# Different Mutation Frequencies and Spectra among Organs by *N*-Methyl-*N*-nitrosourea in *rpsL* (*strA*) Transgenic Mice

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The frequencies and spectra of *N*-methyl-*N*-nitrosourea (MNU)-induced *in vivo* somatic mutations were determined in *rpsL* (*strA*) transgenic mice. The wild-type *rpsL* gene, which exhibits a strepto-mycin-sensitive (Sm$^S$) phenotype, was used as the rescue marker gene. Studies of mutation spectra among different organs and tissues were simplified using this system because of the short coding sequence (375 bp) of the *rpsL* gene. MNU administration to transgenic mice significantly elevated the mutation frequencies in various adult organs. Two distinctive patterns of mutation spectrum were observed, depending on the organs tested. Mutations derived from labile organs (spleen and thymus) were predominantly G:C to A:T transitions, as expected for MNU mutagenesis. Stable organs like the liver and brain, however, carried many fewer G:C to A:T transitions but signifi-cantly more single base deletions, of which the spectrum was very similar to that of background mutations in the *rpsL* transgenic mice. This spectrum difference among more and less proliferating organs was confirmed by the predominant occurrence of G:C to A:T transitions in fetal liver cells exposed to transplacental MNU treatment. In addition, most (approximately 90%) of the G:C to A:T transitions induced by MNU were detected in the first nucleotide of some 5′-G-(C or G)-3′ sequences, many of which corresponded to the middle guanine residue of 5′-purine-G-(C or G)-3′ sequences. It is thus suggested that at particular sites, the neighboring bases in both the 5′ side and 3′ side seem to influence either the susceptibility to DNA damage or the ability to repair MNU-induced lesions.

Key words:    Transgenic mouse — *rpsL* gene — Shuttle vector — Somatic mutation — *N*-Methyl-*N*-nitrosourea

Chemically induced or spontaneous tumors in various species and organs have mutations in oncogene(s) and/or suppressor oncogene(s). Therefore, it is very important to devise a rapid and easy method of detecting somatic muta-tions *in vivo* in mammals. Transgenic mice carrying an *Escherichia coli* shuttle plasmid vector with a mutation-monitoring marker gene are very useful for *in vivo* mutagenesis studies in mammals. Several transgenic mouse systems have been developed and reported.[1–5] Two transgenic systems, Mutamouse[1] and Big Blue,[2–4] have been well characterized and widely used.[6–9] They are color-screening systems using lambda phage (λ) shuttle vectors that consist of the *E. coli lac*Z gene (Mutamouse) or *lac*I gene (Big Blue) as a mutation monitor gene. In order to increase the sensitivity and to reduce the labor-intensive work involved, several positive detection sys-tems have recently been developed for *in vivo* mutagenesis assays.[10–15]

The *rpsL* (*strA*) transgenic mouse is a novel positive detection system using a plasmid shuttle vector pML4.[14]

The wild-type *rpsL* gene carries the dominant streptomy-cin-sensitive (Sm$^S$) phenotype. The shuttle vector pML4 consists of the *rpsL* gene, kanamycin resistance gene (Kan$^R$) and replication origin. In order to estimate the mutation frequencies using the *rpsL* transgenic mouse sys-tem, the shuttle vector is rescued from the transgenic genome by restriction and self-ligation and then is intro-duced into the appropriate *E. coli* host cells by electropora-tion. A part of the transformed *E.coli* cells are spread onto plates containing kanamycin (Km) and all the rest onto plates containing both Km and Sm. The ratio of the num-ber of the Km$^R$ and Sm$^R$ colonies to that of the Km$^R$ colo-nies is thus a measure of the mutation frequencies.[14] In addition to this simplified detection system for *in vivo* mutations, studies on the mutation spectra of the monitor gene are much easier in the *rpsL* transgenic mouse than in other transgenic systems. Firstly, the coding sequence of the *rpsL* gene (375 bp) is much smaller than that of the *lac*I gene (about 1 kb) or the *lac*Z gene (about 3 kb). Sec-ondly, since the shuttle vector is a plasmid DNA in the *rpsL* transgenic system, it can be directly used as a sequencing template. Mutation spectrum studies of the *rpsL* gene have also been conducted on *E. coli*.[16]

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Transgenic mouse models allow us to analyze the mutations of various organs in the same mouse. This is one of the greatest advantages of transgenic systems. Therefore, by using transgenic systems, it is feasible to determine the tissue specificity of spontaneous or induced mutations. *N*-Methyl-*N*-nitrosourea (MNU) is an alkylating agent, which has been shown to induce G:C to A:T transitions by forming $O^6$-methylguanine adducts in prokaryotic and eukaryotic cells, probably through the mismatch pairing of $O^6$-methylguanine with thymidine during DNA replication.[17–19] It has been reported that mutation frequencies were increased by MNU in a tissue-specific manner by using MNU-treated *lac*I transgenic mice and that the predominant mutations in MNU-treated mice were G:C to A:T transitions.[20, 21] We herein describe MNU-induced mutations in various organs of not only adults but also fetuses of the *rpsL* transgenic mice. The mutation frequencies were increased in a tissue-specific manner, as reported,[20, 21] but two distinctive patterns of mutation spectrum, dependent on the rate of the cell division, were observed for the first time.

## MATERIALS AND METHODS

**Transgenic mice**  One of the *rpsL* transgenic lines, HIT017[14] was used. HIT017 carries approximately 350 copies of the pML4 shuttle plasmid vector hemizygously in the haploid genome with a form of head-to-tail tandem arrangement.[14] The HIT017 founder male mouse was crossed and propagated with nontransgenic (C57BL/6J×DBA/2J)F1 (BDF1) female mice. The *rpsL* hemizygous transgenic offspring were identified by Southern blot analyses as previously described.[14] Only the transgenic mice were used for the subsequent mutagenesis analyses.

**Mutagen treatments and tissue isolation**  MNU (Nakalai Tesque Inc., Kyoto) was resolved in a 0.1 *M* citrate buffer, pH 6.0 just prior to administration. When the transgenic mice were killed, all organs were immediately frozen in liquid nitrogen and stored at −80°C. They were thawed only once just prior to DNA isolation as described below.

**Treatment of adult mice**  Pairs of one male and one female mouse, about 8 weeks of age, were treated daily with MNU (0, 10 or 40 mg/kg body weight) intraperitoneally for three consecutive days. Six mice were killed two weeks after the MNU treatment, and the brain, thymus, lung, liver and spleen from each mouse were isolated.

**Treatment of fetuses**  In order to investigate the mutation frequencies in fetuses, 20 BDF1 oocytes were *in vitro* fertilized with a male HIT017 hemizygous transgenic mouse and transferred into the oviducts of pseudo-pregnant ICR female mouse. Two such pregnant ICR female mice were treated intraperitoneally with a single dose of MNU (0 or 75 mg/kg body weight) on day 9.5 of gestation. The fetuses were obtained from the pregnant female mice on day 18.5 of gestation. The liver and brain were isolated. The genotype of each fetus was determined by a Southern blot analysis of the DNA extracted from their limbs and tail. Two and three transgenic fetuses were obtained from the pregnant mice treated with MNU (0 and 75 mg/kg), respectively.

**Genomic DNA preparation**  The detailed procedures of genomic DNA preparation for electroporation have been described previously.[14] The genomic DNA was digested with restriction enzyme, *Ban*II (TaKaRa, Tokyo), in order to excise the integrated shuttle vector at unit size. The *Ban*II-digested DNA was then treated with T4 DNA ligase to self-circularize the transgene.[14]

**Mutagenesis assay**  The detailed procedures for the mutagenesis assay and the calculation of the mutation frequency have also been described previously.[14] Briefly, the whole transgenic DNA after *Ban*II- and T4 DNA ligase treatments was transformed into *E.coli*, RR1 [*supE44, hsds20, ara-14, proA2, lacY1, galk2, rpsL20, xyl-5, mtl-1*] by electroporation with Cell Porator *E. coli* System (Gibco-BRL, Rockville, MD) according to the manufacturer's instructions. After expression in the SOC medium, a small aliquot of the SOC culture was plated onto LB agar plates containing Km (50 $\mu$g/ml) while the rest was plated onto LB plates containing both Km (50 $\mu$g/ml) and Sm (100 $\mu$g/ml).

**Sequencing**  Each mutant colony ($Sm^R$ and $Km^R$) was independently transferred to 1 ml of fresh TB broth[22] containing Km (final concentration of 50 $\mu$g/ml) in each well of a 24-well dish (Corning, Acton, MA). The dish was incubated in a moisture chamber at 37°C overnight under 100 rpm rotation to amplify the plasmid DNA. Then the plasmid DNA was extracted by the alkaline lysis method.[22] The extracted plasmid DNA was suspended in 100 $\mu$l of TE (10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0). To the 50 $\mu$l of plasmid DNA suspension, 0.5 $\mu$l of 1 mg/ml RNase (Sigma, St. Louis, MO) was added and the mixture was incubated for 5 min at room temperature. The DNA was then precipitated at 0°C by adding 30 $\mu$l of the PEG (20% polyethylene glycol, 2.5 *M* NaCl) solution and the pellet after centrifugation was rinsed with 1 ml of 70% ethanol. The DNA was resuspended in 15 $\mu$l of TE. Then, 5 $\mu$l of the DNA (approximately 1 g) was used as a sequencing template for amplification with a dideoxyterminator cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The DNA sequences were determined by using a 373A Autosequencer (Applied Biosystems, Foster City, CA). The primer DNAs for the sense and the antisense strands were oligonucleotides extending from positions −36 to −17 (5′-GTTTACGAAGCAAAAGCTAA-3′)[16] and +433 to +416 (5′-GGCATGGAAATACTCCGTTG-3′) of the *rpsL* sequence, respectively. For the analysis of the promoter

region, an oligonucleotide from position −263 to −243 (5′-GCGCACATTTCCCCGAAAAGT-3′) was also used as a primer for the sense strand.

## RESULTS

**MNU treatment in adult mice** MNU administration to adult *rpsL* transgenic mice induced mutations in a dose-dependent manner, except in the brain. The mutation spectrum study showed that G:C to A:T transitions, which were expected to be induced by MNU administration, were predominantly observed in the spleen, thymus and lung, but not in the liver. The results for each organ are described below.

**Spleen, thymus and lung** The average mutation frequencies in adult mice, that were treated with 40 mg/kg/day for three consecutive days and assayed two weeks after the administration, were $17.1\pm1.0\times10^{-5}$, $12.6\pm1.9\times10^{-5}$ and $11.2\pm0.3\times10^{-5}$ in the spleen, thymus and lung, respectively (Table I). Compared to the untreated controls, the

mutation frequencies were increased by approximately 3-fold, 8-fold and 3-fold in spleen, thymus and lung, respectively (Table I). The mutation frequencies were significantly different between the treated and untreated mice (spleen, $t=5.56$, d.f. (degree of freedom)=2, $P<0.05$; thymus, $t=7.73$, d.f.=2, $P<0.02$; lung, $t=8.88$, d.f.=2, $P<0.02$), in spite of the small sample sizes. The overall mutation frequencies increased dependent on the amount of MNU administered, as shown in Fig. 1. Furthermore, the mutation spectra were studied by the direct DNA sequencing of the whole *rpsL* coding sequence in the shuttle vector from randomly chosen mutant colonies on the Km and Sm plates. The predominant mutations in the MNU-treated mice were G:C to A:T transitions, accounting for 66.7% (22/33), 73.7% (14/19) and 59.1% (13/22) in the spleen, thymus and lung, respectively (Table II). On the other hand, more than half of all mutations derived from the untreated controls were frameshift mutations caused by a single base deletion, that accounted for 52.9% (9/17), 42.9% (3/7) and 61.5% (8/13) in the spleen, thy-

Table I. Mutation Frequencies (MF) in the *rpsL* Gene of pML4 Shuttle Vector Rescued from Adult and Fetal Organs after MNU Treatment

| Organs | MNU dose (mg/kg)×days | No. of vectors screened (×10⁵)[a] | No. of mutants | Average MF±SD (×10⁻⁵) |
|---|---|---|---|---|
| Spleen | 0 | 5.956 | 27 | 5.1±2.9 |
| | 10×3 | 4.633 | 30 | 6.4±0.5 |
| | 40×3 | 5.459 | 95 | 17.1±1.0 |
| Thymus | 0 | 5.927 | 8 | 1.6±0.6 |
| | 10×3 | 7.358 | 19 | 2.6±2.1 |
| | 40×3 | 5.169 | 68 | 12.6±1.9 |
| Lung | 0 | 5.994 | 22 | 3.5±1.2 |
| | 10×3 | 4.611 | 22 | 4.2±3.5 |
| | 40×3 | 2.946 | 33 | 11.2±0.3 |
| Liver | 0 | 4.466 | 29 | 6.3±0.8 |
| | 10×3 | 4.212 | 56 | 13.6±3.2 |
| | 40×3 | 2.032 | 51 | 25.0±2.9 |
| Fetal liver | 0 | 3.284 | 21 | 6.3±0.4 |
| | 75×1 | 2.604 | 89 | 24.8±19.0 |
| Brain | 0 | 2.308 | 15 | 6.9±3.2 |
| | 10×3 | 3.058 | 15 | 4.8±2.3 |
| | 40×3 | 3.437 | 23 | 6.3±1.5 |
| Fetal brain | 0 | 3.609 | 11 | 3.6±1.9 |
| | 75×1 | 7.863 | 32 | 4.3±2.7 |

*a*) The screened number of pML4 shuttle vectors was estimated from the number of Km^R colonies multiplied by the dilution ratio[14]. The mean of the counted Km^R colonies per one tested organ was 1190.9 (at least 219).
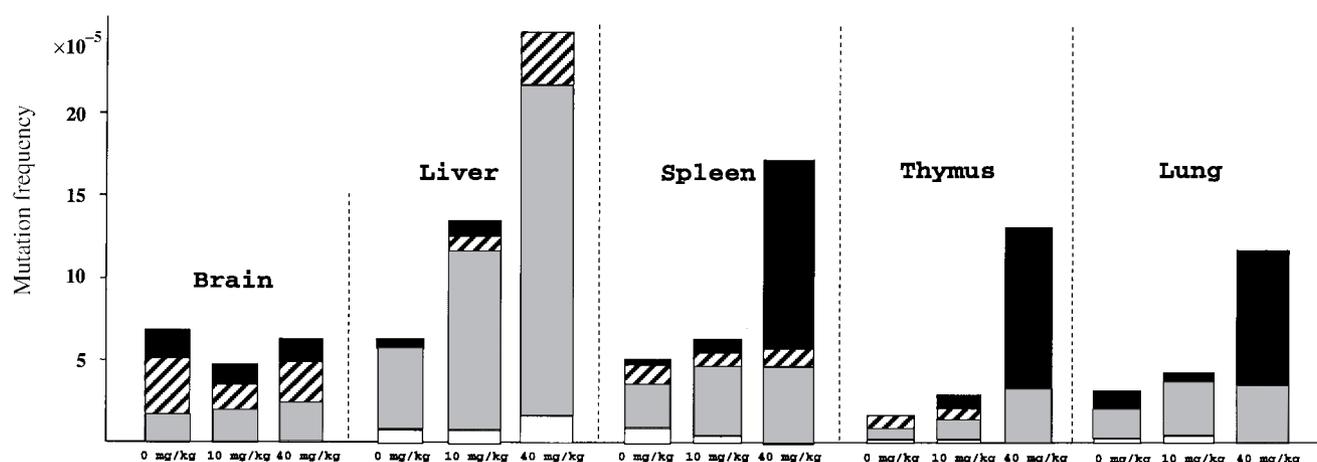
Fig. 1. Schematic presentation of the mutation frequencies and spectra in the MNU mutagenesis study for the spleen, thymus, lung, liver and brain of adult mice. In this figure, the proportion of each mutational type is schematically shown in the bar that indicates the mutation frequency for each organ analyzed. The mutation frequencies and spectra are derived from Tables I and II, respectively.

Table II. Mutation Spectra of Adult Organs Two Weeks after MNU Treatment

| Mutation class | Untreated control mice | | | | | MNU-treated mice (40 mg/kg×3 days) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Spleen | Thymus | Lung | Liver | Brain | Spleen | Thymus | Lung | Liver | Brain |
| Base substitution | 29.4% | 42.9% | 30.8% | 8.3% | 75.0% | 72.7% | 73.7% | 63.6% | 12.9% | 61.1% |
| G:C→A:T | 1 | 0 | 4 | 2 | 4 | 22[a] | 14[a] | 13 | 0 | 4 |
| Others | 4 | 3 | 0 | 0 | 8 | 2 | 0 | 1 | 4 | 7 |
| Single base deletion | 52.9% | 42.9% | 61.5% | 83.3% | 25.0% | 27.3% | 26.3% | 36.4% | 77.4% | 38.9% |
| | 9 | 3 | 8 | 20 | 4 | 9 | 5 | 8 | 24 | 7 |
| Two or more base deletions | 17.6% | 14.3% | 7.7% | 8.3% | 0% | 0% | 0% | 0% | 9.7% | 0% |
| | 3 | 1 | 1 | 2 | 0 | 0 | 0 | 0 | 3 | 0 |
| Total | 17 | 7 | 13 | 24 | 16 | 33 | 19 | 22 | 31 | 18 |

The mutation spectra from the untreated mice and mice treated with 40 mg MNU/kg/day for three days are summarized. The percentages of the mutation classes are indicated above the numbers of mutations.
a) $P<0.005$ (spleen, $\chi^2=16.7$, d.f.=1; thymus, $\chi^2=11.2$, d.f.=1) in comparison with untreated control mice. A $P$ value of less than 0.05 means that the proportion in the total mutations was statistically significantly different.

mus and lung, respectively (Table II). It is noteworthy that such single base deletions of the *rpsL* gene were rarely observed in the mutagenesis studies of *E. coli*.[16]

**Liver** As summarized in Table I, the average mutation frequency in MNU-treated adult mouse liver was also significantly increased by approximately 4-fold in comparison with untreated controls ($t=8.81$, d.f.=2, $P<0.02$). In spite of the significant induction of mutations by MNU, the mutation spectrum was rather similar to that of the untreated controls; namely, the predominant mutations were single base deletions in both the control mice (83.3%, 20/24) and the MNU-treated mice (77.4%, 24/31) (Table II).

**Brain** The average mutation frequency in the adult mouse brain was $6.3\pm1.5\times10^{-5}$ in MNU-treated mice (Table I). Compared to the controls, there was no significant increase of mutation frequency ($t=0.24$, d.f.=2, $P>0.5$). The mutation spectrum was also similar between the control mice and the MNU-treated mice; the G:C to A:T transitions accounted for only 25.0% (4/16) and 22.2% (4/18) in the control mice and the MNU-treated mice, respectively (Table II).

**MNU treatment in fetuses** Compared to the untreated controls, the increase in average mutation frequency of the MNU-treated fetal liver was approximately 4-fold, although this was not statistically significant ($t=1.69$,

Table III. Mutation Spectra of Fetal Organs with MNU Treatment

| Mutation class | Untreated fetuses | | MNU-treated fetuses | |
|---|---|---|---|---|
| | Liver | Brain | Liver | Brain |
| Base substitution | 44.4% | 68.8% | 93.3% | 93.1% |
| G:C→A:T | 0 | 2 | 14[a] | 21[a] |
| Others | 4 | 9 | 0 | 6 |
| Single base deletion | 44.4% | 25.0% | 6.7% | 6.9% |
| | 4 | 4 | 1 | 2 |
| Deletion two or more bases | 0% | 6.3% | 0% | 0% |
| | 0 | 1 | 0 | 0 |
| Addition | 11.1% | 0% | 0% | 0% |
| | 1 | 0 | 0 | 0 |
| Total | 9 | 16 | 15 | 29 |

This table shows the mutation spectra of the analyzed mutations of the liver and brain from two untreated fetuses and three MNU-treated fetuses.

a) $P<0.005$ (liver, $\chi^2=20.1$, d.f.=1; brain, $\chi^2=14.8$, d.f.=1) in comparison with the untreated fetuses.

d.f.=3, $P>0.10$) (Table I). An analysis of the spectrum showed a drastic increase of G:C to A:T transitions (93.3%, 14/15) in comparison with the untreated controls (0%, 0/9) (Table III). In the MNU-treated fetal brain, the increase in the mutation frequency was small, but the majority of the mutations were G:C to A:T transitions (72.4%, 21/29) in contrast with the untreated controls (12.5%, 2/16) (Table III). The increases in the G:C to A:T transitions by MNU were statistically significant with $P<0.005$ both in fetal liver ($\chi^2=20.1$, d.f.=1) and fetal brain ($\chi^2=14.8$, d.f.=1). These mutation spectra thus indicate a drastic difference of MNU mutagenesis between the adult and fetus in the liver and the brain.

**Distribution and incidence of MNU-induced G:C to A:T transitions** The MNU-induced G:C to A:T transitions in the spleen, thymus and lung of adult mice and the fetal liver and brain described above are summarized in Fig. 2. The 84 G:C to A:T transitions were distributed at 12 sites of the *rpsL* sequences. Seven of the 12 sites corresponded to the first nucleotide of 5′-G-(C or G)-3′ with 92.9% (78/84) of G:C to A:T transitions. Of these, 78.2% (61/78) were observed at positions that corresponded to the middle guanine residue in 5′-purine-G-(C or G)-3′,
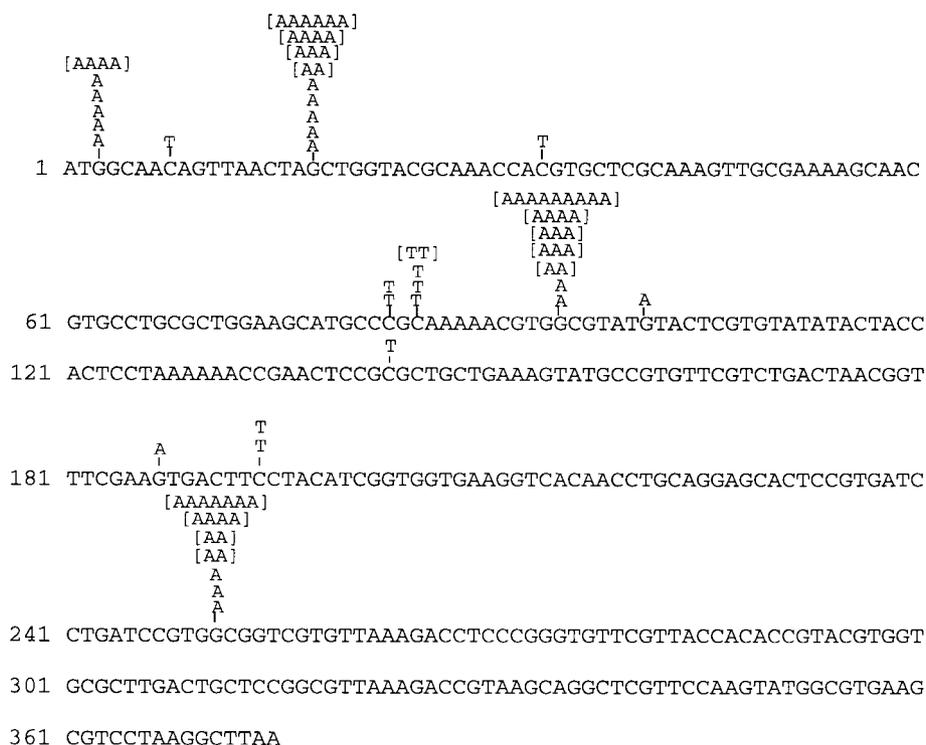


Fig. 2. The distributions and incidences of MNU-induced G:C to A:T transitions. G:C to A:T transitions derived from the spleen, thymus and lung of adult mice treated with 40 mg MNU/kg/day, and from the fetal liver and brain treated with 75 mg MNU/kg are depicted. The nucleotide positions are shown on the left of the sequence, with 1 being the first position of the start codon (ATG) and 375 being the third position of the termination codon (TAA). The mutations derived from the same organ of one mouse, namely, potentially clonally expanded mutations, are grouped in brackets [ ].

such as positions 18, 95 and 251. Thus, these positions seem to be mutation "hot spots" for MNU. Another possibility would be the clonal expansion of a few founder mutations due to cell division during the two week post-treatment period. If all the identical DNA sequence changes observed in one organ of the same mouse were derived from one founder mutation, only 42 founder mutations would have given rise to the 84 G:C to A:T transitions. The putative founder mutations are indicated in brackets in Fig. 2. In this case, 85.7% of the founder mutations (36/42) were still observed at 5'-G-(C or G)-3', and 23 of them corresponded to the middle guanine residues in 5'-purine-G-(C or G)-3'. Thus, even if we assume that the identical mutations were the consequence of expansion from one mutation, there remain evident "hot spots" for MNU-induced mutagenesis. It is noteworthy that, among background mutations, a total of 13 G:C to A:T transitions were observed at 8 sites in the *rpsL* sequence (Fig. 3). Five of the 8 sites corresponded to the first nucleotide of 5'-G-(C or G)-3', and 76.9% of all the G:C to A:T transitions (10/13) in background mutations (if clonal expansions were considered, 72.7% or 8/11) were observed at

these five sites. Four (position 3, 18, 83, and 251) of the 5 sites were the same as the positions at which the MNU-induced mutations were detected (see Fig. 2 and Fig. 3).

**Spectra of the background mutations** In the spleen, thymus, lung and liver, single base deletions were predominant among the mutations from the untreated mice (background mutations). In untreated adult and fetal brains, base substitutions were a major mutational type and consisted of 12/16 (75.0%) and 11/16 (68.8%), respectively, but single base deletions still accounted for a significant portion (25%) (Table II and Table III). Among all the background mutations, single base deletions comprised 51.0% (52/102). The next most common mutational type was base substitutions (40.1%, 41/102), and there were a few deletions of two or more bases (7.8%, 8/102). The distribution is shown in Fig. 3 for these single base deletions and base substitutions. Single base deletions were widely distributed in 27 sites of the *rpsL* sequence. Eighteen of 27 sites (66.7%) corresponded to sequences with a run of several identical residues; for example, at positions 127–132 (5'-AAAAAA-3') and 270–272 (5'-CCC-3'). Base substitutions, 23 transitions and 18 transversions,
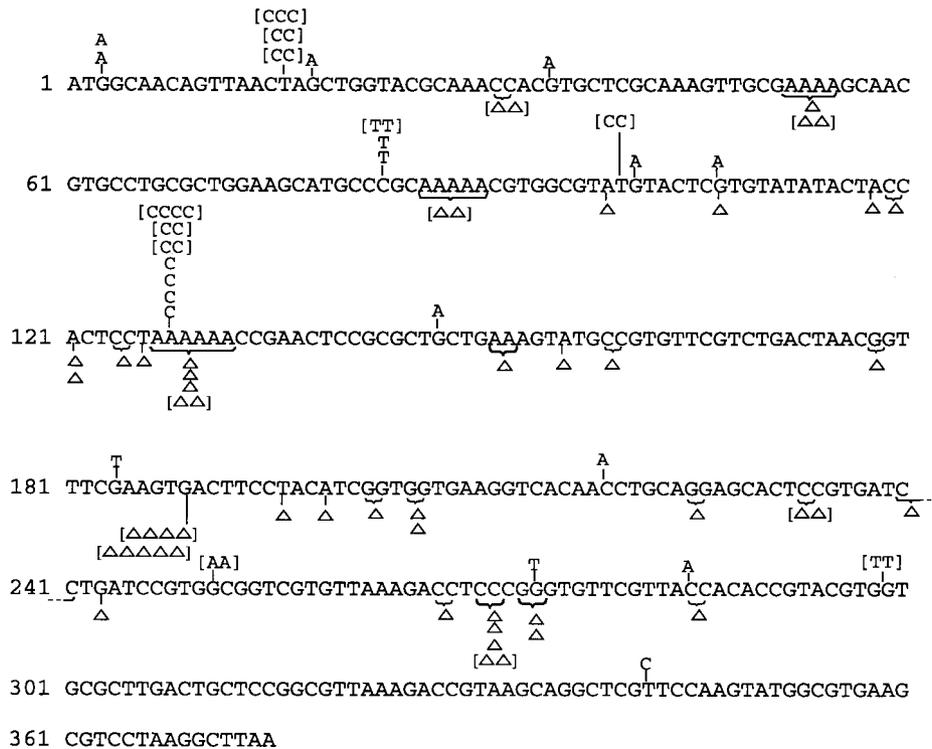


Fig. 3. Distribution of base substitutions and single base deletions in the background mutations. All background mutations except for deletions of two or more base pairs are shown. The mutations are derived from Tables II and III. The nucleotide positions are shown on the left of the sequence, with 1 being the first position of the start codon (ATG) and 375 being the third position of the termination codon (TAA). The sequence changes due to base substitutions are shown above the *rpsL* sequence. Single base deletions are shown as ( △ ) below the sequence. Mutations derived from the same organ of one mouse, namely, potentially clonally expanded mutations, are grouped in brackets [ ].

were identified at 17 sites in the *rpsL* sequence. Interestingly, all 12 A:T to C:G transversions were identified at position 128, and seven of them concomitantly exhibited A:T to G:C transitions at position 16. Even considering clonal expansions, seven founder mutations were identified at position 128 and three founders of "double mutations" at both position 16 and 128 were observed.

In eight deletions of two or more bases, the numbers of deleted nucleotides were 2 bases (3/8), 3 bases (1/8), 4 bases (1/8), 15 bases (1/8) and 194 bases (2/8). In six of the 8 mutations, there were short direct repeats (i.e., 5′-CGCAAA-3′ and 5′-CGTG-3′) flanking the deleted sequences, as if homologous recombination between the direct repeats had caused the deletion (data not shown).

## DISCUSSION

In *rpsL* transgenic mice, we could positively detect *in vivo* somatic mutations, and subsequently characterize the mutational spectra by direct sequencing. We found clear differences in MNU mutagenicity among the tested organs, in the frequency as well as the spectrum. The mutation frequencies of MNU-treated mice were significantly increased in a dose-dependent manner, except in the brain, as has been reported.[20, 21] The findings in the brain were also in agreement with those of MNU mutagenesis studies using *lac*I transgenic mice.[20, 21] Since MNU is believed to be distributed efficiently to all tissues including the brain,[23, 24] the small response in the brain cannot be explained by the exposure level.

In the proliferating organs including the fetal liver and the brain, the predominant mutations were G:C to A:T transitions, as expected from previous studies. MNU is known to induce G:C to A:T transitions in *E. coli*[25, 26] and in eukaryotic cells *in vitro*[27, 28] as well as *in vivo*.[20, 21] On the other hand, the MNU-induced mutations detected in the less proliferating organs in this experiment, such as the adult liver, were predominantly single base deletions, which were also the major mutational type among the background mutations found in untreated *rpsL* transgenic mice. We thus conclude that cell division is necessary for the induction and fixation of G:C to A:T transition by MNU. This view is supported by the mutation spectra of the *rpsL* transgenic mice treated with a much higher dose of MNU with a minimum time of incubation. In a previous study,[14] 100 mg/kg MNU was administered to the *rpsL* transgenic mice for 3 consecutive days. The brain, liver and spleen were immediately retrieved and tested. In this case, all the organs including the spleen exhibited the same mutation pattern as the background mutations (data not shown).

As regards the nature of single base deletions found in background mutations as well as in MNU-treated less-proliferating organs, two possibilities can be considered.

DNA methylations are primarily repaired by methyltransferase.[29, 30] When the degree of methylation overwhelms the activity of methyltransferase, the nucleotide excision repair (NER) system seems to operate.[31, 32] Thus, the first possibility is that single base deletions may result from NER, which might include error-prone DNA replications. The other possibility is that single base deletions might reflect degradation of genomic DNA in the damaged cells. When DNA damaged by methylation far exceeds the NER capacity, such cells are eliminated from the organism by apoptosis, causing DNA fragmentation and cell death.[33–35]

Predominance of frameshift mutations among the background mutations has also been reported in several other studies. For instance, frameshift mutations were predominantly observed in the *gpt* gene of a chromosomally integrated shuttle vector[36] and human *hprt* cDNA integrated in mouse chromosomal DNA[37] as background mutations. In contrast, the major background mutational events are base substitutions in such transgenic systems as the Mutamouse[1] and Big Blue,[4] and in the endogenous *aprt* gene of cultured CHO cells.[38] Thus, the reporter gene used may influence the mutational spectra. Indeed, the pattern of mutation varies even within the *lac*I gene in *E. coli*; for example, the ratio of base substitution to deletion/insertion mutation is 1.4:2 in the amino-terminal DNA binding domain, whereas deletion/insertion is increased by approximately 14-fold (if hotspots of frameshift mutation are deleted, approximately 2.5-fold) in the remaining domains.[9, 39] However, other factors, including the effect of chromosomal integration sites of monitor genes and the number of copies of transgenes, may also affect the spectrum of mutations.

It must be noted that the background mutation spectra in the *rpsL* transgenic mouse system were different from the spectra of the *rpsL* mutations in the *E. coli* system[16]; about half of *rpsL* mutations in *E. coli* were insertions of a transposon and almost all of the others were base substitutions. The difference in the mutational spectra between mice and *E. coli* suggests that the background mutations in our transgenic system are not likely to be artifacts, but probably represent authentic changes in the mouse DNA. Furthermore, we confirmed that the *ex vivo* artifact during enzymatic manipulation to retrieve the shuttle vectors from mouse genome was minimal; details of this finding will be described elsewhere (Gondo, Shioyama and Katsuki, in preparation).

The difference of mutation spectra between this study and previous studies using the Big Blue system[20, 21] might be due to the choice of the rescue method, selection system, and/or monitor gene. Recently, by using another transgenic system with *gpt*-based positive selection in a λ shuttle vector, about half of the background mutations were found to be short deletions/insertions, whereas eth-

ylnitrourea-induced mutations were base substitutions,[40] which is very similar to our results. Therefore, it is necessary to conduct more extensive mutation spectrum studies to elucidate the nature of these mutation assay systems by using various transgenic systems.

Approximately 90% of MNU-induced G:C to A:T transitions are located at the first guanine residue of 5′-G-(C or G)-3′ in the *rpsL* sequence, especially at sites corresponding to the middle guanine residue in 5′-purine-G-(C or G)-3′. A preference for the 5′-purine-G-3′ sequence in MNU- or MNNG (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine)-induced mutations was previously reported in eukaryotic cells[28, 41, 42] and in *E. coli*.[43, 44] Furthermore, the site specificity for the middle G in the 5′-purine-G-N-3′ sequence has been shown to be due primarily to the preferential MNU-reaction at this site to produce $O^6$-methylguanine.[45] Our results are not only consistent with their previous studies, but also suggest that the 3′-flanking base may influence the mutability as well. In our case, the 3′-flanking base may affect the ability to repair rather than the susceptibility to DNA damage, because many G:C to A:T transitions (60–70%) among the background mutations were also observed at the first guanine residue of 5′-G-(C or G)-3′. However, the sequence 5′-G-(C or G)-3′ or 5′-purine-G-(C or G)-3′ seems to be a necessary but not a sufficient condition for hotspots of G:C to A:T transition induced by MNU, because there were many such sequences in which G:C to A:T transitions were not induced in the *rpsL* sequence.

Above all, the target organs of the MNU-induced base-substitutions described here and those of MNU-induced carcinogenesis[46–51] seem to be well correlated. The thymus,[46–48] lung[48, 49] and forestomach[49] are known to be labile target organs of MNU-induced tumorigenesis in the mouse. In addition, a partial hepatectomy study with MNU treatment induced hepatocarcinogenesis in the rat.[50] Various neoplasia were also induced by transplacental exposure to MNU in the rat.[51]

The *rpsL* transgenic system has been proven to be a powerful tool in the study of *in vivo* mutagenesis. In particular, the results in the fetal liver clearly indicated that an increased proportion of mutations compared with the control was G:C to A:T transitions (Fig. 1). Such a sensitive analysis has become possible because of the quick and easy identification of many mutations by streptomycin selection and the availability of a mass sequencing system to analyze such mutations.

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