Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 3749–3753, April 1994
Biochemistry

N-Nitroso-*N*-methylurea-induced rat mammary tumors arise from cells with preexisting oncogenic *Hras1* gene mutations

RITA S. CHA, WILLIAM G. THILLY, AND HELMUT ZARBL*

Division of Toxicology, Center for Environmental Health Sciences, Whitaker College of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA 02139

Communicated by Vernon R. Young, January 3, 1994 (received for review October 23, 1992)

ABSTRACT **GGA to GAA mutations in the 12th codon of the *Hras* gene are frequently observed in rat mammary tumors induced by *N*-nitroso-*N*-methylurea (NMU). We developed an assay to measure point mutations present in tissues at a frequency of 10^{-5} and have now applied this assay to measure the specific G to A transition of the *Hras* gene in rat mammary epithelium. We find that (*i*) 70% of untreated rats contain detectable levels of *Hras* mutants; (*ii*) these mutants are clustered within the gland as sectors in a manner consistent with their origin as a mutation arising during early organ development; and (*iii*) treatment with a carcinogenic dose of NMU did not result in a significant increase in the number of such mutants, the fraction of organ sectors with mutant cells, or the fraction of animals containing detectable levels of *ras* mutants. We conclude that the NMU-induced mammary tumors carrying the G to A transition at the 12th codon of the *Hras* gene arose from preexisting *ras* mutants and that an independent effect of NMU was directly or indirectly responsible for tumor formation.**

To determine whether chemicals cause tumors by mutating cellular oncogenes, Zarbl *et al.* (1) characterized and compared the type of oncomutations found in *N*-nitroso-*N*-methylurea (NMU)- and 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced rat mammary tumors to the known† (or suspected‡) mutational specificity of the inducing carcinogens. A specific G to A transition in the *Hras* gene was found to be reproducibly associated with NMU-induced rat mammary tumors. This mutation was absent in DMBA-induced mammary tumors, some of which harbored an A to T transversion at the 61st codon of the *Hras* gene. Based on the observations that the same type of tumors induced by different chemicals carried different oncomutations, each of which reflected the suspected mutational specificity of the inducing agents, it was hypothesized that the oncomutations found in tumors were directly caused by the inducing chemicals. The findings of Zarbl *et al.* (1) were corroborated by Quintanilla *et al.* (5), who reported a specific A to T transversion in the *Hras* gene among DMBA-induced mouse skin tumors. Studies of this type (6) have provided a putative mechanistic link between environmental exposure and cancer risk and have had an impact on regulatory policy formulation (7).

However, such data are also consistent with an alternative hypothesis. The mutation observed in tumors could have existed before chemical exposure. Such exposure could induce unobserved complementary mutations or physiological effects within the *Hras* mutants that result in tumor formation. Consistent with this possibility is the observation that while the total number of mammary tumors induced per animal increases as a function of NMU dose, the number of tumors harboring the specific *Hras* gene mutation reaches a stable maximum of ≈0.7 per animal (8). It has also been noted that the presence of G to A transitions in *ras* genes among NMU- or ionizing radiation-induced mouse lymphomas corresponded with the genetic background of the mice rather than the mutational specificity of these inducing agents (9, 10).

In raising this alternative hypothesis, we do not imply that carcinogens do not cause any of the recurring specific oncomutations observed in tumors. Clearly, the fact that a variety of oncomutations appear in association with a variety of carcinogens argues that some of them are most probably induced by the carcinogens (11–14). However, in the case of *Hras* gene activation in NMU-induced mammary tumors, we believe that no evidence yet exists to differentiate between these two possibilities (i.e., the carcinogen induced the observed oncomutation or it did not). We have therefore applied the mismatch amplification mutation assay (MAMA) (15) to measure the number of specific mutant *Hras* alleles present among the mammary epithelial cells of 50-day-old female Fischer 344 rats before and after NMU exposure. In the current communication, we show that the specific *ras* oncogenes found in NMU-induced mammary tumors were not directly mutated by NMU.

MATERIALS AND METHODS

Animals. Female Fischer 344 rats (Charles River Breeding Laboratories) were treated with 30 mg of NMU per kg of body weight (Sigma) via tail vein injection at 50 days of age. Six to 10 animals were euthanized before and at 1, 3, 7, 30, 60, 90, 150, 210, and 280 days after treatment. The first macroscopic mammary lesion appeared 90 days after the treatment and a total of 12 lesions were collected during subsequent time points. The entire macroscopically normal mammary gland tissue, mammary lesions, and a 1-g sample of liver were collected from each animal.

Organ Cell Numbers and DNA Isolation. Cell preparations highly enriched for mammary epithelial cells were obtained by previously reported protocols (16). The number of cells was determined by Coulter Counter before isolation of genomic DNA (17). For isolation of liver DNA, 1 g of liver was collected and genomic DNA was isolated by the phenol/chloroform extraction method (18). DNA was quantified by UV spectrometry. At 50 days of age, the mammary epithelium was found to contain ≈3.5×10^7 cells. When mammary

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: NMU, *N*-nitroso-*N*-methylurea; DMBA, dimethylbenz[*a*]anthracene; MAMA, mismatch amplification mutation assay.

*To whom reprint requests should be addressed.

†NMU causes primarily G to A transitions in both bacterial (2) and mammalian (3) cells.

‡Although DMBA forms potentially mutagenic adducts at adenine residues (thus, the possibility of inducing A to T transversion exists), it also forms potentially mutagenic adducts at guanine residues (4). Currently, it is not known which DMBA adducts give rise to mutations.

glands were divided into five sectors, each contained on average 7×10^6 cells (see Fig. 2*A*).

MAMA. MAMA is based on the observation that a PCR primer carrying double mismatches at the 3′ end is extended by *Taq* DNA polymerase at a much lower efficiency than a primer carrying a single mismatch at the ultimate or penultimate 3′ position (15). The limit of detection of MAMA stems from occasional extension by *Taq* polymerase of double mismatch primers (R.S.C., unpublished observation). For the rat genomic DNA, the experimental conditions used here, and the primer specific for the *Hras* 12th codon G to A transition, this limit (10^{-5}) is equivalent to about five mispriming events in the first few cycles of primer extension.

MAMA was performed as described in detail elsewhere (15). To have a useful single DNA sample to use as a concurrent negative control, we took advantage of our early observation that rat liver DNA samples gave uniformly lower MAMA results than most mammary DNA samples. We thus prepared large batches of rat liver DNA and with each day's studies included an aliquot of liver DNA as a control for unintended variation in technique, assay conditions, or the possibility of laboratory cross contamination. Quantitative positive control sets for each experiment were prepared by mixing 5 μg of genomic DNA (1.5×10^6 copies of the haploid rat genome) isolated from untreated liver tissue with tumor DNA containing 0, 15, 150, or 1500 copies of the specific G to A mutation. A 10-μl aliquot from each MAMA reaction mixture was analyzed on a polyacrylamide gel, and radioactivity present as the amplified fragment was measured by PhosphorImager analysis (Molecular Dynamics).

Samples were assayed on at least two separate occasions with concurrent positive and negative control samples. Student's *t* test was used to determine whether the mean sample signals (cpm) recorded on the PhosphorImager were significantly greater than the mean concurrent negative control values at the 95% ($P < 0.05$) confidence limit (19). This definition was rigorously applied to determine whether a tissue or tissue sector contained a signal (cpm) significantly greater than the concurrent negative control.

RESULTS

Analysis of 50-Day-Old Untreated Animals. Genomic DNA samples extracted from all of the mammary epithelial cells isolated from each of five 50-day-old untreated rats were subjected to MAMA. Three of five rats had a positive MAMA response, each corresponding to a *Hras* mutant fraction of 1×10^{-5} (Fig. 1). Two of five showed no significant numbers of mutants relative to concurrent negative controls. Since we could reproducibly isolate $\approx 3.5 \times 10^7$ mammary epithelial cells from the mammary tissue of a 50-day-old rat, the MAMA analysis indicated that three of five untreated rats carried ≈350 *ras* mutants among the mammary cells prior to carcinogen exposures. When 1-g liver samples from the same five untreated animals and three additional animals were examined, only one of eight yielded a statistically significant positive signal (Fig. 1*B*, lane 7). However, because 10^9 diploid cell equivalents of liver cell DNA were isolated in 1 g of tissue, this single positive liver sample contained $\approx 10^4$ *ras* mutants. Subsequent assays of 72 liver samples yielded no additional example of a positive *Hras* MAMA.

MAMA Analysis of Sectored Mammary Tissue from Untreated Animals. If each of the 350 mutant *Hras* alleles detected in the mammary epithelium of a 50-day-old rat arose as a result of an independent mutational event, the rate of single-base-pair mutations (10^{-5} mutations per bp × generation) would have to be several orders of magnitude above the spontaneous rates of mutations detected in other genes (10^{-9}–10^{-10} mutations per bp × generation) (20). Alternatively, these mutant cells could have originated from a single

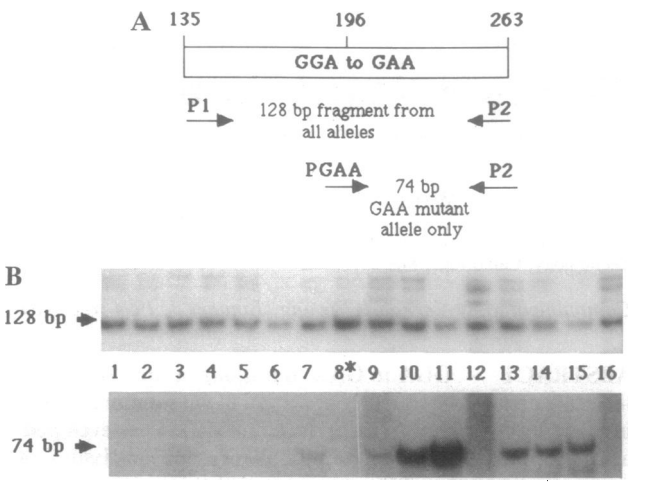


FIG. 1. Detection of mutant *Hras* alleles in liver and mammary glands of untreated 50-day-old rats. (*A*) For the allele-specific amplification, the mutant allele-specific PGAA primer and a downstream P2 primer were used. In a separate reaction, the P1 and P2 primers were used to amplify both the wild-type and the mutant alleles present in each DNA sample and served as controls for PCR efficiency. The expected sizes of PCR products using P1 and P2, or PGAA and P2, are 128 or 74 bp, respectively. The primary sequences of primers P1, P2, and PGAA are reported elsewhere (15). (*B*) Autoradiogram of MAMA reaction products. Genomic DNAs were subjected to standard MAMA reaction and the ^{32}P-labeled products were analyzed on an 8% polyacrylamide gel. Lanes: 1–8, liver genomic DNA samples from eight different rats; 12–16, mammary epithelial cell genomic DNA samples isolated from five different rats; 8–11, quantitative control sets representing the *Hras* gene mutant fractions of 0, 10^{-5}, 10^{-4}, and 10^{-3}, respectively. Liver genomic DNA sample 8 was used as the control wild-type DNA.

cell that acquired the mutation during an early stage of mammary development and gave rise to a colony of mutant cells during organ growth. These two possibilities could be distinguished by examining the pattern of *ras* mutant distribution in the mammary epithelium in the manner introduced by Luria and Delbruck (21) to distinguish among analogous possibilities in phage mutagenesis. Independent mutations would distribute uniformly throughout the organ, whereas a single early developmental mutation would yield a mutant cell cluster within a tissue sector. We divided the mammary tissue of six additional 50-day-old rats into five sectors, each containing $\approx 7 \times 10^6$ epithelial cells (Fig. 2*A*; Table 1). The DNA of each sector was subjected to MAMA. As shown in Fig. 2*B*, 8 of 30 sectors analyzed had a statistically significant MAMA signal ($P < 0.05$), consistent with the notion that the *ras* mutants arose as a result of early developmental mutations. Among 15 additional 1-g liver sectors derived from three of these animals, no positive signals were detected (data not shown).

MAMA Analysis of NMU-Treated Animals. DNA was isolated from mammary epithelial cells, including those in incipient tumors, at 1, 3, 7, 30, 60, 90, 150, 210, and 280 days after NMU treatment. In Fig. 3, the logarithm of the average number of mutants observed per rat from all treated animals is plotted as a function of time. The number of mutants immediately after the treatment (1 and 3 days) was not significantly greater than in untreated animals, indicating that NMU did not induce a detectable number of *ras* mutants. Observations on 42 treated rats, distributed over days 7–90 after treatment, indicated that the average number of mutant mammary cells per animal increased from 200 to ≈1000 (i.e., 5-fold increase). The number of total mammary epithelial cells increased from 3.5×10^7 to 4.5×10^7 (i.e., 1.3-fold) during this period. Thus, it appeared that the preexisting *ras* mutants in the mammary epithelium acquired a selective

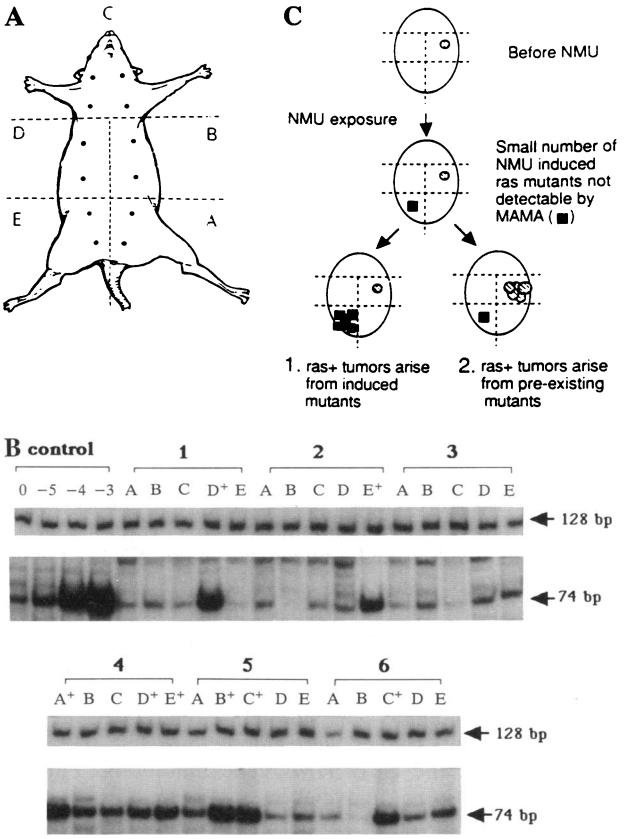


FIG. 2. MAMA analysis of sectored mammary tissues from 50-day-old untreated rats. (*A*) Mammary tissue from each animal was divided into five roughly equivalent sectors. Sectors A, B, D, and E contained two individual mammary glands while sector C contained four mammary glands. Each sector on average contained $\approx 7 \times 10^6$ epithelial cells. (*B*) Autoradiograms of MAMA reaction products (see Fig. 1) obtained from samples of genomic DNA isolated from 30 sectors isolated from six different rats. Analyses were carried out in duplicate; + denotes those samples with a *ras* mutant number that was significantly higher ($P < 0.05$) than the background (control 0; see *Materials and Methods*). For each positive sample, mutant number was determined by comparing the radioactivity incorporated into the mutant-specific 74-bp band in each sample to that of the quantitative control set (also see Table 1). The 128-bp band in each sample represents the control PCR product using primers P1 and P2 (see Fig. 1). (*C*) Alternative pathways of acquiring NMU-induced mammary lesions containing the *ras* mutation. Tumors arise from a small number of *ras* mutants directly induced by NMU (pathway 1) or from preexisting *ras* mutants (pathway 2).

growth advantage as a result of the NMU treatment. At 150 days posttreatment and thereafter, a more rapid increase in mutant cell number occurred and coincided with the appearance of macroscopic tumors, most of which (7/12) carried the 12th codon G to A mutation (data not shown). The *ras* mutant fraction in 220-day-old untreated animals (0.7×10^{-5}) was comparable to that of the 50-day-old untreated animals (0.5×10^{-5}), indicating that the outgrowth of the *ras* mutants is a NMU-mediated effect (unpublished data). Among 54 1-g liver tissue samples isolated from 54 animals sacrificed between 1 and 90 days after NMU exposure, no statistically significant positive signal was detected (data not shown).

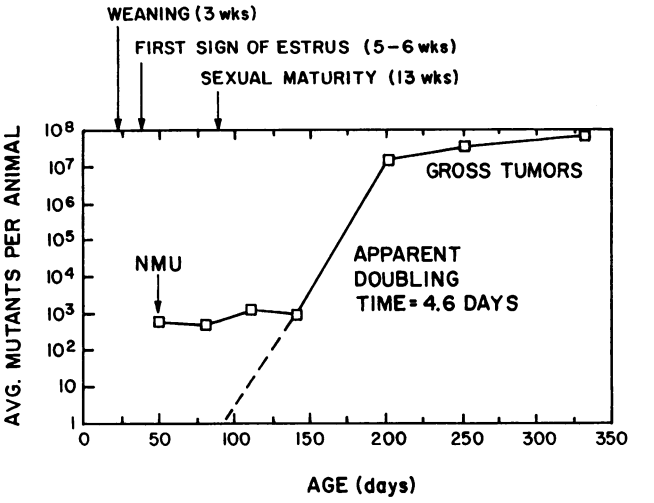
The fraction of animals with detectable *ras* mutants in their mammary epithelium was ≈0.7 (8/11) before NMU treatment and remained unchanged at 0.7 (46/68) through 280 days posttreatment (Fig. 3). The fraction of *ras*-positive animals was thus found to be unaffected by the treatment, suggesting that the majority of tumors with *Hras* mutations arose in animals with preexisting mutations.

Table 1. Distribution of *Hras* mutant cells in sectored mammary tissue of 50-day-old untreated Fischer 344 rats

Animal	Sector*	Mutant fraction × 10^{-5}	Mutants per sector†	Mutants per animal‡
50-1	D	2.1	150	150
50-2	E	1.4	100	100
50-4	A	1.6	110	
	D	0.7	50	
	E	1.2	80	240
50-5	B	2.8	200	
	C	2.1	150	350
50-6	C	1.7	120	120
		Average no. of mutants	32§	162
		ras-positive sectors	8/30 (26.3%)	

*Sectors containing MAMA signals that were significantly higher ($P < 0.05$) than that of the background (see Fig. 2*B*).
†Mutant fraction × (7×10^6 cells). (Also see Fig. 2*A* legend.)
‡Sum of mutants per sector within a given animal.
§Average number of mutants per sector including both positive and negative mammary tissue sectors. This number is calculated by the sum of *ras* mutants in six animals divided by 30 sectors.

MAMA of Sectored Mammary Tissue from NMU-Treated Animals. We could not, however, rule out the possibility that NMU directly induced a small number of *ras* mutants that were not detectable by the MAMA but were the actual tumor precursors. If this were the case, the number of *Hras*-positive sectors must increase as the newly induced mutants grow to



Days after NMU treatment	0*	1†	3†	7†	30	60	90	150	210	280
Avg. mutant fraction (x 10^{-5})	0.5	0.3	0.6	1.1	1.4	3.0	2.0	-	-	-
Avg. mutants per animal‡	183	104	197	380	510	1230	900	1.4 e7	3.4 e7	6.7 e7
Fraction of ras+ animal§	8/11	5/9	6/10	8/9	7/9	3/6	4/6	4/6	3/6	6/7

FIG. 3. Expansion of the *Hras* mutant cell population after NMU exposure. The logarithm of the average mutant cell number per animal before and after NMU treatment is plotted as a function of age. The average mutant number per animal represents the sum of the *ras* mutant alleles present in both the macroscopically normal mammary tissues and the gross tumors within the same animal. The mutant fraction of tumor tissue samples was determined by denaturant gradient gel electrophoresis (22, 23). Also indicated are the average time points for several relevant developmental milestones of the rat (24). *, Time point before NMU treatment (50-day-old rats). Data shown here include results from five untreated rats shown in Fig. 1 and six additional untreated rats analyzed in Fig. 2*B*. †, These three points were omitted from the graph due to space constraints. ‡, Average *ras* mutants per animal was calculated by (average mutant fraction) × (total number of mammary epithelial cells per rat at each time point). §, Presented are the number of animals without any detectable *Hras* mutant cells in both macroscopically normal and cancerous mammary tissue over the total number of animals analyzed at each time point.

form tumors (Fig. 2C, pathway 1). On the other hand, if NMU-induced tumors arose only from colonies of the preexisting *ras* mutants, the number of sectors containing the mutant *Hras* allele should remain constant before and after NMU treatment (Fig. 2C, pathway 2). MAMA was thus performed on sectored mammary tissue of seven NMU-treated animals 160 days after NMU exposure, and the results were compared to those of the untreated animals (Table 2). The average number of mutants per sector increased >10-fold during this period, but the fraction of mutant sectors did not increase (8/30 in untreated animals versus 8/34 in treated animals). The probability that all tumors arose in sectors with preexisting *Hras* mutants by chance is negligible. These results indicated that NMU-induced tumors containing the specific *ras* mutation assayed arose from cells with preexisting *ras* gene mutants (Fig. 2C, pathway 2) and not from NMU-induced *ras* mutants.

DISCUSSION

To test the hypothesis that the specific G to A transition in codon 12 of the *Hras* gene detected in NMU-induced rat mammary tumors was caused by NMU, we applied MAMA (15) to measure the number of *ras* mutant alleles in the target mammary epithelium before and after NMU exposure. We found that the specific oncogenic *ras* mutants existed before NMU treatment and that treatment did not increase the average number of *ras* mutants (250 mutants per animal), the fraction of animals carrying *ras* mutants (70%), or the fraction of tissue sectors with detectable levels of *ras* mutants (25%).

Because our findings are in direct contradiction to the previous observations of Kumar *et al.* (25) and Lu and Archer (26), who reported zero mutants in untreated animals, we accepted a special responsibility to explore the possibility that the positive signals we detected in mammary samples from untreated animals represented procedural artifacts. One potential source of false-positive signals would be the presence of a GGA to AGA mutation at the 12th codon of the *Hras* gene in untreated animals. G to A mutations at either the first (AGA) or the second (GAA) guanine residue of codon 12 will create a single mismatch with the mismatch primer PGAA and would therefore be amplified in MAMA. To test this possibility, we designed a mismatch primer that would amplify only the AGA and not the GAA allele. Subsequent MAMA analysis with this primer demonstrated that there were no detectable AGA mutants in GAA positive samples (data not shown). Thus, the positive signals detected in mammary cells were not the result of AGA mutations in the 12th codon. A second and ever-present hazard with any PCR-based assay is contamination of untreated tissue samples with previously amplified mutant *ras* gene sequences. Part of our quality assurance plan for this research was assay of concurrent duplicate negative controls (>100) and 72 duplicate liver samples. Only one of these samples yielded a positive result (see Fig. 1, lane 7), while some 70% of whole mammary glands and 25% of mammary sectors were found to be positive. Since all the tissue analyses were concurrently performed in the same laboratory in the same manner, these data are inconsistent with the notion that the positive mammary tissue signals were coming from random contamination of our untreated DNA samples. Finally, although *Taq* is a relatively error prone polymerase (with a measured error rate of 10^{-4} per base incorporation), this polymerase induces primarily A to G transitions (27).

Not finding any compelling reason to reject our observations, we next considered why our results could appear to directly contradict the cited earlier reports. Kumar *et al.* (25) induced mammary tumors containing the 12th codon G to A transition of the *Hras* gene by treating rats with NMU at 2 days of age. We estimate that ≈200 breast epithelial cell precursors existed[§] at this stage. Had NMU mutated the *Hras* gene in even one of these 200 cells, the induced mutant fraction would have been 1/200 or 0.5%, a number much greater than the observed mutant fractions even for forward mutations induced by high concentrations of NMU in mammalian cells (2, 3, 28).

Lu and Archer (26) reported no *Hras* mutations in untreated rats even though the assay used had a sensitivity comparable to that of the MAMA (15). This discrepancy might be explained by the fact that the equivalent of only three untreated animals was studied, leaving open the possibility that the rats studied were among those (30%) that had not experienced the early *Hras* gene mutations. Furthermore, these authors analyzed on average only 8 of 12 mammary glands from any given rat. Since our data indicated that mutants are not distributed uniformly in the mammary tissue but clustered in about one sector per rat, it is possible that some of the glands that were not analyzed contained *ras* mutants. Finally, the authors used only 1 μg of genomic DNA for each analysis. A mutant fraction of 10^{-5} in this case would represent three mutant copies, which according to the Poisson distribution would be expected to vary from 0 to 7 (95% confidence limits). It is therefore possible that the assay would not reproducibly score a sample containing a mutant fraction of 10^{-5} as a positive sample. It is noteworthy that Lu and Archer (26) reported a clear clonal expansion of the *Hras* mutant cells starting 30 days after the NMU treatment as we have observed.

Our current findings reopen questions regarding the mechanisms of NMU-induced rat mammary tumorigenesis. Since there are only 200–300 *ras* mutants in the mammary epithelium of a 50-day-old rat, the NMU-mediated effects must occur at a high frequency in order that at least one of these cells may acquire the additional event(s) necessary for carcinogenesis. Since NMU is a potent mutagen (2, 3, 28), NMU could conceivably have directly mutated other critical target genes that complemented the tumorigenic potential of the *ras* mutation. However, as noted above, a mutant fraction of 1 in 200 or 300 cells is well above that expected for forward mutations in mammalian cells (2, 3, 28). The targets of NMU-induced mutagenesis might conceivably be a large set

Table 2. Fractions of animals and sectors containing *ras* mutants before and after NMU treatment

	Positive sectors	Mutants per sector		Positive animals	Mutants per animal	
		+*	±†		+*	±†
Before NMU‡	8/30 (26.3%)	120	32	8/11 (73%)	220	162
After NMU§	8/34 (23.5%)	>1600	>388	4/7 (57%)	>3200	>1880

*Number of mutants per positive sectors (animals) only. See Fig. 2B and Table 1.
†Number of mutants per positive and negative sectors (animals).
‡At 50 days of age.
§At 160 days after NMU treatment at the age of 50 days.

§The number of susceptible epithelial cells in the mammary tissue of a 2-day-old rat was estimated based on the number of target cells at 14 days of age (25) and the observation that both the weight and the surface area of mammary glands increased at a rate comparable to the total body weight and surface area during the first 3 weeks of life (24). It was also assumed that the number of cells in an organ is directly proportional to the weight of the organ. The average body weight of a rat at 1, 10, 15, and 20 days of age was 6, 21, 32, and 44 g, respectively (24). Using a linear regression analysis, the average weights at 2 and 14 days of age were estimated to be 7 and 30 g, respectively. Since there are 600–1200 target cells at the age of 14 days, multiplying this number by the ratio of the weights, 0.23, gives 140–280 target cells in mammary tissue of a 2-day-old rat.

of oncogenes or suppressor genes, some mutants of which can function as dominant negatives (29). Alternatively, the tumorigenic effects of NMU on the *ras* mutants may be mediated via nonmutagenic mechanisms. Although the half-life of NMU *in vivo* is <30 min, the NMU-induced methylation and carbamoylation of cellular macromolecules can persist for days (30, 31) and mediate a number of other biological effects including (*i*) inhibition of critical cellular enzymatic activities such as DNA repair enzymes (32) and DNA polymerases (33), (*ii*) heritable hypomethylation of daughter cell DNA (34), and (*iii*) increased cell proliferation due to cytotoxicity of NMU (28). Given that a mutant *Hras* allele expressed at normal levels is not transforming (35), NMU-mediated effects leading to overexpression of the mutated *Hras* genes are likely mechanisms for NMU-induced carcinogenesis in this tumor model.

The detection of the G to A transition at the 12th codon of the *Hras* gene in the mammary epithelium of 50-day-old untreated rats directly demonstrates that oncogenic mutations observed in chemically induced tumors arose as background mutations. This result underscores the importance of endogenous mutagens and/or spontaneous mutations in the etiology of human cancer (36, 37). G to A transitions represent one of the most frequently observed spontaneous mutations (38), which may arise as a result of cytosine deamination (39) or from exposure to endogenously produced methylating agents such as nitrosamines (40). If such mutations occur early during development, they could give rise to colonies of cells carrying the activated oncogene (41). If these mutant cells have an increased probability of acquiring a cancer phenotype, then tumors carrying this particular mutation would be observed most frequently.

The type of *Hras* mutation detected in a small fraction of DMBA-induced mammary tumors was not the same as in NMU-induced tumors (1). It is thus likely that in DMBA-induced carcinogenesis, the *Hras* gene is a critical target for mutation by DMBA. The development of a MAMA, which measures this specific 61st codon mutation in the *Hras* gene, should make it feasible to address this question.

Readers should note that our approach—direct measurement of mutants in untreated tissues—will not be applicable if the number of preexisting oncomutants is below the limit of detection of the method used. For instance, if there had been only one or two cells in the preexisting *Hras* mutant sectors, we could not have detected them with MAMA and could not have drawn our present conclusions.

In conclusion, we have demonstrated that NMU does not induce the 12th codon GGA to GAA transition in the *Hras* gene in NMU-induced mammary tumors. This current finding is also in agreement with Mironov *et al.* (42), who have recently reported the absence of a mutagenic O^6-methylguanosine adduct at the 12th codon of the *Hras* gene in the rat genomic DNA treated with NMU *in vitro*. Our observations, however, should not be interpreted to mean that there is no association between oncomutations and carcinogen in other specific cases. The current findings do not contest the importance of *Hras* gene mutations in tumorigenesis or the importance of chemicals in the etiology of animal and human cancers. However, they clearly illustrate the pitfalls associated with attempts to establish etiology by comparing the types of oncogene mutations seen in tumors with the mutagenic specificity of the suspected carcinogen measured in other cells and DNA sequences.

We thank Ms. Kris Hewes for her help in administering NMU via tail vein injection. This work was supported by National Institute of Environmental Health Sciences Grants PO1ES05622-02 (Molecular Biomarkers) and PO1ESO3926-08 (Genetics and Toxicology) and the Office of Health and Environmental Research, U.S. Department of Energy Grant DE-FG02-86ER60448.

1. Zarbl, H., Sukumar, S., Arthur, A. V., Martin-Zanca, D. & Barbacid, M. (1985) *Nature (London)* **315**, 382–385.
2. Richardson, K. K., Richardson, K. K., Crosby, R. M., Swenberg, J. A. & Skopek, T. R. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 344–348.
3. DuBridge, R. B., Tang, P., Hsia, H. C., Leoning, P. M., Miller, J. H. & Calos, M. P. (1987) *Mol. Cell. Biol.* **7**, 379–387.
4. Dipple, A., Pigott, M., Moschel, R. C. & Constantino, N. (1983) *Cancer Res.* **43**, 4132–4134.
5. Quintanilla, M., Brown, K., Ramsden, M. & Balmain, A. (1986) *Nature (London)* **322**, 78–80.
6. Balmain, A. & Brown, K. (1988) *Adv. Cancer Res.* **51**, 147–182.
7. Reynolds, S. J., Stowers, S. J., Patterson, R. M., Maronpot, R. R., Aaronson, S. A. & Anderson, M. W. (1987) *Science* **237**, 1309–1312.
8. Zhang, R., Haag, J. D. & Gould, M. N. (1990) *Cancer Res.* **50**, 4286–4290.
9. Guerrero, I., Corces, V. & Pellicer, A. (1984) *Nature (London)* **225**, 1159–1162.
10. Newcomb, E. W., Diamond, L. E., Sloan, W. R., Corominas, M., Guerrerro, I. & Pellicer, A. (1989) *Environ. Health Perspect.* **81**, 33–37.
11. You, M., Wang, Y., Lineen, A. M., Gunning, W. T., Stoner, G. D. & Anderson, M. W. (1992) *Carcinogenesis* **13**, 1583–1586.
12. You, M., Wang, Y., Stoner, G., You, L., Maronpot, R., Reynolds, S. H. & Anderson, M. W. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 5804–5808.
13. You, M., Candrian, U., Maronpot, R. R., Stoner, G. D. & Anderson, M. W. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 3070–3074.
14. Belinsky, S. A., Devereux, T. R., Maronpot, R. R., Stoner, G. D. & Anderson, M. W. (1989) *Cancer Res.* **49**, 5305–5311.
15. Cha, R. S., Zarbl, H., Keohavong, P. & Thilly, W. G. (1992) *PCR Methods Appl.* **2**, 14–20.
16. Richards, J. R., Larson, L., Yang, J., Guzman, R., Tomooka, Y., Osborn, R., Imagawa, W. & Nandi, S. (1983) *J. Tissue Cult. Methods* **8**, 31–37.
17. Qiagen (1990) *The Qiagenologist: Applicative Protocols* (Qiagen, Inc., Chatsworth, CA).
18. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
19. Hogg, R. V. & Tanis, E. A. (1983) *Probability and Statistical Inference* (Macmillan, New York), pp. 422–427.
20. Aidoo, A., Lyn-Cook, L. E., Mittelstaedt, R. A., Heflich, R. H. & Casciano, D. A. (1991) *Environ. Mol. Mutagen.* **17**, 141–151.
21. Luria, S. E. & Delbruck, M. (1943) *Genetics* **28**, 491–511.
22. Fischer, S. G. & Lerman, L. S. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 1579–1583.
23. Sheffield, V. C., Cox, D. R., Lerman, L. S. & Myer, R. M. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 232–237.
24. Sinha, Y. N. & Tucker, H. A. (1966) *Am. J. Physiol.* **210**, 601–605.
25. Kumar, R., Sukumar, S. & Barbacid, M. (1990) *Science* **248**, 1101–1104.
26. Lu, S.-J. & Archer, M. C. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 1001–1005.
27. Keohavong, P. & Thilly, W. G. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 9253–9257.
28. Jenson, J. C. & Thilly, W. G. (1986) *Mutat. Res.* **160**, 95–102.
29. Levine, A. J., Momand, J. & Finlay, C. A. (1991) *Nature (London)* **351**, 453–456.
30. Cox, R. & Irving, C. C. (1979) *Cancer Lett.* **6**, 273–278.
31. Lu, S. J., Chaulk, E. J. & Archer, M. C. (1992) *Carcinogenesis* **13**, 857–861.
32. Fornace, A. J., Kurt, W. K. & Kann, H. E. (1978) *Cancer Res.* **38**, 1064–1069.
33. Chuang, R. Y., Laszlo, J. & Keller, P. (1976) *Biochim. Biophys. Acta* **425**, 463–468.
34. Boehm, T. L. J. & Drahovsky, D. (1981) *Carcinogenesis* **2**, 39–42.
35. Finney, R. E. & Bishop, J. M. (1993) *Science* **260**, 1524–1527.
36. Loeb, L. A. (1989) *Cancer Res.* **49**, 5489–5496.
37. Ames, B. N. (1989) *Mutat. Res.* **214**, 41–46.
38. Bains, W. & Bains, J. (1987) *Mutat. Res.* **179**, 65–74.
39. Bird, A. P. (1980) *Nucleic Acids Res.* **8**, 1499–1504.
40. Lijinsky, W. & Greenblatt, M. (1972) *Nature (London)* **237**, 177.
41. Thilly, W. G. (1989) *IARC Monogr.* **89**, 486–492.
42. Mironov, N. M., Bleicher, F., Martel-Planche, G. & Montesano, R. (1993) *Mutat. Res.* **288**, 197–205.