DNA Damage and Repair in γ-Glutamyltranspeptidase-Positive and Negative Hepatocytes in Primary Culture from Carcinogen-Treated Rats (43162)

JIN-SHENG ZHANG,[1] YI-HUA XU, GERALD L. SATTLER, AND HENRY C. PITOT[2]
Departments of Oncology and Pathology, McArdle Laboratory for Cancer Research, The University of Wisconsin, Madison, Wisconsin 53706

Abstract. **Chemically induced DNA fragmentation and unscheduled DNA synthesis were determined in γ-glutamyltranspeptidase (GGT)-positive and GGT-negative hepatocytes isolated from rat livers subjected to a multistage hepatocarcinogenesis regimen (Solt-Farber), which included 0.05% phenobarbital promotion for 6 weeks (early) or 6 months (late). The results indicated that there was DNA damage in untreated GGT-positive and GGT-negative hepatocytes with either period of promotion compared with normal hepatocytes; however, no statistical difference could be seen between GGT-positive and GGT-negative hepatocytes. DNA damage induced *in vitro* by the activation-dependent carcinogen dimethylnitrosamine was much less in GGT-positive hepatocytes than in GGT-negative hepatocytes or normal hepatocytes. No significant difference in DNA damage was seen in both GGT-positive and GGT-negative cell populations following treatment with the activation-independent carcinogen ethylnitrosourea (ENU), although DNA damage of GGT-positive hepatocytes was less than that of normal hepatocytes. The background of unscheduled DNA synthesis in both GGT-positive and GGT-negative hepatocytes at either time of promotion was higher than that of normal hepatocytes. The capacity for DNA repair in GGT-positive hepatocytes appeared to be lower than that in GGT-negative hepatocytes. GGT-negative hepatocytes exhibited a lower capacity for DNA repair than that of normal hepatocytes in terms of the rate of unscheduled DNA synthesis elicited by dimethylnitrosamine and ethylnitrosourea *in vitro*.**
[P.S.E.B.M. 1991, Vol 196]

Multistage hepatocarcinogenesis involves at least three consecutive stages—initiation, promotion, and progression (1, 2). DNA damage and repair are primary events in relation to initiation (3–5). Furthermore, DNA-bound adducts and DNA single-strand breaks occurring during the stage of initiation persist for a considerable time after treatment with chemical carcinogens (6–9). Hepatocytes in the stages of promotion and/or progression lose much of their capacity to metabolize chemical carcinogens to their ultimate reactive forms and to repair DNA damage caused by carcinogenic agents (10, 11). However, few if any studies have been conducted on DNA damage and repair elicited by treatment with chemical carcinogens *in vivo* or *in vitro* by comparing initiated hepatocytes with those in the stage of promotion.

In several experimental models of hepatocarcinogenesis in the rat, preneoplastic focal lesions may be detected by several enzyme markers. The more commonly used markers are γ-glutamyltranspeptidase (GGT) and the placental isozyme of glutathione *S*-transferase (12, 13). Utilizing the cell surface marker GGT, Xu *et al.* (14) have developed technology allowing for the separation of GGT-positive hepatocytes, which are in the stage of promotion, from GGT-negative hepatocytes, which represent predominantly uninitiated cells. These two cell populations can then be cultured and DNA damage and related properties stud-

[1] Present address: Department of Pathology, Shanghai Medical University, Shanghai 200032, People's Republic of China.

[2] To whom correspondence should be addressed.

Received May 7, 1990. [P.S.E.B.M. 1991, Vol 196]
Accepted July 16, 1990.

0037-9727/91/1961-0047$2.00/0

ied with the alkaline elution technique as described (15, 16).

Determination of unscheduled DNA synthesis (UDS) as described by Hsia *et al.* (17) may be employed for evaluation of the capacity of cells to repair DNA damage. Using these techniques, we have studied the DNA damage induced by a variety of agents *in vitro* and the capacity to repair such damage in GGT-positive and GGT-negative hepatocytes in order to determine whether differences in DNA damage and repair exist between these two cell populations.

Materials and Methods

Reagents. All of the reagents were purchased from Sigma Chemical Co. (St. Louis, MO) except that Leibovitz L15 tissue culture medium was purchased from KC Biological Inc. (Lenexa, KS); insulin-transferrin-selenium was obtained from Collaborative Research Inc. (Lexington, MA); dexamethasone was purchased from Elkins-Sinn, Inc. (Cherry Hill, NJ); Hepes was obtained from Research Organics, Inc. (Cleveland, OH); collagenase A was purchased from Boehringer Mannheim Corp. (Indianapolis, IN); Percoll was obtained from Pharmacia (Uppsala, Sweden); glutaraldehyde was purchased from Ted Pella, Inc. (Redding, CA); octadecylamine, tetraethylammonium hydroxide, and 3,5-diaminobenzoic acid dihydrochloride were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI); perchloric acid was purchased from MCB Manufacturing Chemists, Inc. (Cincinnati, OH); dimethylnitrosamine (DMN) was purchased from Eastman Organic Chemicals (Rochester, NY); [(*methyl*-^{3}H] TdR was obtained from Dupont Co. (Wilmington, DE); polyvinylidene difluoride (SVLP 025) and mixed esters of cellulose (SSWP 025) filter membranes were purchased from Millipore Corp. (Bedford, MA); tissue culture dishes (60 mm) and flasks (25 cm^2) were obtained from Corning Co. (Corning, NY); petri dishes were purchased from Fisher Scientific Co. (Itasca, IL); and rabbit anti-rat GGT serum was a generous gift from Dr. David G. Beer (Kansas City, KS).

Animals. Female Fischer 344/N rats (100 g) were obtained from Harlan Sprague-Dawley, Inc. (Madison, WI). The rats were given diethylnitrosamine (DEN) at a dose of 200 mg/kg body wt; 2 weeks after DEN treatment, 0.02% of 2-acetylaminofluorene (2-AAF) was added to the diet for an additional 2 weeks (18). At the midpoint of 2-AAF administration, the rats were subjected to a 70% partial hepatectomy. Phenobarbital (PB), 0.05%, was added to the diet 4 weeks after the DEN treatment. Rats were maintained on the PB diet and used for experiments after 6 weeks of PB promotion (early period) or 6 months of PB promotion (late period).

Hepatocyte Isolation and Purification of GGT-Positive Hepatocytes. Isolated hepatocytes were obtained by means of the collagenase perfusion technique (19), which has been modified by Xu *et al.* (14). GGT-positive hepatocytes were separated from GGT-negative hepatocytes by a modification of the panning technique originally described by Hanigan and Pitot (19), as improved by Xu *et al.* (14). In short, the isolated hepatocytes were allowed to attach to rabbit anti-rat GGT antibody-coated petri dishes (100 mm) in L15 medium supplemented with 0.2% bovine serum albumin, 18 m*M* Hepes, insulin-transferrin-selenium, 100 μg of streptomycin/ml, 100 units of penicillin/ml, 1 mg of galactose/ml, and 10^{-6} *M* dexamethasone. After 25 min of incubation at room temperature, unattached cells were removed, and the adhering cells were detached by forceful pipetting of the cell suspension and collected as GGT-positive hepatocytes. The unattached cells were subjected to a second panning procedure, and then unattached cells were collected as GGT-negative hepatocytes in order to allow a better comparison between GGT-positive and GGT-negative hepatocytes. Both isolated GGT-positive and GGT-negative hepatocytes were purified by a second isodensity Percoll centrifugation (20) to remove any dead cells produced during the panning procedure. The viability of the final cell preparation, as described by trypan blue exclusion of the cells, was higher than 90%. Hepatocytes derived from adult untreated female F-344 rats and plated on the uncoated petri dishes in order to mimic the panning technique procedure were the untreated controls.

Determination of DNA Single-Stranded Breaks. Purified GGT-positive and GGT-negative hepatocytes were plated in 60-mm Corning tissue culture dishes at a density of 1,000,000 cells/dish in L15 medium supplemented as described above. The hepatocytes were treated with ethylnitrosourea (ENU) or DMN, which had been freshly dissolved in ultrapure water. After incubation in a humidified 100% air incubator for a given time (3 hr for ENU treatment, 1.5 hr for DMN treatment), cells were collected gently with a rubber policeman, and the alkaline elution of DNA was performed as described by Cavanna *et al.* (16). Briefly, approximately 1,000,000 cells were loaded onto a Millipore polyvinylidene difluoride filter (25 mm in diameter, 5 μm in pore size), washed with cold phosphate-buffered saline containing 0.02 *M* EDTA, and lysed with 5 ml of lysis buffer (2% sodium dodecyl sulfate/0.025 *M* EDTA/0.1 *M* glycine, pH adjusted to 10 with 2 *N* NaOH). The lysis buffer was kept in a syringe for at least 15 min before it was allowed to flow through the filter by gravity. Single-stranded DNA was eluted in the dark with 20 ml of eluting solution (0.02 *M* EDTA, the pH being adjusted to 12 with tetraethylammonium hydroxide) at a peristaltic pump speed of 0.07 ml/min on a multichannel fraction collection system (21). The content of DNA in the collected fractions and those remaining on the filters was deter-

Table I. Effect of Different Doses of ENU on DNA Replicative Synthesis and Cell Growth of Rat Hepatocytes in Primary Culture

ENU (m*M*)		Hours in culture				
		24	48	72	120	144
0	a[a]	297 ± 54	1898 ± 528	239 ± 48	106 ± 36	44 ± 3
	b	100	115 ± 5	79 ± 4	34 ± 7	ND[b]
0.1	a	219 ± 91	1042 ± 168	251 ± 146	198 ± 125	75 ± 23
	b	100	114 ± 1	104 ± 11	32 ± 10	ND
0.5	a	230 ± 92	1339 ± 599	348 ± 24	23 ± 6	29 ± 15
	b	100	113 ± 7	109 ± 4	52 ± 4	ND
2	a	127 ± 45	48.7 ± 10	28 ± 14	88 ± 25	267 ± 243
	b	100	103 ± 2	102 ± 4	85 ± 4	ND
4	a	21 ± 11	0	9 ± 9	0	1 ± 1
	b	100	31 ± 2	3 ± 1	0	
10	a	0				
	b	0				

Note. Hepatocytes were isolated from a female adult F-344 rat by collagenase perfusion and plated in 35-mm Corning tissue culture dishes at a cell density of 200,000/dish in L15 medium. Cells were then cultured in Dulbecco's modified Eagle's medium-F12(1:1) medium supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), dexamethasone (0.0001 m*M*), galactose (1 mg/ml), proline (30 μg/ml), insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), 0.2% bovine serum albumin, and 5 ng/ml EGF. At each time point, [^{3}H]TdR (0.45 μCi/ml) was added to each dish; 2.5 hr later the cells were harvested. [^{3}H]TdR incorporation was determined (31). Protein content was measured according to the method of Lowry *et al.* (32). Cell counts were based on four microscopic view fields in two dishes of each group. ENU was diluted with the medium mentioned above, and the ENU medium was renewed every day for the first 72 hr.

[a] a = cpm/mg protein (mean value of three dishes ± SD); b = cell count, % of cell number at 24 hr (mean value of three dishes ± SD).

[b] ND, not determined.

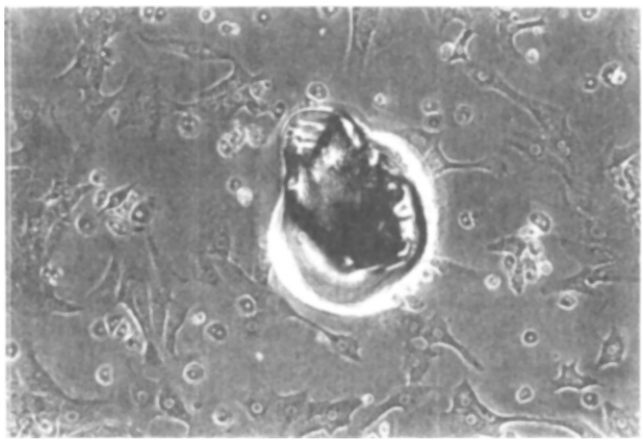

Figure 1. Hepatocytes isolated from a female adult F-344 rat and treated *in vitro* with 2 m*M* ENU exhibit normal morphology after 5 days in culture in Dulbecco's modified Eagle's medium-F12 (1:1) medium (see footnote to Table 1). The central amorphous body is a marker used in counting the cell number (Table I).

mined by a modification of the microfluorometric technique of Kissane and Robins (22). In short, the DNA was precipitated with trichloroacetic acid and washed with absolute ethanol, and the pellets were air-dried at room temperature overnight and then at 45°C for 1 hr. Following that procedure, 0.05 ml of 40% 3,5-diaminobenzoic acid dihydrochloride, which has been absorbed with 50 mg/ml of charcoal before use, was added to each sample. The samples were incubated in a 70°C water bath with shaking for 30 min. After the sample had cooled, 2 ml of 0.6 *N* perchloric acid were added to each tube. The fluorescence was read at 520 nm with an excitation wavelength of 436 nm. The blank readings were made from tubes containing 1 ml of eluting solution subjected to the same procedures. The elution profiles were plotted with respect to the total DNA left on the filter versus the eluted volume, and the results were expressed as elution rate constant (K):

$K = Ln$ (the fraction of DNA retained on the filter)$/V$ (the eluted volume in ml).

The elution rate constant was computed on the third fraction to avoid any artifact due to the prolonged alkaline conditions that might induce additional breaks in the DNA molecule (23).

Assay of Unscheduled DNA Synthesis (UDS). UDS was determined by means of a DNA membrane retention technique (17). Hepatocytes (300,000/ml) were incubated in L15 medium containing [^{3}H]thymidine (0.3 μCi/ml) and hydroxyurea (15 m*M*) in a 25-cm^2 Corning flask in the dark, in an incubator shaker set at 50 oscillations/min at 37°C (Orbit Incubator Shaker; Lab-Line Instruments, Inc.). After 1 hr the carcinogens were added, and the incubation was continued for another 3 hr. Then the flasks were placed on ice and 10 ml of ice-cold phosphate-buffered saline containing thymidine (0.5 mg/ml) was added to each sample. The hepatocytes were loaded onto Millipore filters (3-μm pore size) and lysed in the dark with 10 ml of lysis buffer (see above) containing thymidine (0.5 mg/ml) and proteinase K (0.15 mg/ml). The lysed samples were washed with 3 ml of the same lysis buffer

Table II. DNA Fragmentation Caused *In Vitro* by DMN in GGT(+) and GGT(−) Cells Derived from Rat Livers during Hepatocarcinogenesis

Rats	Cells	DNA Fragmentation		
		Untreated ($K_c \times 10^4$)	DMN (5 m*M*) ($K_t \times 10^4$)	($K_t - K_c$)
Normal	Hepatocyes	163 ± 30	1680 ± 332	1518.5 ± 312
Early	GGT(+)	536 ± 127	821.5 ± 207	210 ± 88[ab]
period	GGT(−)	362 ± 179	1432.5 ± 247	1070.8 ± 182
Late	GGT(+)	606 ± 299	803 ± 147	196.8 ± 174[ab]
period	GGT(−)	418 ± 130	1440 ± 396	1022.3 ± 283

Note. GGT(+) and GGT(−) cells were isolated from rat livers at either early or late periods of hepatocarcinogenesis. Cells were treated with DMN *in vitro* for 1.5 hr, and DNA fragmentation was determined by alkaline elution. *K* is elution rate constant:

$$K = Ln\left(\frac{1}{\text{fraction on filter}}\right) \times \frac{1}{V}$$

V = eluted volume of first three fractions;
K_c = elution rate constant of untreated cells; K_t = elution rate constant of treated cells. The numbers in the table represent the mean value of four experiments ± SD.
[a] $P < 0.05$ when compared with normal hepatocytes; the statistical significance was calculated by means of the Wilcoxon two-sample test (two tailed).
[b] $P < 0.05$; the statistical significance of the difference of ($K_t - K_c$) between GGT(+) and GGT(−) cells was calculated by the same method.

Table III. DNA Fragmentation Elicited *In Vitro* by ENU in GGT(+) Cells and GGT(−) Cells Derived from Rats at the Early and Late Periods of Hepatocarcinogenesis

Rats	Cells	DNA Fragmentation		
		Untreated ($K_c \times 10^4$)	ENU (2 m*M*) ($K_t \times 10^4$)	$K_t - K_c$
Normal	Hepatocytes	163 ± 28	1559 ± 242	1396 ± 221
Early	GGT(+)	536 ± 127	1052 ± 197	515.8 ± 90[a]
stage	GGT(−)	362 ± 179	1020 ± 98	659 ± 81[a]
Late	GGT(+)	606 ± 299	1580 ± 154	974 ± 166[a]
stage	GGT(−)	418 ± 130	1680 ± 336	1262.5 ± 240

Note. GGT(+) and GGT(−) cells were isolated from rat livers at either the early or late period of hepatocarcinogenesis. Cells were treated with ENU *in vitro* for 3 hr, and DNA fragmentation was determined by alkaline elution. K_c and K_t are defined in the footnote to Table II. The numbers in the table represent the mean value of four experiments ± SD.
[a] $P < 0.05$, when compared with normal hepatocytes; the statistical significance was calculated by means of the Wilcoxon two-sample test (two-tailed).

but without proteinase K. The samples were allowed to drip to dryness. Loading, lysis, and washing were all carried out without applying any vacuum to the samples. Then each filter membrane was carefully removed, inverted, and placed in a scintillation vial to which 0.4 ml of 1 *N* HCl was added. The sealed vials were placed in a water bath at 60°C with shaking for 1 hr. After that, 0.5 ml of 2 *N* NaOH was added, and the vials were shaken vigorously for another 1 hr at room temperature. Finally, 0.5-ml aliquots were taken for liquid scintillation counting, and 0.2-ml aliquots were used to determine the DNA concentration, with calf thymus DNA as a standard (24).

Results

Two different carcinogens were used to induce DNA damage of cells *in vitro*. DMN, which is activation dependent, was employed at a concentration of 5 m*M* (11); ENU, which is activation independent, was used at several levels in the medium. A study of the effects of several different concentrations of ENU on normal hepatocytes in primary culture is seen in Table I. ENU inhibited the replicative DNA synthesis of hepatocytes at 48 hr in culture, and this effect was dose dependent. Furthermore, a dose of 2 m*M* ENU induced a significant inhibitory effect without a significant reduction in the proportion of viable cells while maintaining normal cellular morphology (Fig. 1). Doses above 2 m*M* caused considerable cell death; this suggests that ENU may be used at concentrations up to 2 m*M* for experiments on DNA damage.

In Table II, the DNA elution rate constant, *K*, was computed on the third fraction. As a first approximation, *K* is directly proportional to the number of single-stranded breaks of DNA (15). The untreated GGT-positive and GGT-negative hepatocytes that were isolated from the livers at both early and late periods of hepatocarcinogenesis (see Materials and Methods) had increasing K_c values compared with normal hepatocytes ($P < 0.05$). This indicates that the Solt-Farber regimen followed by PB promotion induced DNA damage in all liver cells. The K_c value of GGT-positive hepatocytes appeared to be greater than that of GGT-negative hepatocytes; however, the differences were not statistically significant. The differences between K_t and K_c represent the single-strand breaks of DNA elicited by *in vitro* treatment with carcinogens. That the activation-de-

Table IV. UDS Elicited *In Vitro* by DMN in GGT(+) and GGT(−) Cells Derived from Rat Livers during Hepatocarcinogenesis

Rats	Cells	Radioactivity (dpm/μg DNA)[a]		$\frac{Ut - Uc}{Uc} \times 100\ \%$
		Untreated (Uc)	DMN (5 m*M*) (Ut)	
Normal	Hepatocytes	387 ± 90	988 ± 81	149
Early	GGT(+)	797 ± 132	1180 ± 177[b]	48
period	GGT(−)	771 ± 57	1398 ± 105	81
Late	GGT(+)	566 ± 7	872 ± 9.7	54
period	GGT(−)	428 ± 100	865 ± 114	102

Note. GGT(+) and GGT(−) cells were isolated from rat livers at either early or late periods of hepatocarcinogenesis. DMN treatment and UDS measurement were performed as described in Materials and Methods. Uc, UDS of untreated cells; Ut, UDS of treated cells.
[a] The mean value of five flasks ± SD.
[b] $P < 0.05$, when compared with GGT(−) cells.

Table V. UDS Elicited *In Vitro* by ENU in GGT(+) and GGT(−) Cells Derived from Rat Livers during Hepatocarcinogenesis (following the Solt-Farber Protocol)

Rats	Cells	Radioactivity (dpm/μg DNA)[a]		$\frac{Ut - Uc}{Uc} \times 100\%$
		Untreated (Uc)	ENU (2 m*M*) (Ut)	
Normal	Hepatocytes	387.2 ± 91	977.1 ± 185	151
Early	GGT(+)	738.6 ± 93	1254.0 ± 118[b]	70
stage	GGT(−)	835.1 ± 111	1628.0 ± 237	95
Late	GGT(+)	656.8 ± 86	899.1 ± 192[b]	37
stage	GGT(−)	709.1 ± 95	1347.1 ± 374	90

Note. GGT(+) and GGT(−) cells were isolated from rat livers at either early or late periods of hepatocarcinogenesis. ENU treatment and UDS measurement were performed as described in Materials and Methods. Uc, UDS of untreated cells; Ut, UDS of treated cells.
[a] The mean value of five flasks ± SD.
[b] $P < 0.05$, when compared with GGT(−) cells.

pendent carcinogen DMN induced less DNA damage in GGT-positive hepatocytes than in normal and GGT-negative hepatocytes can also be seen in Table II. Although the value of $(K_t - K_c)$ is higher in normal hepatocytes than in GGT-negative hepatocytes, this difference is not statistically significant. On the other hand, as seen in Table III, there was no significant difference between GGT-positive and GGT-negative hepatocytes in the degree of DNA damage elicited by ENU, although less DNA damage was seen in GGT-positive hepatocytes than in normal hepatocytes. GGT-negative hepatocytes exhibited less DNA damage from ENU than normal hepatocytes only at the early period of carcinogenesis, but not at 6 months of PB promotion.

The results obtained from evaluation of UDS by the technique of membrane retention of DNA indicate that the background of UDS in untreated GGT-positive and GGT-negative hepatocytes derived from either early or late periods was generally higher than that of normal hepatocytes (Tables IV and V). However, the difference of UDS background between GGT-negative hepatocytes at the late period and normal hepatocytes did not reach statistical significance (Table IV). This is in agreement with the data in Table II, which indicate that GGT-positive and GGT-negative hepatocytes exhibited DNA damage at much higher levels than normal hepatocytes. The absolute value of UDS induced by DMN in GGT-negative hepatocytes was higher than in GGT-positive hepatocytes only at the early stage of carcinogenesis (Table IV), whereas UDS induced by ENU in GGT-negative hepatocytes only at the late period was higher than that of GGT-positive hepatocytes (Table IV). However, if we compare the increased rate of UDS elicited by DMN and ENU, the capacity of DNA repair was highest in normal hepatocytes, lowest in GGT-positive hepatocytes, and intermediate in GGT-negative hepatocytes.

Discussion

In the present study, neither GGT-positive nor GGT-negative untreated hepatocytes had higher K_c values than did normal hepatocytes *in vitro*. Furthermore, the background of UDS in both GGT-positive and GGT-negative hepatocytes was higher than in normal hepatocytes from untreated animals at either the late or early periods of PB promotion. These data suggest that DNA fragmentation of liver cells is constantly present during PB promotion after the Solt-Farber regimen (18). Brambilla *et al.* (11, 25) reported that DNA

fragmentation of hepatocytes existed not only in the selection but also in the promotion phases of a similar resistant hepatocyte model. The occurrence of the persistent presence of DNA fragmentation during the development of liver hyperplastic lesions has also been observed with other hepatocarcinogenic regimens. Stout and Becker (9) reported the liver DNA damage progressed either after 2-AAF treatment or in rats given a single dose of DEN after partial hepatectomy and subsequent exposure to PB. The promoting and hepatocarcinogenic effects of a choline-methionine-deficient diet have also been related to DNA damage (26–28).

The demonstration that there is no difference between the DNA fragmentation of untreated GGT-positive and GGT-negative hepatocytes is in accord with earlier findings by Stout and Becker (29, 30) demonstrating progressive DNA damage in livers of animals treated with only a single dose of DEN or several cycles of 2-acetylaminofluorene feeding. Such changes occurred well after the cessation of administration of the carcinogen, both of which are employed in the Solt-Farber model (18). These authors have theorized that an explanation of this continuous expression of DNA damage may be the result of faulty replication resulting from altered DNA ligation or other processes necessary for cell division. Data from our laboratory (31) have demonstrated the persistence of altered karyotypes of both GGT-positive and GGT-negative hepatocytes isolated from the Solt-Farber protocol. On the other hand, the responses to *in vitro* treatment of GGT-positive and GGT-negative hepatocytes with carcinogens are quite different. The activation-dependent carcinogen DMN was able to induce more DNA damage in normal and GGT-negative hepatocytes than in GGT-positive hepatocytes, whereas the activation-independent carcinogen ENU elicited almost the same degree of DNA damage in GGT-positive and GGT-negative hepatocytes. This evidence strongly suggests that the metabolic conversion of the procarcinogen to its ultimate form is responsible for the difference of induced DNA fragmentation between these two cell populations. These findings are in accord with the demonstration by several laboratories of a reduced level of Phase I enzymes of xenobiotic metabolism in hyperplastic nodules (32, 33) and in enzyme-altered foci (34), as well as GGT-positive cells isolated by techniques developed in our laboratory (35).

The data reported by Brambilla *et al.* (11) suggested that the relative resistance to the DNA-damaging activity of both activation-dependent and activation-independent carcinogens should be considered as a property acquired by all hepatocytes, or at least by the large majority of them. Our data showed that the amount of DNA damage induced by DMN and ENU *in vitro* in GGT-positive and GGT-negative hepatocytes was lower than that of normal hepatocytes; however, the difference in DNA damage between GGT-negative and normal hepatocytes was not statistically significant except that GGT-negative hepatocytes isolated from rats after 6 weeks of PB administration treated with ENU had less DNA damage than normal hepatocytes. This may be due to the much shorter period of carcinogen exposure in our experiments (1.5–3 hr) than in those of Brambilla *et al.* (20 hr).

The data in Tables IV and V demonstrate that the level of UDS elicited in GGT-positive hepatocytes by either the activation-dependent or the activation-independent carcinogen was lower or the same as that in GGT-negative hepatocytes of the same liver. This is in agreement with the observation made by Mori *et al.* (36). The degree of [^{3}H]TdR incorporation induced by carcinogen treatment in normal hepatocytes was not higher than that of GGT-positive or GGT-negative hepatocytes. This is different from the results reported by Brambilla *et al.* (11), which indicated that normal hepatocytes had higher [3H]TdR incorporation into the nuclei on autoradiography. If, however, we compare the normal hepatocytes with GGT-positive and GGT-negative hepatocytes in terms of the increased rate of UDS induced by carcinogens ($U_t - U_c/U_c \times 100\%$), it can be seen that the capacity of DNA repair is highest in the normal hepatocytes and lowest in the GGT-positive hepatocytes, with the GGT-negative hepatocytes between the other two cell populations. The relatively greater reduction of DNA repair in GGT-positive than in GGT-negative hepatocytes suggests that GGT-positive hepatocytes might be more readily transformed into the malignant state (the stage of progression) than GGT-negative hepatocytes as a result of their lowered DNA repair mechanisms.

The processes of DNA damage and repair are continuous in the livers of rats subjected to the Solt-Farber regimen (18) followed by PB promotion. Altered hepatic foci as represented by GGT-positive hepatocytes are more resistant to treatment with an activation-dependent carcinogen than are GGT-negative hepatocytes. These data, along with the relatively lower capacity to repair DNA lesions in GGT-positive hepatocytes than in GGT-negative hepatocytes, could be important for the transformation of the preneoplastic lesions to the malignant state, i.e., the transition from the stage of promotion to progression.

1. Pitot HC, Campbell HA. Quantitative studies on multistage hepatocarcinogenesis in the rat. In: Langenbach R, Barret JC, Elmore E, Eds. Tumor Promoters: Biological Promoters for Mechanistic Studies and Assay Systems. New York: Raven Press, pp79–95, 1988.
2. Pitot HC. Progression: The terminal stage in carcinogenesis. Jpn J Cancer Res **80**:599–607, 1989.
3. Damjanov I, Cox R, Sarma DSR, Farber E. Patterns of damage

and repair of liver DNA induced by carcinogenic methylating agents *in vivo*. Cancer Res **33**:2122–2128, 1973.
4. Stewart BW, Farber E. Strand breakage in rat liver DNA and its repair following administration of cyclic nitrosamines. Cancer Res **33**:3209–3215, 1973.
5. Stewart BW, Farber E, Mirvish SS. Induction by a hepatic carcinogen, 1-nitroso-5,6-dihydrouracil, of single and double strand breaks of liver DNA with rapid repair. Biochem Biophys Res Commun **53**:773–779, 1973.
6. Singer B. *N*-Nitroso alkylating agents: Formation and persistence of alkyl derivatives in mammalian nucleic acid as contributing factors in carcinogenesis. J Natl Cancer Inst **62**:1329–1339, 1979.
7. Floot BGJ, Philippus EJ, Hart AAM, Den Engelse L. Persistence and accumulation of (potential) single strand breaks in liver DNA of rats treated with diethylnitrosamine or dimethylnitrosamine: correlation with hepatocarcinogenicity. Chem Biol Interact **25**:229–242, 1979.
8. Stout DL, Becker FF. Progressive DNA damage in hepatic nodules during 2-acetylaminofluorene carcinogenesis. Cancer Res **40**:1269–1273, 1980.
9. Stout DL, Becker FF. Occurrence of progressive DNA damage coincident with the appearance of foci of altered hepatocytes. Carcinogenesis (Lond) **3**:599–602, 1982.
10. Den Engelse L, Jenner AAJ, Menkveld GJ, Scherer E. Immunocytochemical and chromatographical analysis of the formation and stability of (m)ethylated products in DNA of different rat tissues including precancerous liver foci. In: Myrnes B, Korkan H, Eds. Repair of DNA Lesions Induced by N-Nitroso Compounds. Oslo: Norwegian University Press, pp257–283, 1986.
11. Brambilla G, Martelli A, Pino A, Robbiano L. Reduction of DNA repair efficiency during the initial phase of rat liver carcinogenesis. Anticancer Res **8**:605–610, 1988.
12. Pitot HC, Sirica AE. The stages of initiation and promotion in hepatocarcinogenesis. Biochim Biophys Acta **605**:191–215, 1980.
13. Hendrich S, Pitot HC. Enzymes of glutathione metabolism as biochemical markers during hepatocarcinogenesis. Cancer Metastasis Rev **6**:155–178, 1987.
14. Xu YH, Sattler GL, Pitot HC. A method for the comparative study of replicative DNA synthesis in GGT-positive and GGT-negative hepatocytes in primary culture isolated from carcinogen-treated rats. In Vitro Cell Dev Biol **24**:995–1000, 1988.
15. Kohn KW, Erickson LC, Ewiq RAG, Friedman CA. Fraction of DNA from mammalian cells by alkaline elution. Biochemistry **15**:4629–4637, 1976.
16. Cavanna M, Parodi S, Robbiano L, Pino A, Sciaba L, Brambilla G. Alkaline elution assay as a potentially useful method for assessing DNA damage induced in vivo by diazoalkanes. Gann **71**:251–259, 1980.
17. Hsia MTS, Kreamer BL, Dolara P. A rapid and simple method to quantitate chemically induced unscheduled DNA synthesis in freshly isolated rat hepatocytes facilitated by DNA retention of membrane filters. Mutat Res **122**:177–185, 1983.
18. Solt D, Farber E. New principle for the analysis of chemical carcinogenesis. Nature **263**:701–703, 1976.
19. Hanigan MH, Pitot HC. Isolation of γ-glutamyltranspeptidase positive hepatocytes during the early stages of hepatocarcinogenesis in the rat. Carcinogenesis (Lond) **3**:1349–1354, 1982.
20. Kreamer BL, Staecker JL, Sawada N, Sattler GL, Hsia MTS, Pitot HC. Use of a low-speed, iso-density percoll centrifugation method to increase the viability of isolated rat hepatocyte preparations. In Vitro Cell Dev Biol **22**:201–211, 1986.
21. Kreamer BL, Hsia MTS. A multichannel fraction-collection system designed for the use in DNA-damage studies via the alkaline-elution technique. Anal Biochem **129**:224–227, 1983.
22. Kissane JM, Robins E. The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. J Biol Chem **233**:184–188, 1958.
23. Bolognesi C, Cesarone CF, Santi L. Evaluation of DNA damage by alkaline elution technique after in vivo treatment with aromatic amines. Carcinogenesis **2**:265–268, 1981.
24. Labarca C, Paigen K. A simple, rapid, and sensitive DNA assay procedure. Anal Biochem **102**:344–352, 1980.
25. Brambilla G, Martelli A, Pino A, Robbiano L. Sequential analysis of DNA damage and repair during the development of carcinogen-induced rat liver hyperplastic lesions. Cancer Res **46**:3476–3481, 1986.
26. Mikal YB, Hoover KL, Creasia D, Poirier LA. Hepatocarcinogenesis in rats fed methyl-deficient, amino acid-deficient diets. Carcinogenesis (Lond) **4**:1619–1629, 1983.
27. Ghoshal AK, Farber E. The induction of liver cancer by dietary deficiency of choline and methionine without added carcinogens. Carcinogenesis (Lond) **5**:1367–1370, 1984.
28. Yokoyama S, Sells MA, Reddy TV, Lombardi B. Hepatocarcinogenesis and promoting action of a choline-devoid diet in the rat. Cancer Res **45**:2834–2842, 1985.
29. Stout DL, Becker FF. Occurrence of progressive DNA damage coincident with the appearance of foci of altered hepatocytes. Carcinogenesis **3**:599–602, 1982.
30. Stout DL, Becker FF. Progressive DNA damage in hepatic nodules during 2-acetylaminofluorene carcinogenesis. Cancer Res **40**:1269–1273, 1980.
31. Sargent L, Xu Y-H, Sattler GL, Meisner L, Pitot HC. Ploidy and karyotype of hepatocytes isolated from enzyme-altered foci in two different protocols of multistage hepatocarcinogenesis in the rat. Carcinogenesis **10**:387–391, 1989.
32. Roomi MW, Ho RK, Sarma DSR, Farber E. A common biochemical pattern in preneoplastic hepatocyte nodules generated in four different models in the rat. Cancer Res **45**:564–571, 1985.
33. Eriksson LC, Blanck A, Bock KW, Mannervik B. Metabolism of xenobiotics in hepatocyte nodules. Toxicol Pathol **15**:27–42, 1987.
34. Kunz HW, Buchmann A, Schwarz M, Schmitt R, Kuhlmann WD, Wolf CR, Oesch F. Expression and inducibility of drug-metabolizing enzymes in preneoplastic and neoplastic lesions of rat liver during nitrosamine-induced hepatocarcinogenesis. Arch Toxicol **60**:198–203, 1987.
35. Hanigan MH, Pitot HC. Activities of benzphetamine *N*-demethylase and aryl hydrocarbon hydroxylase in cells isolated from γ-glutamyl transpeptidase-positive foci and surrounding liver. J Natl Cancer Inst **75**:1107–1112, 1985.
36. Mori H, Sugie S, Ohbayashi F, Shima H, Yoshimi N, Takahashi M, Williams GM. Reduced DNA repair response of cells from carcinogen-induced rat liver altered foci exposed to chemical carcinogens in culture. Carcinogenesis (Lond) **6**:1087–1090, 1985.