Table 1
Mutant frequencies induced by BaP and 10-azaBaP in eight organs of Muta™Mouse

Tissue	Treatment	*lacZ* assay				*cII* assay			
		Individual animal data			Average ± S.D.	Individual animal data			Average ± S.D.
		No. of phages analyzed	No. of mutants	MF × 10^6	MF × 10^6	No. of phages analyzed	No. of mutants	MF × 10^6	MF × 10^6
Liver	Control (olive oil)	422000	21	49.8		378000	15	39.7	
		602000	39	64.8		1394000	37	26.5	
		194500	10	51.4		938000	48	51.2	
		136000	8	58.8		1168000	32	27.4	
		333000	18	54.1	55.8 ± 5.5	987900	31	31.4	35.2 ± 9.2
	BaP	1495500	403	269.5		349200	17	48.7	
		211000	63	298.6		225900	36	159.4	
		1054500	561	532.0		103800	8	77.1	
		1457000	602	413.2		323400	31	95.9	
		483000	267	552.8	413.2 ± 116.1**	286500	26	90.8	94.3 ± 36.4*
	10-AzaBaP	1116500	80	71.7		1515900	153	100.9	
		148000	27	182.4		368700	21	57.0	
		369000	28	75.9		555300	34	61.2	
		155500	31	199.4		643800	33	51.3	
		236500	37	156.4	137.2 ± 53.5*	489300	22	45.0	63.1 ± 19.7*
Spleen	Control (olive oil)	634500	30	47.3		745200	29	38.9	
		781000	40	51.2		846900	17	20.1	
		221000	19	86.0		366000	10	27.3	
		771500	43	55.7		1016700	37	36.4	
		680500	48	70.5	62.1 ± 14.3	442200	15	33.9	31.3 ± 6.8
	BaP	276500	170	614.8		672300	88	130.9	
		194000	101	520.6		579600	77	132.9	
		882000	731	828.8		204900	37	180.6	
		1024000	354	345.7		849000	128	150.8	
		136050	122	896.7	641.3 ± 201.5**	61550	13	211.2	161.3 ± 30.7**
	10-AzaBaP	226000	11	48.7		1532100	30	19.6	
		452500	25	55.2		609600	11	18.0	
		322000	24	74.5		366600	19	51.8	
		240000	17	70.8		346800	11	31.7	
		729500	93	127.5	75.4 ± 27.8	1024800	25	24.4	29.1 ± 12.3
Lung	Control (olive oil)	473000	21	44.4		743700	41	55.1	
		211500	21	99.3		424200	17	40.1	
		460500	28	60.8		816900	47	57.5	
		191000	11	57.6		400200	18	45.0	
		293000	55	187.7	90.0 ± 52.2	278100	10	36.0	46.7 ± 8.4
	BaP	181000	43	237.6		415200	39	93.9	
		127500	71	556.9		314700	46	146.2	
		138000	63	456.5		372000	25	67.2	
		76500	27	352.9		210900	92	436.2	
		41500	16	385.5	397.9 ± 106.4**	150600	10	66.4	162.0 ± 140.1
	10-AzaBaP	172500	31	179.7		515700	24	46.5	
		214500	16	74.6		471000	13	27.6	
		516500	25	48.4		909000	100	110.0	
		196000	13	66.3		610200	21	34.4	
		102000	8	78.4	89.5 ± 46.3	327900	28	85.4	60.8 ± 31.7

Table 1 (*Continued*)

Tissue	Treatment	*lacZ* assay				*cII* assay			
		Individual animal data			Average ± S.D.	Individual animal data			Average ± S.D.
		No. of phages analyzed	No. of mutants	MF × 10^6	MF × 10^6	No. of phages analyzed	No. of mutants	MF × 10^6	MF × 10^6
Kidney	Control (olive oil)	269000	32	119.0		802800	34	42.4	
		299000	19	63.5		687600	37	53.8	
		695500	49	70.5		1512600	54	35.7	
		843500	63	74.7		1579200	116	73.5	
		818500	70	85.5	82.6 ± 19.5	1654200	122	73.8	55.8 ± 15.6
	BaP	243500	53	217.7		821400	86	104.7	
		355500	73	205.3		1071000	84	78.4	
		685500	129	188.2		1394400	56	40.2	
		393000	162	412.2		1596000	161	100.9	
		516000	116	224.8	249.6 ± 82.2**	1236000	167	135.1	91.9 ± 31.5
	10-AzaBaP	859500	58	67.5		442200	31	70.1	
		192000	18	93.8		476700	19	39.9	
		500500	66	131.9		1330200	90	67.7	
		427500	47	109.9		1339800	98	73.1	
		450000	29	64.4	93.5 ± 25.5	1216800	55	45.2	59.2 ± 13.8
Bone marrow	Control (olive oil)	934500	180	192.6		672300	30	44.6	
		248500	15	60.4		395400	11	27.8	
		197000	11	55.8		417300	6	14.4	
		57000	3	52.6		178500	3	16.8	
		391500	24	61.3	84.6 ± 54.1	321300	19	59.1	32.6 ± 17.0
	BaP	313500	202	644.3		554400	136	245.3	
		130500	101	773.9		318900	48	150.5	
		12000	9	750.0		112500	11	97.8	
		57000	50	877.2		276300	36	130.3	
		121500	139	1144.0	837.9 ± 170.0**	44400	14	315.3	187.8 ± 80.5**
	10-AzaBaP	241000	15	62.2		569400	13	22.8	
		115000	6	52.2		282300	7	24.8	
		11500	1	87.0		115200	5	43.4	
		56500	4	70.8		18300	1	54.6	
		372000	3	8.1	56.0 ± 26.6	81900	2	24.4	34.0 ± 12.8
Colon	Control (olive oil)	619500	70	113.0		332700	14	42.1	
		676500	56	82.8		385500	10	25.9	
		521200	33	63.3		1084900	26	24.0	
		913500	79	86.5		389700	15	38.5	
		365000	18	49.3	79.0 ± 21.7	619200	15	24.2	30.9 ± 7.7
	BaP	784000	1241	1582.9		310800	75	241.3	
		396000	529	1335.9		627900	170	270.7	
		201000	417	2074.6		363000	72	198.3	
		484000	654	1351.2		1005900	676	672.0	
		81900	196	2393.2	1747.6 ± 418.9**	434500	199	458.0	368.1 ± 176.0**
	10-AzaBaP	503000	74	147.1		277050	14	50.5	
		259500	33	127.2		523500	27	51.6	
		125000	21	168.0		954000	32	33.5	
		582000	53	91.1		247800	10	40.4	
		129500	19	146.7	136.0 ± 25.9**	1070400	32	29.9	41.2 ± 8.7

Table 1 (*Continued*)

Tissue	Treatment	*lacZ* assay				*cII* assay			
		Individual animal data			Average ± S.D.	Individual animal data			Average ± S.D.
		No. of phages analyzed	No. of mutants	MF × 10^6	MF × 10^6	No. of phages analyzed	No. of mutants	MF × 10^6	MF × 10^6
Stomach	Control (olive oil)	399500	17	42.6		705000	13	18.4	
		363000	18	49.6		559800	17	30.4	
		506500	36	71.1		1060200	76	71.7	
		604000	62	102.6		1090200	34	31.2	
		341500	39	114.2	76.0 ± 28.3	703800	26	36.9	37.7 ± 18.0
	BaP	402000	175	435.3		705900	79	111.9	
		275500	80	290.4		448800	33	73.5	
		153000	114	745.1		366600	87	237.3	
		226000	126	557.5		715200	104	145.4	
		559500	264	471.8	500.0 ± 149.9**	847200	84	99.2	133.5 ± 56.9**
	10-AzaBaP	403500	62	153.7		515100	17	33.0	
		1065000	62	58.2		589800	14	23.7	
		380000	41	107.9		964200	123	127.6	
		313500	25	79.7		787200	29	36.8	
		390500	43	110.1	101.9 ± 32.2	723600	13	18.0	47.8 ± 40.4
Forestomach	Control (olive oil)	129500	20	154.4		334500	14	41.9	
		205000	12	58.5		303000	11	36.3	
		566500	40	70.6		453900	18	39.7	
		430500	25	58.1	85.4 ± 40.2	446400	17	38.1	39.0 ± 2.0
	BaP	36500	102	2794.5		111600	75	672.0	
		114000	693	6078.9		316800	392	1237.4	
		55500	190	3423.4		167400	161	961.8	
		469500	697	1484.6		645900	333	515.6	
		321000	80	249.2	2806.1 ± 1968.6*	339600	36	106.0	698.6 ± 386.0*
	10-AzaBaP	65500	11	167.9		197700	5	25.3	
		55000	8	145.5		243600	20	82.1	
		232500	34	146.2		297600	15	50.4	
		364000	26	71.4		502500	40	79.6	
		334000	33	98.8	126.0 ± 35.4	399600	12	30.0	53.5 ± 23.9

* $P < 0.05$ (significantly different from the control group).
** $P < 0.01$ (significantly different from the control group).

PCB-induced rat liver S9 [12]. To investigate the difference between the in vivo and in vitro mutagenicity, BaP and 10-azaBaP were re-tested for in vitro mutagenicity by Ames test using liver homogenates from different sources.

Mutagenicities were tested with *S. typhimurium* TA100. In all cases using liver homogenates, BaP and 10-azaBaP showed more than twice the number of background revertants. The results are summarized in Table 6, where the number of revertants per nmol, a measure of mutagenic potency, was calculated from the linear portion of the dose–response curve.

It is well known that BaP and 3-MC strongly induce CYP1A1, one of the enzymes necessary to convert BaP to the ultimate form according to the bay-region theory [6–9]. We prepared CYP1A-inducer-treated rats, and CYP1A-inducer-treated and non-treated mice for the Ames test. Furthermore, we carried out an Ames test using pooled human liver S9 [27] to examine species differences in metabolic activation.

It was obvious that, in the Ames test using a non-treated mouse liver homogenate, 10-azaBaP showed about the same mutagenicity as BaP, but in the Ames test using a CYP1A-inducer treated rodent liver

Table 2
Sequences of *cII* mutations in the liver of 10-azaBaP-treated Muta™Mouse

Mutant no.	Position	Mutation	Sequence			Amino acid change
A1	188–189	+T	GTC	GTT	GAC	Frameshift
A2	128	G to A	AGG	TGG	AAG	Trp to Stop
A3	161	T to G	ATG	CTG	CTT	Leu to Arg
A4	64	G to A	ATC	GCA	ATG	Ala to Thr
A5[a]	64	G to A	ATC	GCA	ATG	Ala to Thr
A6	113	C to G	AAG	TCG	CAG	Ser to Trp
A7	241–246	+2A	AAA	AAA	CGC	Frameshift
A7	247–249	−CGC	AAA	AAA	CGC	Frameshift
A8	89	C to T	ACA	GCG	GAA	Ala to Val
A9	34	C to T	CTA	CGA	ATC	Arg to Stop
A10[a]	34	C to T	CTA	CGA	ATC	Arg to Stop
A11	46	G to C	AGT	GCG	TTG	Ala to Pro
A12	91	+C	GCG	GAA	GCT	Frameshift
A13	34	C to T	CTA	CGA	ATC	Arg to Stop
A14[a]	34	C to T	CTA	CGA	ATC	Arg to Stop
A15	132	G to T	TGG	AAG	AGG	Lys to Asn
A16	103	G to T	GGC	GTT	GAT	Val to Phe
A17	179–184	−G	TGG	GGG	GTC	Frameshift
A18[a]	179–184	−G	TGG	GGG	GTC	Frameshift
A19	101	G to T	GTG	GGC	GTT	Gly to Val
A20	28	G to T	GAG	GCT	CTA	Ala to Ser
A21	35	G to C	CTA	CGA	ATC	Arg to Pro
A22	41	A to T	ATC	GAG	AGT	Glu to Val
A23	185	T to G	GGG	GTC	GTT	Val to Gly
A24	281–282	AG to TA	ATC	CAG	ATG	Gln to Leu
A25	180	G to T	GAA	TGG	GGG	Trp to Cys
A26	202	G to A	ATG	GCT	CGA	Ala to Thr
A27	62	T to A	AAA	ATC	GCA	Ile to Asn
A28	25	G to A	AAC	GAG	GCT	Glu to Lys
A29[a]	25	G to A	AAC	GAG	GCT	Glu to Lys
A30	40	G to A	ATC	GAG	AGT	Glu to Lys
A31	196	G to A	GAC	GAC	ATG	Asp to Asn
A32	57	C to A	CTT	AAC	AAA	Asn to Lys
A33	101	G to T	GTG	GGG	GTT	Gly to Val
A34	34	C to T	CTA	CGA	ATC	Arg to Stop
A35	205	C to T	GCT	CGA	TTC	Arg to Stop
A36	233	T to A	ATT	CTC	ACC	Leu to His
A37	117	G to T	TCG	CAG	ATC	Gln to His
A38	196	G to A	GAC	GAC	ATG	Asp to Asn
A39	34	C to T	CTA	CGA	ATC	Arg to Stop
A40	212	C to T	TTG	GCG	CGA	Ala to Val
A41	193	G to A	GAC	GAC	GAC	Asp to Asn
A42	25	G to A	AAC	GAG	GCT	Glu to Lys
A43	178	T to A	GAA	TGG	GGG	Trp to Arg
A44	179–184	+G	TGG	GGG	GTC	Frameshift
A45	89	C to T	ACA	GCG	GAA	Ala to Val
A46	51	G to T	GCG	TTG	CTT	Leu to Phe
A47	233	T to C	ATT	CTC	ACC	Leu to Pro
A48	212	C to A	TTG	GCG	CGA	Ala to Glu
A49	103	G to T	GGC	GTT	GAT	Val to Phe

[a] Ascribable to the same mutation obtained in an identical mouse.

Table 3
Sequences of *cII* mutations in the liver of BaP-treated Muta™Mouse

Mutant no.	Position	Mutation	Sequence			Amino acid change
B1	39	C to G	CGA	ATC	GAG	Ile to Met
B2	179–184	+G	TGG	GGG	GTC	Frameshift
B3	38	T to A	CGA	ATC	GAG	Ile to Asn
B4	89	C to A	ACA	GCG	GAA	Ala to Glu
B5	224	C to A	GTT	GCT	GCG	Ala to Asp
B6	252–253	−G	CGC	CCG	GCG	Frameshift
B7	193	G to T	GAC	GAC	GAC	Asp to Tyr
B8	73	G to T	CTT	GGA	ACT	Gly to Stop
B9	125	G to T	AGC	AGG	TGG	Arg to Met
B10	71	T to A	ATG	CTT	GGA	Leu to His
B11	180	G to T	GAA	TGG	GGG	Trp to Cys
B12	115	C to A	TCG	CAG	ATC	Gln to Lys
B13[a]	115	C to A	TCG	CAG	ATC	Gln to Lys
B14	103	G to C	GGC	GTT	GAT	Val to Leu
B15	25	G to A	AAC	GAG	GCT	Glu to Lys
B16	125–126	−G	AGC	AGG	TGG	Frameshift
B17	25	G to T	AAC	GAG	GCT	Glu to Stop
B18	101	G to C	GTG	GGC	GTT	Gly to Ala
B19	103	G to T	GGC	GTT	GAT	Val to Phe
B20	212	C to A	TTG	GCG	CGA	Ala to Glu
B20	215	G to A	GCG	CGA	CAA	Arg to Gln
B21	103	G to A	GGC	GTT	GAT	Val to Ile
B22	241–246	−A	AAT	AAA	AAA	Frameshift
B23	179	G to T	GAA	TGG	GGG	Trp to Leu
B24	166	G to T	CTT	GCT	GTT	Ala to Ser
B25	205	C to T	GCT	CGA	TTG	Arg to Stop
B26	88	G to T	ACA	GCG	GAA	Ala to Ser
B27	117	G to T	TCG	CAG	ATC	Gln to His
B28	89	C to A	ACA	GCG	GAA	Ala to Glu
B29	103	G to T	GGC	GTT	GAT	Val to Phe
B30	179–184	−G	TGG	GGG	GTC	Frameshift
B31	190–198	−GAC	GAC	GAC	GAC	Deletion
B32	146	C to A	ATT	CCA	AAG	Pro to Gln
B33	196	G to T	GAC	GAC	ATG	Asp to Tyr
B34	179	G to T	GAA	TGG	GGG	Trp to Leu
B35	134–136	−G	AAG	AGG	GAC	Frameshift
B36	88	G to A	ACA	GCG	GAA	Ala to Thr
B37	169	G to T	GCT	GTT	CTT	Val to Phe

[a] Ascribable to the same mutation obtained in an identical mouse.

homogenate, BaP had a greater mutagenicity than 10-azaBaP. In the Ames test using pooled human liver S9, 10-azaBaP showed a higher mutagenicity than BaP.

4. Discussion

BaP is known to be a typical potent environmental mutagen. BaP is also known to bind to the aryl hydrocarbon receptor [28] and activates its *trans*-activating gene such as *CYP1A1* [6–8]. It is known that BaP is activated by CYP1A1 by the bay-region mechanism to induce mutations [9]. On the other hand, 10-azaBaP would not be activated by the bay-region mechanism because 10-azaBaP has the nitrogen atom at position 10 of the BaP skeleton. However, the in vitro mutagenicity of 10-azaBaP was nearly equal to that of BaP as previously reported [12]. Next we investigated the mutagenicity of 10-azaBaP in vivo and in vitro in comparison with BaP.

Table 4
Sequences of *cII* mutations in the liver of control Muta™Mouse

Mutant no.	Position	Mutation	Sequence			Amino acid change
C1	74	G to T	CTT	GGA	ACT	Gly to Val
C2	150	G to T	CCA	AAG	TTC	Lys to Asn
C3	212	C to T	TTG	GCG	CGA	Ala to Val
C4[a]	212	C to T	TTG	GCG	CGA	Ala to Val
C5	133	A to T	AAG	AGG	GAC	Arg to Trp
C6	46	G to C	AGT	GCG	TTG	Ala to Pro
C7	64	G to A	ATC	GCA	ATG	Ala to Thr
C8	113	C to T	AAG	TCG	CAG	Ser to Leu
C9	146	C to T	ATT	CCA	AAG	Pro to Leu
C10	52	C to T	TTG	CTT	AAC	Leu to Phe
C11	40	G to A	ATC	GAG	AGT	Glu to Lys
C12	34	C to T	CTA	CGA	ATC	Arg to Stop
C13[a]	34	C to T	CTA	CGA	ATC	Arg to Stop
C14	40	G to T	ATC	GAG	AGT	Glu to Stop
C15	233	T to A	ATT	CTC	ACC	Leu to His
C16	112	T to A	AAG	TCG	CAG	Ser to Thr
C17	196	G to A	GAC	GAC	ATG	Asp to Asn
C18	179–184	+G	TGG	GGG	GTC	Frameshift
C19	73	G to T	CTT	GGA	ACT	Gly to Stop
C20	64	G to A	ATC	GCA	ATG	Ala to Thr
C21[a]	64	G to A	ATC	GCA	ATG	Ala to Thr
C22	214	C to T	GCG	CGA	CAA	Arg to Stop
C23	179–184	+G	TGG	GGG	GTC	Frameshift
C24[a]	179–184	+G	TGG	GGG	GTC	Frameshift
C25	179–184	−G	TGG	GGG	GTC	Frameshift
C26	103	G to A	GGC	GTT	GAT	Val to Ile
C27	40	G to A	ATC	GAG	AGT	Glu to Lys
C28	211–212	GC to AT	TTG	GCG	CGA	Ala to Met
C29	214	C to T	GCG	CGA	CAA	Arg to Stop
C30	89	C to T	ACA	GCG	GAA	Ala to Val
C31	71	T to C	ATG	CTT	GGA	Leu to Pro
C32	42	G to T	ATC	GAG	AGT	Glu to Asp
C33	64	G to A	ATC	GCA	ATG	Ala to Thr
C34[a]	64	G to A	ATC	GCA	ATG	Ala to Thr
C35	25	G to A	AAC	GAG	GCT	Glu to Lys
C36	212	C to T	TTG	GCG	CGA	Ala to Val
C37	103	G to A	GGC	GTT	GAT	Val to Ile

[a] Ascribable to the same mutation obtained in an identical mouse.

The total dose of 625 mg/kg (125 mg/kg × 5 days) of BaP was high enough to show carcinogenicity and mutagenicity for Muta™Mouse as previously reported [2]. In this study, it was observed that BaP remarkably increased the mutant frequencies in all organs as previously reported; however, 10-azaBaP slightly increased the mutant frequency only in the liver and colon at the above dose. Therefore, it is obvious that 10-azaBaP has a much less in vivo mutagenicity in Muta™Mouse than BaP in contrast to the in vitro mutagenic activity.

According to the *cII* mutant spectrum analysis in Muta™Mouse liver, the characteristic of spontaneous mutations was the G:C to A:T transition mutation. The majority of BaP-induced mutations were G:C to T:A transversions as previously reported [3]. It is known that the bay-region diol epoxide of BaP forms adducts primarily with the N^2-exocyclic amino group of guanine moieties in DNA, predominantly giving G:C to T:A transversions [29,30]. On the other hand, 10-azaBaP slightly increased the G:C to T:A

Table 5
Summary of *cII* mutation spectra in the liver of Muta™Mouse

Mutation class	Control (%)	BaP (%)	10-AzaBaP (%)
Base substitution	28 (88)	28 (78)	38 (86)
Transitions			
GC to AT	18 (56)	4 (11)	17 (39)
AT to GC	1 (3)	0 (0)	1 (2)
Transversions			
AT to TA	3 (9)	2 (6)	4 (9)
AT to CG	0 (0)	0 (0)	2 (5)
GC to TA	5 (16)	19 (53)	11 (25)
GC to CG	1 (3)	3 (8)	3 (7)
−1 Frameshifts	1 (3)	5 (14)	1 (2)
+1 Frameshifts	2 (6)	1 (3)	3 (7)
Deletion	0 (0)	1 (3)	0 (0)
Insertion	0 (0)	0 (0)	0 (0)
Complex	1 (3)	1 (3)	2 (5)
Total	32 (100)	36 (100)	44 (100)

The same mutations from an identical mouse were counted as a single event.

transversion and slightly decreased the G:C to A:T transition. The results suggest that 10-azaBaP might mainly induce G:C to T:A transversions.

Furthermore, we examined the in vitro mutagenicities of BaP and 10-azaBaP by Ames test using liver homogenates prepared from different sources, i.e. the liver of CYP1A-inducer-treated rats, CYP1A-inducer-treated and non-treated mice, and humans. The results demonstrated that in the presence of a liver homogenate from CYP1A-inducer-treated rodents, BaP showed a greater mutagenicity than 10-azaBaP; however, in the presence of a liver homogenate from non-treated mice, 10-azaBaP induced as many mutations as BaP. Therefore, the CYP1A-induced liver homogenate efficiently enhanced the in vitro mutagenicity of BaP, but not that of 10-azaBaP. These findings suggest that 10-azaBaP might not be converted to the ultimate form by CYP1A and that the potent CYP1A-inducibility of BaP leads to the potent mutagenicity of BaP in Muta™Mice. In the in vivo mutation assay system using the transgenic rodent such as Muta™Mouse, a mutagen which has inducibility of the enzymes necessary to convert itself to the ultimate form, such as BaP, might show a more potent mutagenicity than the risk expected from in vitro mutagenicity tests such as the Ames test.

Table 6
Mutagenicities of BaP and 10-azaBaP in *S. typhimurium* TA100

Species	Liver fraction	Inducer	Revertants/nmol[a]	
			BaP	10-AzaBaP
Rat	S9	PCB[b]	317.5[c]	316.2[c]
Rat	Microsome	3-MC[d]	117.6	41.8
Mouse	Microsome	–	86.0	70.0
Mouse	Microsome	BaP	216.2	45.8
Human	S9	–	126.6	964.8

[a] Calculated from the slope of the linear portion of each curve near the origin. The numbers indicate the means of at least three independent experiments.
[b] Polychlorinated biphenyl.
[c] The data of BaP and 10-azaBaP using PCB-induced rat liver S9 are from the previous report [12].
[d] 3-Methylcholanthrene.

Additionally, the result that 10-azaBaP showed a more potent mutagenicity than BaP in the presence of human liver S9 suggests that the enzymes responsible for the conversion of 10-azaBaP to the ultimate form might be more abundant in human liver homogenates than CYP1A1, which is responsible for the conversion of BaP to the ultimate form.

It is worth mentioning that the hepatocarcinogen quinoline [31], an aza-analog of naphthalene and a partial structure of 10-azaBaP, may be metabolized by the liver microsomal enzymes to the ultimate genotoxic form, enamine epoxide (*N*-4-hydrated 2,3-epoxide), which is responsible for the mutagenic modification of DNA [24,32–34]. Our previous studies reveal that quinoline induces mutations in Muta™Mouse only in the liver, but not in the kidney, lung, bone marrow, or testis [17,32], and support the hypothesis that metabolic oxidation might take place in the pyridine moiety of quinoline in vivo as well as in vitro [32,35]. Because 10-azaBaP showed mutagenicity only in the liver and colon, but not in the kidney, lung or bone marrow, it may be proposed that the genotoxicity of 10-azaBaP might be attributable to formation of a quinoline-type ultimate form, an enamine epoxide.

In conclusion, our results suggest that the metabolic activation pathway of 10-azaBaP, both in vitro and in vivo, may be different from that of BaP which is mediated by CYP1A, and 10-azaBaP might be as much risky a mutagen as BaP in humans. These results indicate that 10-aza-substitution markedly modifies the

mutagenicity of benzo[*a*]pyrene in both in vivo and in vitro mutagenesis assays.

Acknowledgements

We thank Dr. T. Nohmi for his kind gift of *E. coli* NM759 and BHB2688, and Dr. K. Masumura for his technical direction in making packaging extracts. This research is supported in part by the grant from Ministry of the Environment, Japan.

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Dinitropyrenes induce gene mutations in multiple organs of the lambda/*lacZ* transgenic mouse (Muta™ Mouse)

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Received 17 April 2001; received in revised form 28 December 2001; accepted 28 December 2001

Abstract

Dinitropyrenes (DNPs), 1,3-, 1,6- and 1,8-dinitropyrene, are carcinogenic compounds found in diesel engine exhaust. DNPs are strongly mutagenic in the bacterial mutation assay (Ames test), mainly inducing frameshift type mutations. To assess mutagenicity of DNPs in vivo is important in evaluating their possible involvement in diesel exhaust-induced carcinogenesis in human. For this purpose, we used the lambda/*lacZ* transgenic mouse (Muta™ Mouse) to examine induction of mutations in multiple organs. A commercially available mixture of DNPs (1,3-, 1,6-, 1,8-, and unidentified isomer (s) with a content of 20.2, 30.4, 35.2, and 14.2%, respectively) was injected intragastrically at 200 and 400 mg/kg once each week for 4 weeks. Seven days after the final treatment, liver, lung, colon, stomach, and bone marrow were collected for mutation analysis. The target transgene was recovered by the lambda packaging method and mutation of *lacZ* gene was analyzed by a positive selection with *galE*$^-$ *E. coli*. In order to determine the sequence alterations by DNPs, the mutagenicity of the lambda *cII* gene was also examined by the positive selection with *hfl*$^-$ *E. coli*. Since *cII* gene (294 bp) is much smaller than the *lacZ* (3024 bp), it facilitated the sequence analysis. Strongest increases in mutant frequencies (MFs) were observed in colon for both *lacZ* (7.5×10^{-5} to 43.3×10^{-5}) and *cII* (2.7×10^{-5} to 22.5×10^{-5}) gene. Three–four-fold increases were observed in stomach for both genes. A statistically significant increase in MFs was also evident in liver and lung for the *lacZ* gene, and in lung and bone marrow for the *cII* gene. The sequence alterations of the *cII* gene recovered from 37 mutants in the colon were compared with 50 mutants from untreated mice. Base substitution mutations predominated for both untreated (91%) and DNP-treated (84%) groups. The DNPs treatment increased the incidence of G:C to T:A transversion (2–43%) and decreased G:C to A:T transitions (70–22%). The G:C to T:A transversions, characteristic to DNPs treatment, is probably caused by the guanine–C8 adduct, which is known as a major DNA-adduct induced by DNPs, through an incorporation of adenine opposite the adduct ("A"-rule). The present study showed a relevant use of the *cII* gene as an additional target for mutagenesis in the Muta™ Mouse and revealed a mutagenic specificity of DNPs in vivo.

Keywords: 1,3-Dinitropyrene; 1,6-Dinitropyrene; 1,8-Dinitropyrene; *cII*; Muta™ Mouse; Mutation spectrum; G:C to T:A transversion

1. Introduction

Air pollution from diesel exhaust is an increasing concern as an environmental risk factor for

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1383-5718/02/$ – see front matter
PII: S1383-5718(02)00007-4

carcinogenesis. Diesel exhaust is known to induce tumors in experimental animals [1]. Of the compounds present in this complex mixture, various polycyclic aromatic hydrocarbons (PAHs) such as benzo[*a*]pyrene, and nitroarenes such as dinitropyrenes (DNPs) and nitrofluorantenes are potent mutagens and carcinogens. In the *Salmonella* reverse mutation assay (Ames test), DNPs show extremely strong mutagenicity without an exogenous metabolic activation system, in which the frameshift type mutations predominated [2–4]. DNPs are activated by the nitroreductase and the *O*-acetyltransferase of the bacteria to form DNA-adducts; strains deficient in these enzymes show reduced mutagenicity [5]. The ultimate reactive form of DNPs is presumably the nitropyrene-1-nitrenium ion which reacts with guanine to form a major DNA-adduct, 1-*N*-(deoxyguanosine-8-yl)-1-amino-nitropyrene [6]. The DNP–DNA-adduct was identified both in vitro and in vivo [7–11]. Despite a strong mutagenicity in bacteria, little is known about mutagenicity in vivo. To evaluate the mutagenicity of DNPs in vivo, the micronucleus test was performed with the transgenic mouse mutation (TG) assay [12].

Since diesel exhaust contains a various mutagens/carcinogens, the question arises to what extent the DNPs contribute to the mutagenicity and carcinogenicity. Recently, Sato et al. reported that direct exposure of *lacI* transgenic (Big Blue®) rats to diesel exhaust increased the mutation frequency in lung [13]. Comparing the mutation frequency and spectrum of DNPs with those of diesel exhaust, the possible involvement of DNPs in the mutagenicity of diesel exhaust is discussed.

2. Materials and methods

2.1. Chemicals

DNPs, as a mixture of isomers, was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The isomer content was analyzed by the high-performance liquid chromatography (HPLC) and four peaks were detected. Three of them were identified as 1,3-, 1,6-, and 1,8-DNP by the co-chromatography with standard DNPs (Sigma). The fourth peak was revealed as DNP isomer (s) by the UV and mass spectrometry but the exact structure was not determined. The content of each isomer is 20.2% (1,3-DNP), 30.4% (1,6-DNP), 35.2% (1,8-DNP) and 14.2% (unidentified isomer (s)) as a peak area ratio in the HPLC analysis. DNPs were dissolved in olive oil for use. The chemical structures of these isomers are shown in Fig. 1.

2.2. Animals and treatments

Six-week-old male Muta™ Mouse (ca. 25 g body weight) supplied by Covance Research Products (PA, USA) were acclimatized for 1 week before use and divided into five groups. Based on the LD_{50}, 200 or 400 mg/kg (40 and 80% LD_{50}, respectively) DNPs was injected intragastrically at a volume of 10 ml/kg once a week for 4 weeks. The vehicle control (olive oil) group was treated at the same time in the same manner. Six-week-old male ICR mice were supplied by Japan SLC (Shizuoka, Japan) and were used for the micronucleus assay after 1 week of acclimatization.

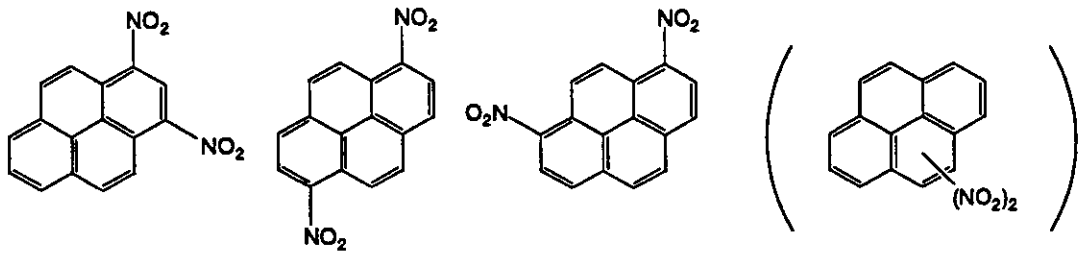

Fig. 1. Structures and contents of DNP isomers.

2.3. Micronucleus test

Forty-eight hours after the first treatment with the Muta™ Mouse, 5 μl of peripheral blood was collected from the tail vein without anti-coagulant. The blood thus collected from each animal was placed on an acridine orange-coated glass slide, covered with a cover slip, and supravitally stained [14]. Type I, II, and III reticulocytes (RETs) with red fluorescent reticulum in the cytoplasm were scored. One thousand RETs were analyzed per animal under a fluorescence microscope within a few days after the slide preparation. The number of RETs with a micronucleus, which fluoresced greenish yellow, was recorded. For a further analysis, five male ICR mice (7-week-old) per group were treated intraperitoneally or intragastrically with 100, 200, 400, and 800 mg/kg DNPs, and micronucleus induction was analyzed in a same manner using peripheral blood. In this experiment, 2000 RETs were analyzed per animal and mitomycin C (0.25 mg/kg) was used as a positive control.

2.4. Tissue collection

Animals were killed 7 days after the last treatment by cervical dislocation. Liver, lung, stomach, bone marrow, and colon were removed, quickly frozen in liquid nitrogen, then stored in a deep freezer at −80 °C until analysis.

2.5. DNA isolation and in vitro packaging

The isolation of total genomic DNA from tissue samples was carried out using the standard phenol/chloroform method (Stratagene manual, 1994). Briefly, homogenized tissues were incubated with RNase and proteinase K, and genomic DNA was extracted using a phenol/chloroform mixture and chloroform. The DNA was precipitated with ethanol and dissolved in TE-4 buffer (10 mM Tris–HCl at pH 8.0 containing 4 mM EDTA).

2.6. In vitro packaging and mutant frequency (MF) determination

The *lacZ* transgene, integrated into the lambda phage vector (lambda gt10), was recovered by in vitro packaging reactions. The DNA solution (10 μl) adjusted to 1 mg DNA/ml was gently mixed with the Transpack packaging extract (Stratagene, La Jolla, CA, USA) and incubated at 37 °C for 1.5 h twice. The *lacZ* MF was determined by a positive selection with *galE*⁻ *E. coli* according to the standard procedure (Corning Hazleton, October, 1996). The MF was also analyzed for the *cII* gene of lambda phage vector. A positive selection for *cII* mutants was performed according to Jakubczak et al. [15] with a slight modification. Briefly, the phage solution was adsorbed to 1 ml of *E. coli* G1225 (*hfl*⁻) at room temperature for 20–30 min. For the titration, appropriately diluted phage solution was mixed with 200 μl of *E. coli* G1225. The phage-*E. coli* solution was mixed with 14 ml (for selection) and 6 ml (for titration) LB top agar (containing 10 mM $MgSO_4$), and plated onto five and two petri dishes (9 cm), respectively, containing 10 ml bottom agar. The plates were incubated for 48 h at 25 °C for the selection of *cII* mutants or at 37 °C for the titer of total phages. Wild-type phage, recovered from Muta™ Mouse, has a *cI*⁻ phenotype, which permits plaque formation with *hfl*⁻ strain at 37 °C but not at 25 °C.

2.7. Sequencing of mutants

The entire lambda *cII* region was amplified directly from mutant plaques by Taq DNA polymerase (Takara Shuzo, Tokyo Japan) with primers P1; 5′-AAAAAGGGCATCAAATTAAACC-3′, and P2; 5′-CCGAAGTTGAGTATTTTTGCTGT-3′. Amplification was done by the Minicycler PTC-150-25 (MJ Research Inc., MA, USA) with an initial heating step at 95 °C for 5 min followed by 30 cycles of denaturing at 95 °C for 20 s, annealing at 53 °C for 30 s, extension at 72 °C for 40 s, and 10 min incubation at 72 °C. A 446 bp PCR product was purified with a microspin column (Amersham Pharmacia, Tokyo, Japan) and then used for a sequencing reaction with the Ampli Taq cycle sequencing kit (PE Biosystems, Tokyo, Japan). The sequencing reaction was done by Minicycler PTC-150-25 with 25 cycles of denaturing at 96 °C for 10 s, annealing at 50 °C for 5 s, and extension at 60 °C for 4 min, with the primer P1. The reaction product was purified by ethanol precipitation and analyzed by the ABI PRISM™ 310 genetic analyzer (PE Biosystems, Tokyo, Japan).

2.8. Statistical analysis

The difference in MFs between control and treated group was evaluated with the one-side test with the Poisson regression using quasi-likelihood. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Micronucleus induction

Results of the micronucleus test 48 h after the first intragastric administration of DNPs in the Muta™ Mouse are shown in Table 1. The mean incidence of the micronucleated RETs (MNRETs) after intragastric injection of 200 or 400 mg/kg DNPs was not different from the vehicle control. To confirm the negative response, male ICR mice were treated intraperitoneally or intragastrically with 200–800 mg/kg DNPs. As shown in Table 2, no increase of MNRETs by DNPs treatment was observed while micronucleus induction did occur with the positive control MMC.

3.2. MF of lacZ and cII genes

DNA was isolated from various organs 7days after the last treatment with DNPs. The MFs of *lacZ* and *cII* genes were analyzed for liver, lung, colon, stomach, and bone marrow. At least 10^5 plaques were analyzed for both genes, which is generally obtained by one packaging reaction, although a few DNA samples with a low packaging efficiency did not reach this number. The individual data are presented in Table 3. Spontaneous MFs were in the range of 3.1×10^{-5} to 7.6×10^{-5} and 1.6×10^{-5} to 5.9×10^{-5} for the *lacZ* and the *cII* gene, respectively. The MF increase above spontaneous levels was most apparent in colon, with respectively six- and eight-fold increases *lacZ* and *cII* MF. Increase was also evident in the stomach for both genes although the increase was not statistically significant at the higher dose for the *cII* gene. A statistically significant increase was observed in liver and lung for the *lacZ* gene but was not evident for liver of the *cII* gene. A few-fold increase was observed in bone marrow for both genes but was statistically significant only for the *cII* gene.

Table 1
Micronucleus induction in peripheral blood of Muta™mice treated with DNP

Treatment	Dose	MNRETs per 1000 RETs					Mean ± S.D. (%)
Intragastric							
Olive oil	10 ml/kg	1	2	1	3	1	0.16 ± 0.09
DNP	200 mg/kg	2	3	2	2	1	0.20 ± 0.07
	400 mg/kg	1	4	1	2	1	0.18 ± 0.13

Table 2
Micronucleus induction in peripheral blood of ICR mice treated with DNP

Treatment	Dose	MNRETs per 2000 RETs				Mean ± S.D. (%)
Olive oil	10 ml/kg (intraperitoneal)	7	5	4	4	0.25 ± 0.06
DNP	200 mg/kg (intraperitoneal)	4	5	6	6	0.26 ± 0.04
	400 mg/kg (intraperitoneal)	2	1	3	7	0.16 ± 0.11
	800 mg/kg (intraperitoneal)	4	4	4	5	0.21 ± 0.02
Olive oil	10 ml/kg (intragastric)	1	7	6	3	0.21 ± 0.12
DNP	200 mg/kg (intragastric)	3	10	5	6	0.30 ± 0.13
	400 mg/kg (intragastric)	3	3	3	5	0.18 ± 0.04
	800 mg/kg (intragastric)	5	4	4	11	0.30 ± 0.15
MMC (positive control)	0.25 mg/kg (intraperitoneal)	7	12	16	12	0.59 ± 0.16*

* $P < 0.05$ in Fisher's exact test.

Table 3
MFs in the *lacZ* and *cII* gene from various organs of Muta™ Mouse treated with DNP

Organ	Treatment	Animal ID	*lacZ*					*cII*				
			Total plaque	Mutants	MF ($\times 10^6$)	Mean ± S.D.	*P* value	Total plaque	Mutants	MF ($\times 10^6$)	Mean ± S.D.	*P* value
Liver	Control	11	238500	7	29.4			663000	16	24.1		
		12	239000	6	25.1			274800	3	10.9		
		13	112500	4	35.6	31.2 ± 4.9		132300	0	0.0	16.1 ± 9.4	
		14	131000	5	38.2			167100	4	23.9		
		15	434500	12	27.6			369600	8	21.6		
		Total	1155500	34	29.4			1606800	31	19.3		
	DNP 400	31	509500	39	76.5			804000	23	28.6		
		32	357000	22	61.6			534000	10	18.7		
		33	334500	17	50.8	82.7 ± 32.5	<0.01	424800	3	7.1	15.8 ± 9.6	0.575
		34	360500	52	144.2			364800	1	2.7		
		35	285500	23	80.6			362400	8	22.1		
		Total	1847000	153	82.8			2490000	45	18.1		
Lung	Control	11	514500	27	52.5			768000	69	89.8		
		12	817000	50	61.2			1344000	60	44.6		
		13	1220000	136	111.5	76.1 ± 21.4		1480800	77	52.0	59.4 ± 16.1	
		14	1174000	104	88.6			1425600	87	61.0		
		15	1338000	89	66.5			1456800	72	49.4		
		Total	5063500	406	80.2			6475200	365	56.4		
	DNP 400	31	1141000	121	106.0			1752000	96	54.8		
		32	2723000	357	131.1			2142000	283	132.1		
		33	391000	64	163.7	133.6 ± 18.3	<0.01	538800	34	63.1	81.7 ± 28.3	0.034
		34	796000	107	134.4			805200	75	93.1		
		35	1275000	169	132.5			1380000	90	65.2		
		Total	6326000	818	129.3			6618000	578	87.3		
Colon	Control	11	424000	24	56.6			416400	14	33.6		
		12	401000	34	84.8			483900	16	33.1		
		13	304500	36	118.2	74.8 ± 24.6		353700	9	25.4	27.2 ± 8.1	
		14	100000	5	50.0			125700	4	31.8		
		15	155000	10	64.5			167100	2	12.0		
		Total	1384500	109	78.7			1546800	45	29.1		
	DNP 200	21	150500	29	192.7			183300	23	125.5		
		22	304500	54	177.3			342000	50	146.2		
		23	187500	31	165.3	173.6 ± 39.6	<0.01	216000	11	50.9	132.5 ± 51.0	0.0014
		24	75500	8	106.0			92700	12	129.4		
		25	128000	29	226.6			189900	40	210.6		
		Total	846000	151	178.5			1023900	136	132.8		
	DNP 400	31	236500	118	498.9			315600	44	139.4		
		32	160000	72	450.0			281100	56	199.2		
		33	688000	308	447.7	432.9 ± 46.8	<0.01	1073700	421	392.1	225.4 ± 104.1	<0.01

Table 3 (*Continued*)

Organ	Treatment	Animal ID	*lacZ*					*cII*				
			Total plaque	Mutants	MF ($\times 10^6$)	Mean ± S.D.	*P* value	Total plaque	Mutants	MF ($\times 10^6$)	Mean ± S.D.	*P* value
		34	106000	38	358.5			112800	12	106.4		
		35	457000	187	409.2			782700	227	290.0		
		Total	1647500	723	438.8			2565900	760	296.2		
Stomach	Control	11	149000	11	73.8			190800	5	26.2		
		12	464500	25	53.8			489600	16	32.7		
		13	283000	9	31.8	54.6 ± 13.7		267000	6	22.5	25.3 ± 8.8	
		14	435000	23	52.9			399000	14	35.1		
		15	115000	7	60.9			198900	2	10.1		
		Total	1446500	75	51.8			1545300	43	27.8		
	DNP 200	21	203000	16	78.8			243300	22	90.4		
		22	191500	23	120.1			219000	17	77.6		
		23	343500	22	64.0	79.5 ± 23.3	0.102	449400	29	64.5	108.9 ± 39.5	<0.01
		24	215500	18	83.5			313200	47	150.1		
		25	196500	10	50.9			210000	34	161.9		
		Total	1150000	89	77.4			1434900	149	103.8		
	DNP 400	31	190500	16	84.0			155400	3	19.3		
		32	142500	36	252.6			226200	21	92.8		
		33	128000	35	273.4	160.7 ± 86.4	<0.01	174900	14	80.0	52.5 ± 30.2	0.130
		34	486000	63	129.6			598800	12	20.0		
		35	140500	9	64.1			238500	12	50.3		
		Total	1087500	159	146.2			1393800	62	44.5		
Bone marrow	Control	11	224500	6	26.7			240300	3	12.5		
		12	432500	11	25.4			536700	4	7.5		
		13	213000	5	23.5	34.7 ± 18.0		170100	4	23.5	14.5 ± 6.0	
		14	588500	16	27.2			822000	8	9.7		
		15	255000	18	70.6			308100	6	19.5		
		Total	1713500	56	32.7			2077200	25	12.0		
	DNP 200	21	127500	7	54.9			130200	1	7.7		
		22	121500	11	90.5			119400	11	92.1		
		23	61500	9	146.3	71.9 ± 42.7	0.088	94200	6	63.7	43.6 ± 30.7	0.0014
		24	309500	12	38.8			392400	14	35.7		
		25	103000	3	29.1			105600	2	18.9		
		Total	723000	42	58.1			841800	34	40.4		
	DNP 400	31	100500	14	139.3			113100	9	79.6		
		32	180500	16	88.6			224100	4	17.8		
		33	388500	8	20.6	69.1 ± 41.5	0.055	390900	14	35.8	38.2 ± 22.2	<0.01
		34	1566000	88	56.2			1350000	51	37.8		
		35	122000	5	41.0			198600	4	20.1		
		Total	2357500	131	55.6			2276700	82	36.0		

Table 4
The *cII* mutations recovered from colon

Mutation type	Treatment	Altered site	Sequence	Amino acid change	No. of mutant[a]
Base substitution					
Transition					
G:C to A:T	Control	25	aac Gag gct	Glu to Lys	3
		34	cta Cga atc	Arg to Stop	3
		40	atc Gag agt	Glu to Lys	2
		64	atc Gca atg	Ala to Thr	3
		89	aca gCg gaa	Ala to Val	1
		103	ggc Gtt gat	Val to Ile	1
		113	aag tCg cag	Ser to Leu	3 (2)
		146	att cCa aag	Pro to Leu	1
		193	gac Gac gac	Asp to Asn	1
		196	gac Gac atg	Asp to Asn	2
		212	ttg gCg cga	Ala to Val	3
		214	gcg Cga caa	Arg to Stop	1
	DNPs	34	cta Cga atc	Arg to Stop	1
		40	atc Gag agt	Glu to Lys	1
		64	atc Gca atg	Ala to Thr	1
		141	gca tgG att	Trp to Stop	1
		196	gac Gac atg	Asp to Asn	1
		212	ttg gCg cga	Ala to Val	1
		214	gcg Cga caa	Arg to Stop	2
A:T to G:C	DNPs	233	att cTc acc	Leu to Pro	1
Transversion					
A:T to T:A	Control	112	aag Tcg cag	Ser to Thr	1
	DNPs	38	cga aTc gag	Ile to Asn	3 (2)
A:T to C:G	Control	110	gat aAg tcg	Lys to Thr	1
	DNPs	149	cca aAg ttc	Lys to Thr	1
G:C to T:A	Control	42	atc gaG agt	Glu to Asp	1
		79	act Gag aag	Glu to Stop	1
		141	gca tgG att	Trp to Cys	1
		167	ctt gCt gtt	Ala to Asp	1
	DNPs	20	aaa cGc aac	Arg to Leu	1
		35	cta cGa atc	Arg to Leu	1
		89	aca gCg gaa	Ala to Glu	1
		94	gaa Gct gtg	Ala to Ser	1
		100	gtg Ggc gtt	Gly to Cys	1
		122	atc aGc agg	Ser to Ile	1
		132	tgg aaG agg	Lys to Asn	1
		134	aag aGg gac	Arg to Met	1
		159	tca atG ctg	Met to Ile	1
		167	ctt gCt gtt	Ala to Asp	1
		179	gaa tGg ggg	Trp to Leu	1
		210	cga ttG gcg	Leu to Phe	1
		212	ttg gCg cga	Ala to Glu	3
		224	gtt gCt gcg	Ala to Asp	1

Table 4 (*Continued*)

Mutation type	Treatment	Altered site	Sequence	Amino acid change	No. of mutant[a]
G:C to C:G	Control	84	gag aaG aca	Lys to Asn	1
	DNPs	44	ctt gGa act	Gly to Ala	1
		74	ctt gGa act	Gly to Ala	1
		179	gaa tGg ggg	Trp to Ser	1
−1 Frameshift					
	Control	179–184	gaa tgg ggg gtc gtt		1
		260–261	gca acc gag		1
	DNPs	179–184	gaa tgg ggg gtc gtt		7 (3)
+1 Frameshift					
	Control	179–184	gaa tgg ggg gtc gtt		1
	DNPs	179–184	gaa tgg ggg gtc gtt		3 (2)
Deletion					
	DNPs	190–198	gtt GAC GAC GAC atg (−GAC)		1

[a] Numbers in parenthesis indicate independent mutations after subtraction of mutations recovered from the identical mouse.

3.3. The *cII* mutation spectrum

Thirty-seven DNPs-induced mutants in the colon were sequenced, together with 33 control mutants. Table 4 lists the types of mutations detected, and the mutation spectra are summarized in Table 5.

Table 5
Summary of *cII* mutations in the colon of control and DNP-treated Muta™ Mouse

Mutation class	Colon	
	Control (%)	DNP (%)
Base substitution	30 (91)	31 (84)
Transitions	23 (70)	9 (24)
G:C to A:T	23 (70)	8 (22)
at CpG sites	22 (67)	1 (3)
A:T to G:C	0 (0)	1 (3)
Transversions	7 (21)	22 (59)
A:T to T:A	1 (3)	2 (5)
A:T to C:G	1 (3)	1 (3)
G:C to T:A	4 (12)	16 (43)
G:C to C:G	1 (3)	3 (8)
−1 Frameshift	2 (6)	3 (8)
+1 Frameshift	1 (3)	2 (5)
Deletion	0 (0)	1 (3)
Insertion	0 (0)	0 (0)
Complex	0 (0)	0 (0)
Total	33 (100)	37 (100)
MF ($\times 10^{-6}$)	29.1	296.2

Spontaneous mutations consisted mainly of base substitutions (30/33). Among them, G:C to A:T transitions (23/30) predominated and almost all of them (22/23) occurred at CpG sites. DNPs-induced mutations also consisted mainly of base substitutions (31/37). Comparing to the control, G:C to A:T transitions decreased (24% versus 70%) and G:C to T:A transversions increased (43% versus 12%). No change was observed for incidences of frameshift mutations. The locations of the mutations, obtained from colon of control and DNP-treated mouse, were shown in Fig. 2.

4. Discussion

There have been increasing concerns on the carcinogenic risk of diesel exhaust. DNPs are important mutagenic components in diesel exhaust, and were proved to be carcinogenic in rodents [16]. A strong mutagenicity of DNPs in bacteria suggested that they may also be mutagenic in vivo. Therefore, we have investigated the in vivo mutagenicity of DNPs by the TG assay, which is a powerful tool for studying chemical mutagenesis in vivo. Because DNPs exist in the diesel exhaust as a mixture, we used a commercially available mixture to test whether any of the isomers possess mutagenic potency in the transgenic system. The mutagenicity of DNPs in vivo is clearly demonstrated by this assay using the *lacZ* and *cII* gene

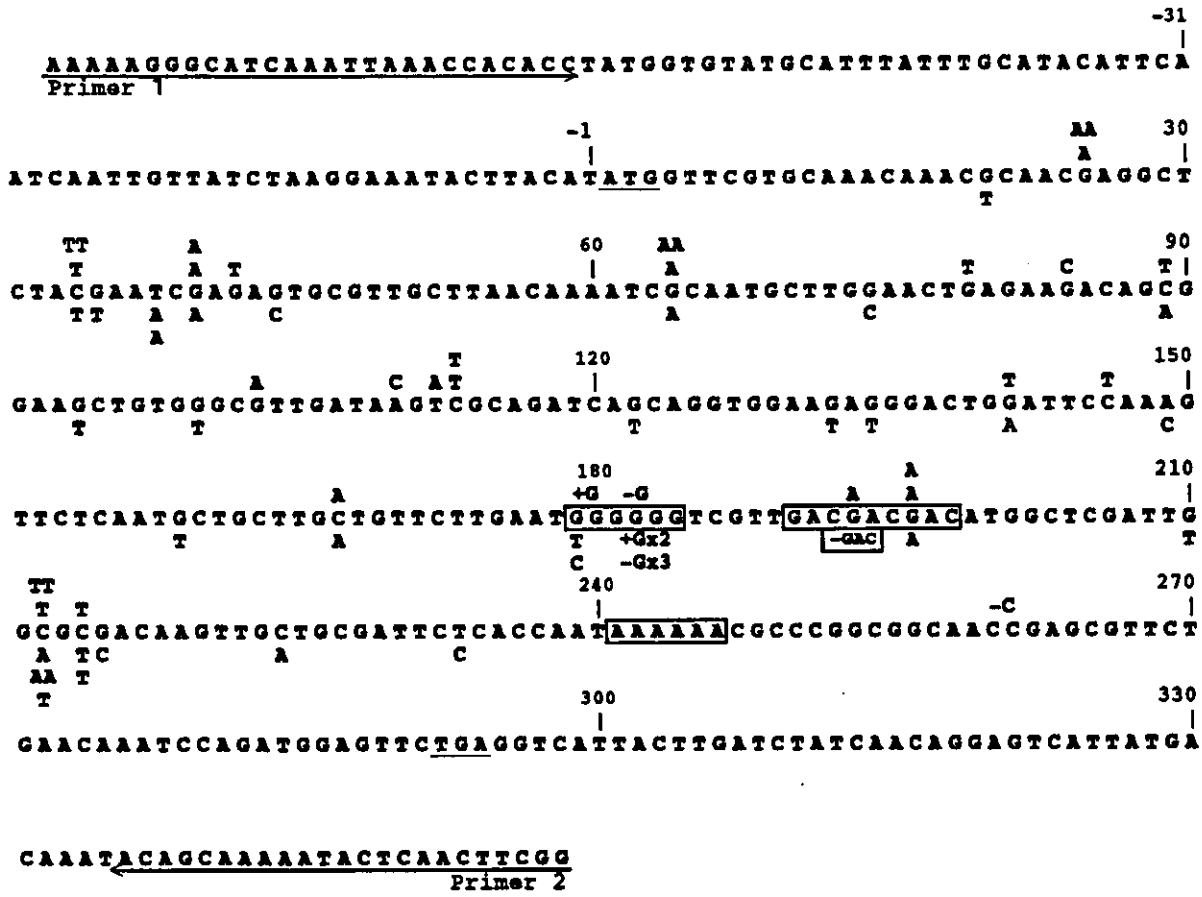

Fig. 2. The *cII* gene mutations in the colon of control and DNP-treated Muta™Mice. The sequence from top to bottom represents the amplified lambda *cII* region. Mutations shown above the sequence were detected in control mice, whereas those below were detected in DNP-treated mice.

as targets, although the micronucleus tests gave negative results. These results demonstrated that the TG assay is important as a complementary or alternative in vivo test to the micronucleus test. Analysis of the *lacZ* and *cII* gene mutations showed organ-specific MF among liver, lung, colon, stomach, and bone marrow. The strongest MF increase was observed in colon followed by stomach and lung > liver > bone marrow although such ordering is not absolute, and is dependent on maximal mutation expression. Accordingly, this relative order might be affected if tissue-specific sub-optimal expression conditions existed in this study. The weakest response, which occurred in bone marrow, might correlate with the negative result in the micronucleus assay. Because DNPs were administered by intragastric injections, organs of contact (stomach and colon) are likely to be the main target organs for mutagenesis. The carcinogenicity of DNPs has previously been observed at the site of administration, lung by intrapulmonary instillation [17,18], sarcoma by subcutaneous injection [19,20], and soft tissues of peritoneal cavity by intraperitoneal injection [11]. In addition, it was reported that formation of DNA-adducts in the lung was 10-fold higher than in the liver after intrapulmonary instillation of 1,6-DNP [7]. The highest MF induction in colon, as was also the case for other mutagens [21–25], suggested that colon is the most sensitive organ for mutagenesis in the TG assay, probably because of its high proliferation rate. It should also be considered that enteric bacteria might have some role in mutagenic activation of DNPs in colon by a nitro-reduction.

The mutagenic response of the *cII* gene is almost similar to that of the *lacZ* gene, although the MF is generally lower in *cII*. There were variations in the MFs among animals and between *lacZ* and *cII* gene in some case, that might be derived from clonal (jackpot) mutations. The possibility of clonal mutations can be checked by sequencing the mutants. The *cII* gene has a great advantage for sequence analysis because the target size is about (1/10) of the *lacZ* gene. The sequence analysis was performed on the mutants recovered from colon where the highest MF was obtained. As has been reported with the *lacZ* and *lacI* genes, the most common spontaneous mutation was G:C to A:T transitions, most of which occurred at CpG sites. The site of mutations was distributed widely among *cII* genes. The DNPs mainly induced base substitution mutations that contrast to the results in the *Salmonella* reversion (Ames) assay, in which DNPs induce mainly frameshift mutations [26]. Similar differences between bacterial and transgenic systems were reported for heterocyclic amines [27,28]. It is rare that a chemical induces predominantly frameshift-type mutations in the TG assays. A possible explanation for the difference is a repair deficiency (*uvrB*$^-$) or the target-sequence context (GC repeats) of bacterial tester strains. The main changes in the mutation spectrum after DNPs treatment were the reduction of G:C to A:T transitions and the induction of the G:C to T:A transversions. The latter correlates with the fact that 1,6- or 1,8-DNPs-induced lung tumors contained G to T transversions in codon 12 of the K-*ras* gene [29,30] and the major type of base substitution mutation in bacteria [34]. The major DNA-adduct formed by 1,6-DNP was reported as 1-*N*-(deoxyguanosine-8-yl)-1-amino-6-nitropyrene [6]. As was also observed with heterocyclic amines [31], guanine–C8 adducts induce G:C to T:A transversion mutations probably by inserting "A" opposite to the uninformative or apurinic bases [32]. There were no apparent hot spots for the DNPs-induced mutations.

Recently, Sato et al. [13] reported that exposure to diesel exhaust increased mutation frequency in lung of Big Blue® rat. This report is important for providing evidence that diesel exhaust, as a mixture, acts as a mutagen in vivo. They reported that the G:C to T:A transversion of the *lacI* gene at the site 211 was a hot spot. The surrounding sequence of this site (gatt**G**gcg) is identical to the *cII* sequence at 206–213 but only one mutation was recovered at the corresponding guanine. In addition, the major mutations induced by diesel exhaust in the *lacI* gene were A:T to G:C and G:C to A:T transitions. Therefore, there was no direct evidence on a contribution of DNPs to the mutagenicity of diesel exhaust although it was reported that DNPs contribute 43% of the total direct mutagenicity of diesel particulate extracts in the Ames test [33]. The different routes of exposure (inhalation or intragastric administration) might gave different mutation spectra between Sato et al. [13] and this study.

In conclusion, the TG assay demonstrated that DNPs, as a mixture, are mutagenic in multiple organs, while micronucleus induction was not observed. Sequence analyses of DNPs-induced *cII* mutants revealed a molecular signature of DNPs-induced mutation as G:C to T:A transversions.

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