pol V$^-$ (YG6126)
G · C → T · A								
Expt I	28.2 ± 3.5	3.66 ± 0.32	2.72 ± 0.20	3.57 ± 0.42				
Expt II	35.6 ± 2.5	2.61 ± 0.12	2.82 ± 0.15	3.23 ± 0.30				
A · T → C · G								
Expt I					12.8 ± 0.61	2.49 ± 0.18	3.24 ± 0.25	2.43 ± 0.15
Expt II					10.0 ± 0.60	2.08 ± 0.16	3.7 ± 0.21	1.86 ± 0.19
Expt III					9.1 ± 0.80	2.38 ± 0.47		

[a] The mutagenicity assay was carried out as described previously (32). Briefly, a single colony was inoculated into 2 ml of M9-glucose minimal medium, and then the overnight culture was diluted 1,000-fold. Eight to twelve diluted cultures were prepared, and they were cultivated overnight. One milliliter of each culture was harvested and washed twice with phosphate buffer (pH 7.4), and then the cell pellet was suspended in phosphate buffer. All of the suspension was spread on one plate for mutation, and a portion of the diluted suspension was used for determining survival. Twenty amino acids were added in the assays (both liquid medium and plates) for growth of *sodAB fur* strains because the production of amino acids is inhibited by oxygen radicals (3). The values are means ± standard errors.

of 2-OH-dATP or 8-OH-dGTP require both DNA Pol IV and DNA Pol V functions.

The dNTP pool and DNA are continuously exposed to a variety of exogenous and endogenous damaging agents, including reactive oxygen species, and the incorporation of oxidized dNTPs into DNA is a major source of spontaneous mutagenesis and carcinogenesis (1). Here we obtained biochemical and genetic evidence that DNA Pol IV and Pol V may be involved in oxidative mutagenesis through misincorporation of altered nucleotides (i.e., 2-OH-dATP and 8-OH-dGTP) during DNA synthesis. This is consistent with the report by Satou et al. (27) that DNA Pol IV promotes mutation of G · C to T · A in *E. coli* when 2-OH-dATP is directly introduced into cells by $CaCl_2$ treatment. It has also been suggested that SOS-inducible polymerases, including Pol IV and Pol V, are involved in mutagenesis caused by increases in the normal levels of dNTPs (33). It has been reported that more than one DNA polymerase is involved in mutagenesis when the Y-family DNA polymerases are involved in TLS. For benzo[a]pyrene-induced mutagenesis, both Pol IV and Pol V are required for a −1 frameshift TLS (23). DNA lesions induced by other chemicals, including 3-methylcholanthrene or dimethylbenzo[a]anthracene, also require both DNA Pol IV and Pol V for a −2 frameshift in a CG repetitive sequence in *Salmonella enterica* serovar Typhimurium (18, 21). Thus, we speculate that DNA Pol IV and Pol V are involved in sequential biochemical steps, such as incorporation and extension of oxidized dNTPs during chromosome replication. One of these polymerases might incorporate oxidized dNTPs into DNA in an erroneous manner, and the other might extend the mutagenic primer termini containing the oxidized deoxynucleoside monophosphate, thereby inducing base substitutions. It is obvious, however, that more experiments are needed to elucidate the precise mechanisms.

DNA Pol IV is controlled by σ^S, and the level of expression of Pol IV in the stationary phase decreases significantly when the *rpoS* gene encoding σ^S is defective (10, 19). Thus, Pol IV appears to be regulated not only by the SOS response but also by the σ^S-dependent stress response. In stationary-phase cells, the amount of cellular mismatch repair proteins decreases at least 10-fold (8). Hence, the error-prone nature of Pol IV is expected to be more significant. In fact, DNA Pol IV is responsible for some of the adaptive mutations in stationary-phase cells (9, 22), Interestingly, adaptive mutagenesis is approximately fourfold more frequent in a *sodA sodB* strain than in the parental strain, and this mutagenesis is suppressed under anaerobic conditions (2). Therefore, DNA Pol IV might be involved in stationary-phase mutagenesis by either incorporation of oxidized dNTPs or extension of primers having oxidized deoxynucleoside monophosphates or both, although it is possible that DNA Pol IV induces mutations by error-prone bypass across oxidized bases in template DNA.

The oxidized nucleotide pools also cause a problem in mammalian cells. Spontaneous tumorigenesis in lungs, livers, and stomachs is enhanced in mice that are deficient in *Mth1* (30). In addition, a recent study suggested that the majority of mutations in human cells that are deficient in mismatch repair do not arise from spontaneous replication errors but from the incorporation of oxidized dNTPs (6). Thus, it might be interesting to examine the roles of mammalian Y-family DNA Pols in genome instability caused by oxidation of the nucleotide pool.

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