Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 6468–6472, July 1993
Medical Sciences

O^6-Methylguanine-DNA methyltransferase protects against nitrosamine-induced hepatocarcinogenesis

(DNA repair/liver tumor/transgenic mouse/*ada* gene)

YOKO NAKATSURU*, SHOICHI MATSUKUMA†, NOBUO NEMOTO‡, HARUO SUGANO§, MUTSUO SEKIGUCHI¶, AND TAKATOSHI ISHIKAWA*‖

*Department of Pathology, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan; Departments of †Cell Biology, ‡Experimental Pathology, and §Pathology, Cancer Institute, Toshima-ku, Tokyo 170, Japan; and ¶Department of Biochemistry, Kyushu University, Fukuoka 812, Japan

Communicated by Richard B. Setlow, April 8, 1993

ABSTRACT **We previously generated transgenic C3H/HeN mice by introducing the *Escherichia coli* O^6-methylguanine-DNA methyltransferase (MGMT, DNA-O^6-methylguanine:protein-L-cysteine *S*-methyltransferase, EC2.1.1.63) gene, *ada*, attached to the Chinese hamster metallothionein I gene promoter. One transgenic mouse line expressing both *ada*-specific mRNA and Ada protein could be propagated over many generations in a homozygous state with respect to the integrated DNA. Liver extracts from transgenic homozygous mice have consistently demonstrated about 3 times the control activity of normal mice. Furthermore, in the transgenic homozygotes treated with $ZnSO_4$, activity is increased to 6–8 times the normal level in mice and is equivalent to that for man. To examine whether these increased levels of MGMT activity can actually decrease the susceptibility of animals to *N*-nitroso compounds, we studied liver carcinogenesis in our transgenic mice expressing high amounts of MGMT. Groups of transgenic and nontransgenic mice, each comprising about 200 suckling animals (14 ± 1 days old), were divided each into eight subgroups, providing paired groups of transgenic and nontransgenic mice. They received an i.p. injection of $ZnSO_4$ to induce MGMT, and 10 hr thereafter were given an i.p. injection of either dimethylnitrosamine or diethylnitrosamine. Liver tumor development was quantitatively assessed at 7–11 months. Here, we report statistically significant reduction of tumor formation in transgenic mice of four of the six paired groups that received treatment. The remaining two demonstrated results in line with dose dependence. Therefore, our data indicate that MGMT can indeed protect animals from low-dose exposure to environmental alkylating carcinogens.**

While xeroderma pigmentosum (XP) is regarded as a convincing human example showing a link between cancer proneness and DNA excision–repair deficiency (1, 2) we do not have any equivalent appropriate animal models to study for clarification of this important area.

Alkylating carcinogens present in the environment produce various kinds of alkylated purine and pyrimidine bases in DNA (3, 4), O^6-methylguanine being regarded as one of the most potent premutagenic lesions. It preferentially pairs with thymine rather than with cytosine, resulting in a G·C to A·T transition mutation (3, 4). This O^6-methylguanine-DNA adduct can be repaired by the enzyme O^6-methylguanine-DNA methyltransferase (MGMT; DNA-O^6-methylguanine:protein-L-cysteine *S*-methyltransferase, EC 2.1.1.63) (5–10), which transfers a methyl group from the O^6-methylguanine moieties of double-stranded DNA to a cysteine residue of the enzyme molecule MGMT itself (11). MGMT can also repair other O^6-alkylguanines, such as O^6-ethylguanine or O^6-butylguanine, although at reduced efficiency. There is evidence from animal and cell culture systems indicating that repair of O^6-alkylguanine protects cells from malignant conversion. For example, carcinogenic *N*-alkyl-*N*-nitrosoureas are known to induce tumors preferentially in tissues with low MGMT activity (12–16), and upon exposure to ethylnitrosourea *in vitro*, rodent cell variants with low MGMT activity undergo malignant conversion with much higher frequency than their high MGMT activity counterpart cells (17). The presence of MGMT proteins has been demonstrated in various organisms including bacteria, yeast, fish, rodents, monkeys, and humans (8, 18–20). The levels of MGMT activity vary greatly among species and also between tissues, the liver having the highest enzyme activity. Enzyme activity is generally several times higher in humans than in rodents (14) and regulated at appreciable levels throughout the lifetime (21).

We previously generated transgenic mice (22) by introducing the *Escherichia coli* MGMT gene, *ada* (9), attached to the Chinese hamster metallothionein I gene promoter. One transgenic mouse line expressing both *ada*-specific mRNA and Ada protein, which could be propagated in a homozygous state with respect to the integrated DNA, has proved highly reproductive over many generations (23). Liver extracts from these transgenic homozygous mice have consistently demonstrated about 3 times the control activity of normal C3H mice. Furthermore, their levels of enzyme activity can be increased up to about 8 times after treatment with zinc, since the metal-responsive metallothionein promoter is attached to the *ada* gene (23). Recently other groups have also reported the production of transgenic mice expressing the *ada* chimeric gene (24, 25) or the human MGMT gene in the liver (26). This raises the interesting possibility of directly examining whether increased levels of MGMT activity can actually decrease the susceptibility of animals to *N*-nitroso compounds for tumor induction using such animals.

In the present investigation, liver carcinogenesis was studied in our transgenic mice expressing high amounts of the *E. coli* gene, *ada*. Here, we report that the transgenic mice do demonstrate significantly reduced rates of development of liver tumors after treatment with dimethylnitrosamine (DMNA) or diethylnitrosamine (DENA), indicating that MGMT can indeed protect animals against nitrosamine-induced hepatocarcinogenesis.

MATERIALS AND METHODS

Characterization of Transgenic Mice. Characterization of our transgenic mice has been reported in detail elsewhere (22, 23). Founder mice of the C3H/HeN strain were obtained

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: DENA, diethylnitrosamine; DMNA, dimethylnitrosamine; MGMT, O^6-methylguanine-DNA methyltransferase.

‖To whom reprint requests should be addressed.

from Japan SLC laboratory (Hamamatsu-shi, Japan). Briefly, four transgenic C3H/HeN offspring with integrated chimeric genes, composed of the *E. coli ada* coding sequence and Chinese hamster metallothionein I gene promoter, were identified. Germ-line transmission was confirmed for two lines, and one of these, no. 708, was selected for further characterization. The chimeric gene copy number in the no. 708 lineage was estimated to be about 100, and Southern blot analysis indicated that the injected DNA was tandemly rejoined in the same orientation at the site of integration. The transgenic mice used in these studies were bred and maintained in our laboratory as a homozygous colony with respect to the integrated *ada* gene, and no reduction in survival, growth, or fecundity was apparent as compared with nontransgenic mice. Liver extracts from transgenic homozygotes showed ≈3 times the control MGMT activity, with a marked increase to about 8 times the nontransgenic control levels being observed at 10 hr and continuing up to 20 hr after zinc treatment (23). All animals were housed in a controlled environment at 23°C and fed on CE-2 diet (CLEA Japan, Tokyo) and water ad libitum.

Production of Suckling Transgenic and Nontransgenic Mice. Transgenic and nontransgenic (normal) mice groups of about 200 suckling mice (C3H/HeN strain) each were used in the present experiments. Parent transgenic mice were checked for gene integration before mating, and several litter mice were confirmed for MGMT activity as described in our previous paper (23). To standardize the experimental conditions, sufficient numbers of suckling mice 14 ± 1 days old, including both sexes, were pooled to constitute eight paired groups of transgenic and nontransgenic mice, and the paired groups were treated concurrently (Table 1).

Carcinogen Treatment. Transgenic and nontransgenic mice (14 ± 1 days old) received an i.p. injection of $ZnSO_4$ (30 mg/kg of body weight) to induce MGMT, and 10 hr thereafter were given an i.p. injection of either DMNA (Tokyo Kasei, Tokyo) (1 or 5 mg/kg), DENA (Tokyo Kasei, Tokyo) (1 or 5 mg/kg) or 0.05 ml of saline (Fig. 1 and Table 1). The 10-hr time span between the two treatments was estimated to be optimal for MGMT expression based on our previous study results (23). DMNA and DENA are both well known as potent hepatocarcinogens, metabolized to active carcinogenic species in the liver. The infant mouse liver has been shown to be particularly sensitive to DMNA or DENA within the first 2 weeks after birth (27, 28). At the ages of 7, 9, and 11 months, groups of animals were sacrificed under anesthesia, these time points being selected based on differences in susceptibilities between male and female mice to the carcinogens. Mice injected with $ZnSO_4$ and saline (groups 1 and 2, Table 1) were common controls for groups 3–8 exposed to DMNA and DENA. Due to the limitation of our animal facilities, the present experiments did not include experimental groups without $ZnSO_4$ pretreatment.

Scoring of Liver Tumors. At necropsy, livers were removed, weighed, and examined for grossly visible lesions. Tumor nodules larger than 1.0 mm in diameter were scored. After fixation in 10% formaldehyde solution, each liver lobe was completely cut into 1.5-mm-thick slices, which were routinely processed for light microscopy.

The term "tumor" is used here without distinction between benign or malignant neoplasms. It was not practical to classify all tumors into adenomas and carcinomas, since there were many borderline cases. However, the numbers of mice bearing unequivocal carcinoma(s) are indicated. Histopathological examination of all slices through liver lobes occasionally revealed tiny adenoma(s) which had escaped gross observation. These examples were also included in the tumor-bearing animal category.

Statistical analyses were made between transgenic and nontransgenic (normal) paired groups (groups 1–8) for numbers of tumor-bearing mice, average numbers of tumors per mouse and number of carcinoma-bearing mice (Table 1). Group differences were assessed for statistical significance by using Student's t test or χ^2 test.

RESULTS

Design for Carcinogenesis Experiments. It is generally known that C3H strain female mice are more refractory than male mice with respect to spontaneous and chemically induced carcinogenesis (29); indeed, male mice produced a few tumors without treatment, whereas under the same conditions, female mice yielded no lesions (groups 1 and 2 in Table 1). Higher concentrations of the carcinogens were required for female mice to obtain the same levels of response attained by male mice. Thus, in the present experiments, two different levels of carcinogens were given to male and female groups.

Table 1. Frequencies of liver tumors in transgenic and nontransgenic (normal) mice after exposure to DMNA or DENA

Treatment				Mice					
	Carcinogen								
Group	*N*-Nitrosamine	Dose, mg/kg	Month of termination	Sex	Type	Exposed, no.	Tumor-bearing, no. (%)	Carcinoma-bearing, no. (%)	Tumors per animal, no. (mean ± SEM)
1	None (saline)	—	11	♀	Normal	38	0 (0)	0 (0)	0
					Transgenic	19	0 (0)	0 (0)	0
2		—	9	♂	Normal	22	3 (14)	0 (0)	0.2 ± 0.5
					Transgenic	21	3 (14)	0 (0)	0.2 ± 0.5
3	DMNA	1	11	♀	Normal	27	6 (22)	0 (0)	0.1 ± 0.5
					Transgenic	25	1 (4)	0 (0)	<0.1
4		5	9	♀	Normal	31	21 (68)*	5 (16)†	1.0 ± 0.9‡
					Transgenic	30	4 (13)*	0 (0)†	0.2 ± 0.6‡
5		1	9	♂	Normal	29	26 (90)*	12 (41)†	4.6 ± 5.4§
					Transgenic	24	9 (38)*	1 (4)†	1.1 ± 2.8§
6		5	7	♂	Normal	25	22 (88)	13 (52)	5.5 ± 4.3
					Transgenic	16	16 (100)	11 (68)	6.8 ± 3.7
7	DENA	5	9	♀	Normal	25	14 (56)¶	1 (4)	0.6 ± 0.7¶
					Transgenic	26	5 (19)¶	0 (0)	0.2 ± 0.4¶
8		1	9	♂	Normal	19	18 (95)	11 (58)†	9.4 ± 5.8‡
					Transgenic	29	25 (86)	7 (24)†	3.7 ± 3.6‡

Significant differences between control and transgenic mice are indicated by the following symbols as superscripts: *, $P < 0.001$; †, $P < 0.05$; ‡, $P < 0.005$; §, $P < 0.01$; ¶, $P < 0.025$.

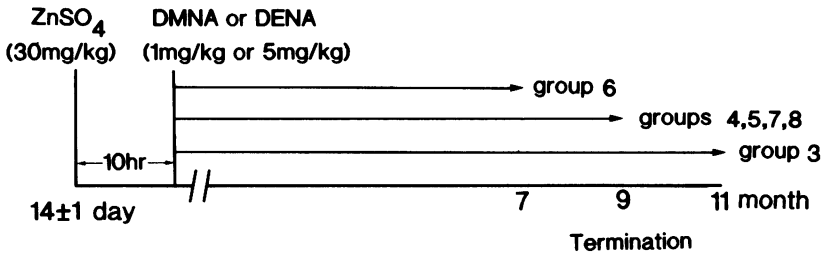

FIG. 1. Protocol for carcinogenesis experiments. To standardize the experimental conditions, sufficient numbers of suckling mice (14 ± 1 days old) were pooled and divided to create eight groups of transgenic and eight groups of nontransgenic mice, providing eight paired groups for concurrent treatment. The mice received an i.p. injection of $ZnSO_4$ (30 mg/kg of body weight) to induce MGMT; 10 hr thereafter the mice were given an i.p. injection of DMNA, DENA, or 0.05 ml of saline. The experimental groups were sacrificed at three different times based on different susceptibilities to the carcinogens of male and female mice.

Although the exact mechanisms for this are still unknown, it has been suggested that high responsiveness of male C3H mice to carcinogenic events may be related to some strain-specific androgen conditions under the control of a hepatocarcinogen sensitivity locus (29).

Tumor Induction by DMNA. When 1 mg of DMNA was administered per kg of body weight, 22% of normal female mice produced tumors, whereas only 4% of transgenic mice yielded tumors (group 3 in Table 1). Although these values are still statistically insignificant, more striking differences between normal and transgenic mice were observed with a group of female mice receiving 5 mg of DMNA per kg (group 4). With respect to both the number of tumor-bearing animals and the average number of tumors per animal, the *ada* transgenic mice showed significantly lower values as compared with those for the normal mice ($P < 0.005$ for the latter

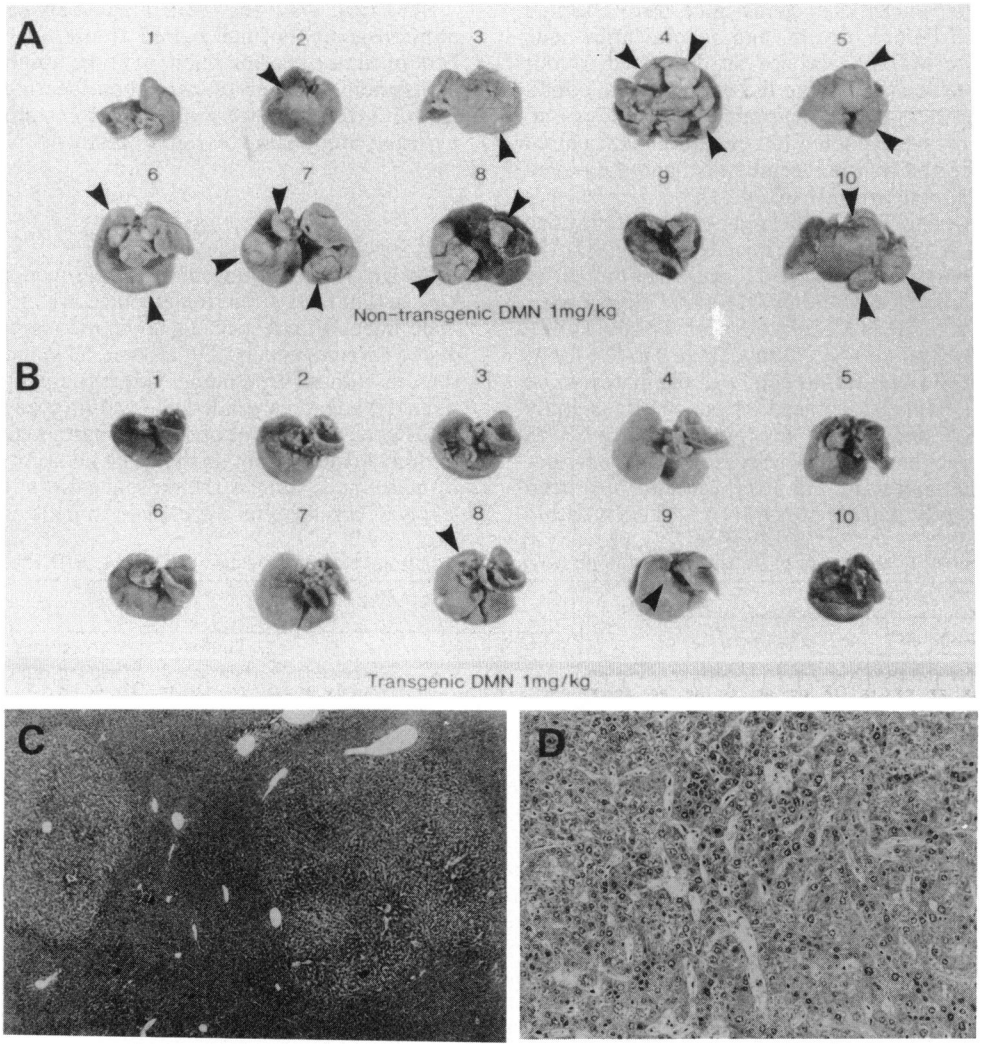

FIG. 2. Examples of liver tumors induced in male transgenic and nontransgenic mice administered 1 mg of DMNA per kg of body weight (group 5 of Table 1). Ten livers from each group autopsied in consecutive order are shown. (*A*) Nontransgenic mice. (*B*) Transgenic mice. Arrowheads indicate tumors, the numbers developing in transgenic mice being clearly less than in nontransgenic mice. (*C*) Histologic appearance of two tiny transgenic mouse liver nodules (1.5–2.0 mm in diameter) illustrating focal growth without atypia. Therefore, they were diagnosed as liver cell adenomas. (Hematoxylin/eosin; ×20.) (*D*) Histologic appearance of a large nontransgenic mouse carcinoma nodule (1.5 cm in diameter) showing typical trabecular patterns of malignant hepatocytes. (Hematoxylin/eosin; ×100.)

mice). No carcinoma-bearing mice were found in the transgenic mice, whereas 16% of normal mice produced carcinomas with this treatment.

A similar result was obtained with male mice which received 1 mg of DMNA per kg (group 5). In this case, too, more carcinomas were produced in normal mice (41%) than in transgenic mice (4%) ($P < 0.05$). Examples of tumor-bearing livers are shown in Fig. 2. Exposure of mice to 5 mg of DMNA per kg yielded large numbers of tumors in both normal and transgenic cases and thus no significant difference was observed (group 6).

Tumor Induction by DENA. To adjust for susceptibilities to carcinogens, different levels of DENA were applied to female and male mice. Female transgenic mice that received 5 mg of DENA per kg yielded significantly less ($P < 0.025$) tumors than did normal mice treated in the same manner (groups 7). In the case of male mice, a lower dose of DENA (1 mg/kg) was applied, and essentially similar results were obtained with respect to both the average number of tumors per animal ($P < 0.005$) and the percentage of carcinoma-bearing animals ($P < 0.05$) (group 8).

DISCUSSION

In the present study application of transgenic mouse technology allowed direct determination of whether increased levels of *E. coli* MGMT activity in mice render them resistant to hepatocarcinogenesis induced by DENA or DMNA. We established an optimization schedule to elicit maximum enzyme activity—6–8 times the normal level and equivalent to that for man—at the time of DMNA or DENA exposure and found a statistically significant reduction of tumor formation in transgenic mice in four of 6 paired groups that received treatment. The results obtained for the remaining two groups were also consistent when viewed with respect to dose–response relationships, since the levels of carcinogen administered might have been too low (group 3) or overwhelmingly high (group 6). Therefore, we consider that our data provide direct evidence that the intracellular level of MGMT may be an important factor in determining susceptibility of animals to tumor induction by alkylating carcinogens.

We cannot completely preclude the possibility that artificial gene integration or the disruption itself used in transgenic or gene-targeting experiments may affect the genetic background, thus influencing tumor induction. However, we could not find any change or abnormality in our transgenic mice strain other than the high expression of repair gene activity and efficient DNA repair (23). Further, we confirmed that several factors (growth pattern, cell proliferation, and various hepatic enzymes including DMNA demethylase) that may modify carcinogenesis did not differ between transgenic and normal mice (unpublished data).

It has been established that the *E. coli* Ada protein carries two distinct methyltransferase activities, one transferring methyl groups from O^6-methylguanine and O^4-methylthymine and the other transferring methyl groups from phosphomethyltriesters, while the corresponding mammalian enzyme mainly acts on methyl groups from O^6-methylguanine (9). The level of repair of O^4-methylthymine in mammalian cells has been considered to be very low or nonexistent (30). However, it should be borne in mind that the tumor inhibition in our transgenic mice could in part have reflected O^4-methylthymine removal by *E. coli* Ada protein, although this is only a minor DNA adduct produced by alkylating carcinogens. The *ada* transgenic mice can be even more resistant to methylating agent than is estimated by analysis of O^6-methylguanine repair. Since it was recently reported that O^6-ethylguanine can be slowly repaired by cooperation between MGMT and the excision repair pathway in human cells (31), this might explain our higher tumor yield and modest inhibition data for the ethylating carcinogen DENA.

The question of whether MGMT also protects chromosomal DNA from naturally occurring alkylating substances remains to be elucidated. In this respect, it will be of interest to determine whether more tumors are formed in mouse tissues with a paucity in methyltransferase activity. Construction of methyltransferase-defective mice by gene targeting is thus awaited.

Note. After this manuscript was submitted our results received support by experiments showing that expression of a human methyltransferase transgene in mouse thymus very efficiently protects these animals from developing thymic lymphomas after the application of a single dose of methylnitrosourea at the age of 6 weeks (32).

We thank Junko Sakurai, Kiyomi Ebina, Hironori Murayama, Hiroaki Mitani, and Keiko Isahaya for excellent technical assistance. The present research was supported by Grants-in-Aid for Cancer Research from the Ministry of Education and Culture, the Smoking Research Foundation, and the Sandoz Foundation for Gerontological Research.

1. Cleaver, J. E. (1968) *Nature (London)* **218,** 652–656.
2. Tanaka, K., Miura, N., Satokata, I., Miyamoto, I., Yoshida, H. C., Satoh, Y., Kondo, S., Yasui, A., Okayama, H. & Okada, Y. (1990) *Nature (London)* **348,** 73–76.
3. Snow, E. T., Foote, R. S. & Mitra, S. (1984) *J. Biol. Chem.* **259,** 8095–8100.
4. Saffhill, R., Margison, G. P. & O'Connor, P. J. (1985) *Biochim. Biophys. Acta* **823,** 111–145.
5. Olsson, M. & Lindahl, T. (1980) *J. Biol. Chem.* **255,** 10569–10571.
6. Foote, R. S., Mitra, S. & Pal, B. C. (1980) *Biochem. Biophys. Res. Commun.* **97,** 654–659.
7. Pegg, A. E., Weist, L., Foote, R. S., Mitra, S. & Perry, W. (1983) *J. Biol. Chem.* **258,** 2327–2333.
8. Hall, J., Bresil, H. & Montesano, R. (1985) *Carcinogenesis* **6,** 209–211.
9. Nakabeppu, Y., Kondo, H., Kawabata, S., Iwanaga, S. & Sekiguchi, M. (1985) *J. Biol. Chem.* **260,** 7281–7288.
10. Shiraishi, A., Sakumi, K., Nakatsu, Y., Hayakawa, H. & Sekiguchi, M. (1992) *Carcinogenesis* **13,** 289–296.
11. Lindahl, T. (1982) *Annu. Rev. Biochem.* **51,** 61–87.
12. Goth, R. & Rajewsky, M. F. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 639–643.
13. Frei, J. V., Swenson, D. H., Warren, W. & Lawley, P. D. (1978) *Biochem. J.* **174,** 1031–1044.
14. Gerson, S. L., Trey, J. E., Miller, K. & Berger, N. (1986) *Carcinogenesis* **7,** 745–749.
15. Belinsky, S. A., Foley, J. F., White, C. M., Anderson, M. W. & Maronpot, R. R. (1990) *Cancer Res.* **50,** 3772–3780.
16. Peterson, L. A. & Hecht, S. (1991) *Cancer Res.* **51,** 5557–5564.
17. Thomale, J., Huh, N., Nehls, P., Eberle, G. & Rajewsky, M. F. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 9883–9887.
18. Waldstein, E. A., Cao, E., Bender, M. A. & Setlow, R. B. (1982) *Mutat. Res.* **95,** 405–416.
19. Nakatsuru, Y., Nemoto, N., Nakagawa, K., Masahito, P. & Ishikawa, T. (1987) *Carcinogenesis* **8,** 1123–1127.
20. Xiao, W., Derfler, B., Chen, J. & Samson, L. (1991) *EMBO J.* **10,** 2179–2186.
21. Nakatsuru, Y., Aoki, K. & Ishikawa, T. (1989) *Mutat. Res.* **219,** 51–56.
22. Matsukuma, S., Nakatsuru, Y., Nakagawa, K., Utakoji, T., Sugano, H., Kataoka, H., Sekiguchi, M. & Ishikawa, T. (1989) *Mutat. Res.* **218,** 197–206.
23. Nakatsuru, Y., Matsukuma, S., Sekiguchi, M. & Ishikawa, T. (1991) *Mutat. Res.* **254,** 225–230.
24. Lim, I. K., Dumenco, L. L., Yun, J., Donovan, C., Warman, B., Gorodetzkaya, N., Wagner, T. E., Clapp, D. W., Hansen, R. W. & Gerson, S. L. (1990) *Cancer Res.* **50,** 1701–1708.
25. Dumenco, L. L., Arce, C., Norton, K., Yun, J., Wagner, T. & Gerson, S. L. (1991) *Cancer Res.* **51,** 3391–3398.

26. Fan, C. Y., Potter, P. M., Rafferty, J., Watson, A. J., Cawkwell, L., Searle, P. F., O'Connor, P. J. & Margison, G. P. (1990) *Nucleic Acids Res.* **18,** 5723–5727.
27. Drinkwater, N. R. & Ginsler, J. J. (1986) *Carcinogenesis* **7,** 1701–1707.
28. Klaunig, J. E., Weghorst, C. M. & Pereira, M. A. (1988) *Cancer Lett.* **42,** 133–139.
29. Hanigan, M. H., Kemp, C. J., Ginsler, J. J. & Drinkwater, N. (1988) *Carcinogenesis* **9,** 885–890.
30. Brent, J. P., Dolan, M. E., Fraenkel-Conrat, H., Hall, J., Karran, P., Laval, F., Margison, G. P., Montesano, R., Pegg, A. E., Potter, P. M., Singer, B., Swenberg, J. A. & Yarosh, D. B. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 1759–1762.
31. Bronstein, S. M., Skopek, T. R. & Swenbery, J. A. (1992) *Cancer Res.* **52,** 2008–2011.
32. Dumenco, L. L., Allay, E., Norton, K. & Gerson, S. L. (1993) *Science* **259,** 219–222.