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Reduction in the Frequency of Activated *ras* Oncogenes in Rat Mammary Carcinomas with Increasing *N*-Methyl-*N*-nitrosourea Doses or Increasing Prolactin Levels[1]

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ABSTRACT

The role of c-Ha-*ras*-1 oncogene activation in the multistage biological process of *N*-methyl-*N*-nitrosourea (NMU)-induced mammary carcinogenesis was investigated. The average yield of NMU-induced mammary tumors in Wistar-Furth rats was altered by modification of either the initiation or promotion/progression stage of carcinogenesis. Initiation was varied by the use of different doses of NMU from 20 to 50 mg/kg. Tumor yield was increased with increasing NMU doses. However, the frequency of mammary tumors with activated c-Ha-*ras*-1 decreased in a linear fashion with increasing NMU doses. Promotion/progression was varied by increasing prolactin levels starting approximately 2 weeks after NMU administration. This hormonal manipulation increased tumor yield, while reducing the frequency of tumors with activated *ras*. It is postulated that *ras* activation represents one of several possible mechanisms by which NMU initiates mammary carcinogenesis. Furthermore, initiated cells without activated *ras* are more dependent on epigenetic promotional events provided by either prolactin or NMU than are *ras*-initiated cells.

INTRODUCTION

Activated cellular protooncogenes have been described in a large variety of both human and animal tumors. While the association between oncogene activation and carcinogenesis is strong, only limited mechanistic information is available that places oncogene activation into the multistage carcinogenic process. An important model with which to approach biological mechanisms of oncogene action was described by Zarbl *et al.* (1), who demonstrated that when Sprague-Dawley, Buffalo, and F344 rats were given the carcinogen NMU,[3] they developed mammary carcinomas. Approximately 75% of these tumors had activated c-Ha-*ras*-1. This activation uniformly occurred through a G to A transition at the second base of the 12th codon. In contrast, when mammary tumors were initiated by the polycyclic aromatic hydrocarbon 7,12-dimethylbenz(a)-anthracene, only about 25% of the resulting tumors had activated c-Ha-*ras*-1 oncogenes. This activation was limited to changes in the 61st codon. Based on this carcinogen specificity and the very short biological half-life of NMU, it was argued that in chemically induced rat mammary tumors, *ras* mutation occurred shortly after carcinogen administration.

It is not yet clear if the mutational activation of *ras* plays a role early in the carcinogenesis process and acts as an initiation lesion or acts later as a postinitiation event or both. If *ras* activation following NMU exposure represents an initiation lesion, it is not known if it is the predominant initiation lesion caused by this carcinogen. In order to investigate the role of *ras* activation in multistage carcinogenesis, we altered mammary tumor yield by modifying either the initiation or promotion/progression stage of the mammary carcinogenesis process, measuring *ras* activation in mammary carcinomas arising in these treated rats. Tumor initiation was modulated by varying the dose of the direct-acting carcinogen NMU. NMU was chosen for both its short biological half-life and high yield of activated *ras*-containing mammary tumors (1, 2). Tumor yield was also enhanced by hormonal manipulation during the promotion/progression stage of carcinogenesis. We have previously demonstrated that increasing prolactin levels while decreasing corticosterone following either chemically (3) or physically (4) initiated mammary carcinogenesis consistently raised the average number of mammary carcinomas per rat while decreasing their latency time. The effects of these alterations in the NMU mammary model on the frequency of carcinomas with activated *ras* are presented here.

MATERIALS AND METHODS

Tumor Induction. Specific-pathogen-free virgin female Wistar-Furth rats were obtained from Harlan Sprague-Dawley, Inc., Madison, WI, and housed under a 12-h light–12-h dark cycle. Rats were fed Teklad Lab Blox chow *ad libitum*. Rats (50–55 days of age) were given i.v. injections of a single dose of NMU (Ash-Stevens, Inc., Detroit, MI). NMU was dissolved in 0.9% NaCl solution acidified to pH 5.0 with acetic acid. Fresh solutions were prepared every 20 min.

In the first experiment, rats were treated with one of three doses (20, 30, or 50 mg/kg) of NMU. The 30-mg/kg NMU dose was the dose used by Sukumar *et al.* (2) to establish this mammary *ras* model. The lowest dose group contained 40 rats while the two higher dose groups contained 30 rats each. In the second experiment, two groups of 25 rats were given 50 mg/kg of NMU. Two weeks following NMU administration, one group was hormonally modified to increase the levels of circulating prolactin and lower the blood levels of glucocorticoids. Increased levels of prolactin were achieved by grafting a single anterior pituitary from an isologous rat adjacent to silicon capsules containing estrone into the spleens of the recipient rats. The estrone directly stimulates release of prolactin from the grafted gland. Prolactin and estrone enter the portal circulation, where one-pass hepatic clearance of estrone occurs. High peripheral prolactin titers are achieved without hyperestrinism (4). Circulating glucocorticoids were lowered by adrenalectomy. Adrenalectomized rats were maintained with weekly injections of 2.5 mg deoxycorticosterone and allowed to drink isotonic saline *ad libitum*.

Rats in both groups were palpated weekly for mammary tumors beginning 4 weeks after NMU injection. Tumors were surgically resected when they reached a size of approximately 1 cm^3 and the rats were returned to the experiment. All remaining mammary tumors were removed at the termination of the experiments. Tumors were rapidly removed and divided for histopathology and molecular analysis. Samples for molecular analysis were flash frozen in liquid nitrogen and stored at −70°C.

***ras* Analysis.** DNA was purified from 285 tumor samples by phenol-chloroform extraction and ethanol precipitation (5). Genomic DNA was digested with *Mnl*I at 37°C overnight under the conditions recommended by the supplier (New England Biolabs). A 231-base pair segment, which flanks codon 12 of the first exon in the H-*ras* gene, was amplified exponentially by means of the PCR (6). The 5′ 20-mer primer (5′TTGCCTACTCAGTGCTGGAG3′) was located in the first

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[3] The abbreviations used are: NMU, *N*-methyl-*N*-nitrosourea; PCR, polymerase chain reaction; RIA, radioimmunoassay.

intron while the 3′ primer (5′CATACTCGTCCACAAAATGG3′) was located downstream of the 12th codon. Twenty μl of PCR product were loaded onto a 4% agarose gel (3% NuSieve and 1% SeaKem) and subjected to electrophoresis. DNA was transferred to Gene Screen nylon filter (DuPont) by the method of Southern (7). DNA was further fixed by baking the filters at 85–90°C for 2–4 h. Filters were prewashed and hybridized under stringent conditions (57°C) for 24 h with a 19-mer oligonucleotide probe (5′GCGCTGAAGGCGTGGGAAA3′) ($1–4 \times 10^5$ cpm/ml) which was 5′-end labeled by phosphorylation with [^{32}P]ATP and T_4 polynucleotide kinase (New England Biolabs) and purified using NAP-10 columns (Pharmacia). The hybridized filters were washed twice in washing buffer (DuPont manual) at room temperature for 5 min. The filters were autoradiographed (Kodak XAR) with an intensifying screen at −70°C for 16 h. Each gel contained normal mammary DNA as a negative control, and DNA from a known codon 12-mutated *ras* mammary tumor and/or the plasmid p-NMU (2), which contains a codon 12-activated rat *ras* gene. In a limited number of tumors, including those presented in Fig. 5, results of this assay were confirmed by the methods of Kumar and Dunn (8).

Activation of K-*ras* by a similar mutation in codon 12 was also analyzed by PCR-allele-specific probing as described above for c-Ha-*ras*. For this assay, the 5′ 20-mer primer was 5′GCCTGCTGAAAATGACTGAG3′ and the 3′ 20-mer primer was 5′TGATTCTGAATTAGCTGTAT3′. The allele-specific 20-mer probe used to detect activated K-*ras* was 5′TTGGAGCTGATGGCGTAGGC3′. Rat normal mammary DNA was used as a negative control. DNA from a NMU-induced rat kidney tumor kindly provided by Dr. S. Sukumar was used as a positive control.

Prolactin RIA. NMU-treated rats (n = 21) that received pituitary grafts and matched control rats (n = 21) were bled at defined morning hours under ketamine anesthesia. Serum samples obtained 16 weeks after pituitary grafting were kept frozen at −70°C until assay. Prolactin RIA was performed in triplicate according to the method of Niswender *et al.* (9). Specific reagents for rat prolactin RIA were obtained from the National Pituitary Agency through the Hormone Distribution Office, National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, NIH (Bethesda, MD).

RESULTS

Evaluation of the Sensitivity of Detection of *ras* Mutations. Mammary carcinomas contain tumor cells with varying but often high levels of normal stromal cells. It is also possible that under varying biological situations mammary tumors may be heterogeneous for the presence of carcinoma cells with activated *ras*. We thus modified previous methods (1, 2) to detect *ras* mutations in tumor samples, in order to increase sensitivity while maintaining both reliability and efficiency for screening large numbers of tumors. After testing several methods, we chose the one described above, which combines predigestion of DNA with *Mnl*I, which cuts between the PCR primer complementary sequences in only nonmutated *ras* DNA, followed by PCR amplification and allele-specific oligonucleotide hybridization. Detection of alterations in the pseudogene c-Ha-*ras*-2 was avoided by placing the upstream PCR primer into a region of the c-Ha-*ras*-1 gene that lacks a high degree of homology with the c-Ha-*ras*-2 gene.

In order to test the sensitivity of our detection method, we purified DNA from a NMU-induced rat mammary carcinoma that contained activated *ras* gene mutated at codon 12. This carcinoma was previously analyzed by comparison to the plasmid p-NMU, which contains a rat H-*ras* gene with a codon 12 mutation (2). This tumor DNA was then serially diluted with DNA from normal mammary gland. Final filters were analyzed both by autoradiography and direct quantitative ^{32}P-scanning with an Ambis detector. Results shown in Fig. 1 suggest that this method can reliably and unambiguously detect mutated ras if present in only 1% of the tumor cell population.

Dose Response Study. Fig. 2 shows that, as the NMU dose was raised from 20 to 50 mg/kg, the average number of tumors per rat increased almost 5-fold. Greater than 95% of all tumors were carcinomas. Fig. 3 shows that as the NMU dose was

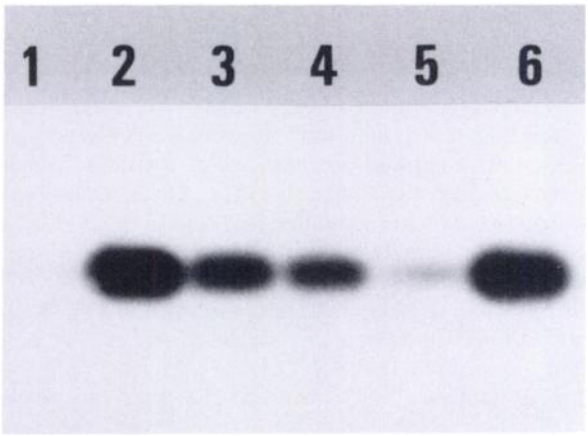

Fig. 1. Sensitivity of the detection assay for H-*ras* mutation. An *Mnl*I digestion-PCR amplification-oligonucleotide hybridization method was used as described in "Materials and Methods." *Lane 1*, normal DNA; *Lane 2*, mixture of normal DNA with H-*ras*-mutated tumor DNA in 1:1 ratio; *Lane 3*, mixture of normal DNA with H-*ras*-mutated tumor DNA in 10:1 ratio; *Lane 4*, mixture of normal DNA with H-*ras*-mutated tumor DNA in 100:1 ratio; *Lane 5*, mixture of normal DNA with H-*ras*-mutated tumor DNA in 1000:1 ratio; *Lane 6*, H-*ras*-mutated tumor DNA.

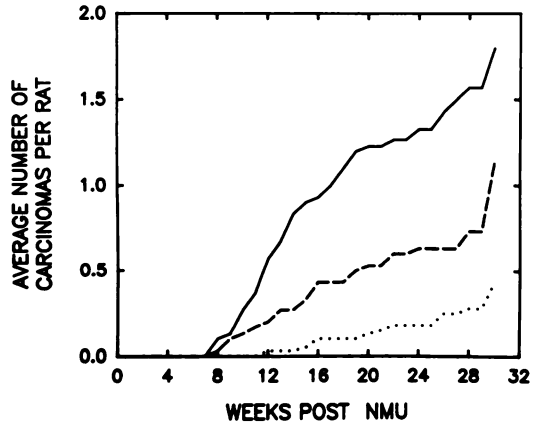

Fig. 2. Average number of mammary tumors developing in WF female rats following different doses of NMU: 50 mg/kg (——, n = 30), 30 mg/kg (– – –, n = 30), 20 mg/kg (· · · · ·, n = 40).

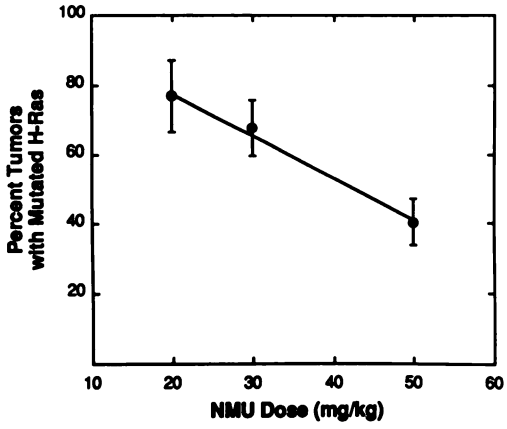

Fig. 3. Relationship of NMU dose and percentage of mammary tumors with c-Ha-*ras*-1 activation. *ras* activation was assayed as described in the text. The data fit an inverse linear model (r = 0.996). *Bars*, SE.

increased, the frequency of mammary tumors with activated *ras* decreased in an inverse linear relationship ($r = 0.996$). However, it is interesting to note that, while the average number of tumors per rat continued to increase with doses of NMU from 20 to 50 mg/kg, the average number of tumors with activated *ras* per rat only increased from a dose of 20 to 30 mg/kg and then remained constant when the dose was raised to 50 mg/kg (Fig. 4).

Twenty-five carcinomas that were negative for H-*ras* activation and 5 that were positive were assayed for activation of K-*ras* (codon 12). All tumors were negative for K-*ras* activation. A representative autoradiogram in which activated H-*ras* and K-*ras* were analyzed is shown in Fig. 5. Detailed data of this dose response study are presented in Table 1.

Effects of Prolactin. Rats were hormonally modified to raise prolactin by pituitary grafting as described above. Treated rats ($n = 21$) were analyzed for serum prolactin levels 16 weeks postgrafting and compared to controls ($n = 21$). The control rats had a serum prolactin level of 2.5 ± 1.8 (SE) ng/ml while pituitary-grafted rats had a level of 31.9 ± 2.5 ng/ml ($P < 0.001$). Pituitary-grafted rats were also adrenalectomized to reduce adrenal-produced corticosterones. No regrowth of adrenal tissue was detected at autopsy. In addition, all mammary tissue in the prolactin-enhanced rats was nonsecretory, indicating a deficiency of circulating glucorticoids.

Rats given 50 mg/kg of NMU and hormonally modified starting 2 weeks after carcinogen administration, by raising serum levels of prolactin while lowering serum levels of corticosterone, developed approximately twice as many tumors when compared to NMU-treated controls (Fig. 6). Greater than 95% of tumors in both groups were carcinomas. Rats receiving hormonal manipulation but not NMU rarely, if ever, developed tumors within a 6-month follow-up (data not shown).

The tumors that developed in the hormone-modified group had a greatly reduced frequency of H-*ras* mutations ($P < 0.025$) (Fig. 7*A*). However, it is important to note that in spite of this frequency shift, both the control and hormonally modified groups had the same ($P > 0.5$) average number of tumors with mutated H-*ras* per rat (Fig. 7*B*). In addition, 25 H-*ras*-negative and 5 H-*ras*-positive tumors were assayed for K-*ras* activation. All were negative (Table 1).

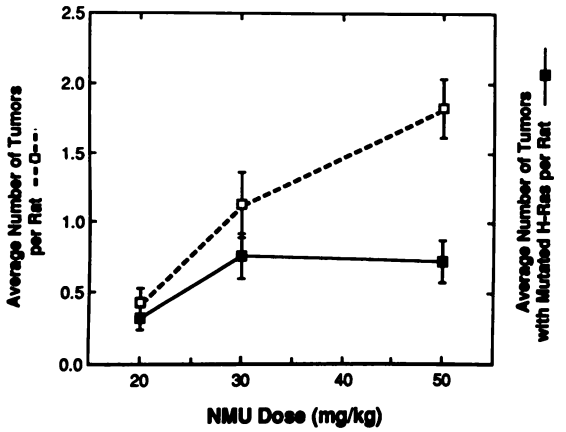

Fig. 4. Effects of NMU dose on mammary tumor formation and H-*ras* activation. Comparison of average number of tumors per rat (□) and the average number of tumors with mutated H-*ras* (■) per rat following different doses of NMU. *Bars*, SE.

DISCUSSION

It is now widely accepted that tumors arise as a result of a multistage process. Delineating the components of this process and their interrelationships is requisite to a mechanistic definition. An important component in the carcinogenesis process is the organ-specific activation of various oncogenes. We have used the NMU-induced mammary carcinoma model (10) to determine the role of *ras* oncogene activation in the biological process of rat mammary carcinogenesis. When the promotion/progression stage of the mammary carcinogenesis process was modified by hormonal manipulation following NMU exposure, the percentage of tumors with activated *ras* was decreased by half (Fig. 7*A*) as a result of an increase in total tumor yield without changing the absolute average number of tumors with activated *ras* per rat (Fig. 7*B*). These data are compatible with a hypothesis suggesting that NMU initiates mammary cells either by activation of *ras* or through other yet undefined heritable molecular events. Furthermore, cells with activated *ras* are more likely to progress than cells with non-*ras* initiation events in rats with normal physiological hormonal levels. Thus many mammary carcinomas in these rats are derived from cells with activated *ras*. However, in the presence of supraphysiological levels of the mammary tumor promoter prolactin, the cells with non-*ras* initiation events are driven to progress to frank carcinomas, while NMU-initiated *ras*-activated cells do not gain additional progression advantage from prolactin exposure. Thus, the absolute number of tumors with *ras* activation remain unchanged while the additional carcinomas arising following prolactin exposure develop from non-*ras*-initiated cells.

The above discussion pertains to experiments in which the NMU mammary carcinogenesis model was modified by hormonal manipulations during the promotion/progression stage. We also investigated the effects of modifying the initiation stage by altering the dose of NMU on the frequency of tumors with activated *ras*. It has been shown (11) and confirmed here that increasing the NMU dose in a single administration increases tumor yield. We also found that as NMU dose was increased, the frequency of tumors with activated *ras* decreased in a linear fashion. A model similar to that used to explain the prolactin-*ras* data may also be applied to the results of this dose

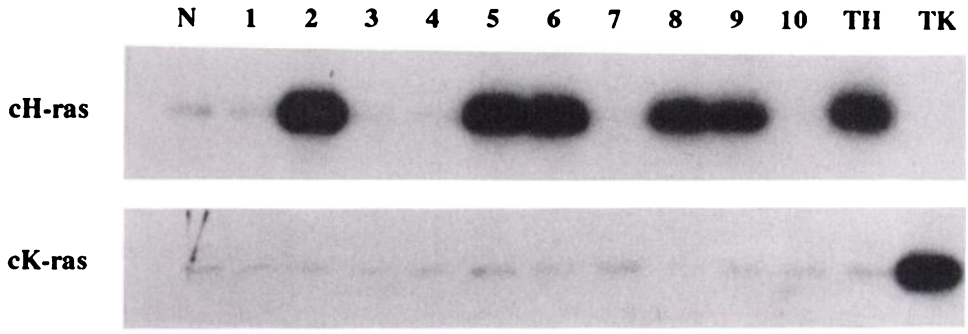

Fig. 5. Allele-specific oligonucleotide probe analysis of activated H-*ras* and K-*ras* in NMU-induced rat mammary carcinomas. DNA was isolated from carcinomas and the regions surrounding codon 12 of the *ras* genes were amplified by PCR, subjected to Southern analysis, and probed with allele-specific probes for mutated *ras* as described in the text. *Lane N*, normal Wistar-Furth rat mammary DNA; *Lanes 1–10*, DNA from NMU (50 mg/kg)-induced rat mammary tumors. *Lane TH*, positive control for H-*ras* activation from a rat mammary tumor induced with NMU (30 mg/kg). *Lane TK*, positive control for K-*ras* activation from a NMU-induced rat kidney tumor.

Table 1 *Summary of ras activation in NMU-induced rat mammary carcinomas*

Group	NMU dose (mg/kg)	Hormonal treatment	Av. no. of carcinomas/rat ± SE (total carcinomas)	No. of carcinomas with activated H-ras/no. of carcinomas assayed	Frequency ± SE (%)	No. of carcinomas with activated K-ras/no. of carcinomas assayed
A_1	50	Intact	1.80 ± 0.21 (54)	22/54	40.7 ± 6.7	0/30[a]
A_2	30	Intact	1.13 ± 0.23 (34)	23/34	67.6 ± 8.1	NA[b]
A_3	20	Intact	0.43 ± 0.10 (17)	13/17	76.5 ± 10.6	NA
B_1	50	Intact	2.72 ± 0.34 (68)	20/68	29.4 ± 5.6	NA
B_2	50	Pit/Adx	4.67 ± 0.45 (112)	17/112	15.2 ± 3.4	0/30[a]

[a] 25 carcinomas without c-Ha-ras activation and 5 with activation.
[b] NA, not assayed; Pit/Adx, pituitary implantation and adrenalectomy.

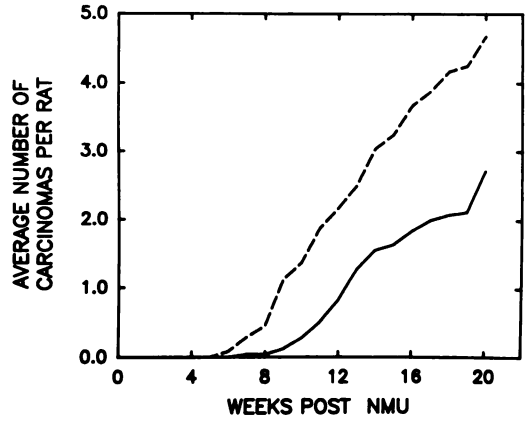

Fig. 6. Effects of increased prolactin levels on tumor incidence. The average number of mammary tumors developing in normal (——, $n = 25$) and prolactin-treated (– – –, $n = 24$) female WF rats following 50 mg/kg NMU administration is compared.

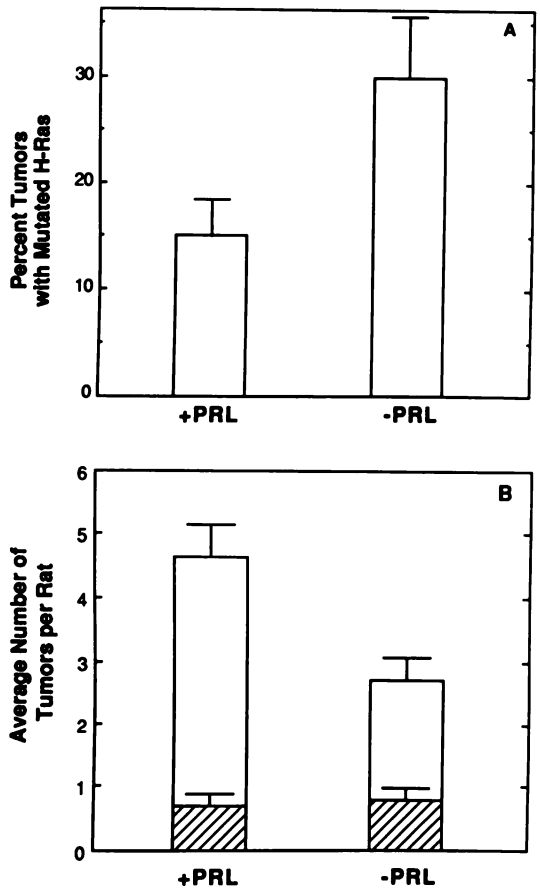

Fig. 7. Effects of increased prolactin levels on mammary tumor formation and H-*ras* activation. *A*, percentage tumors with activated *ras* compared in hyperprolactin (+*PRL*, $n = 24$) and intact (−*PRL*, $n = 25$) female WF rats. Rats were given prolactin treatment as described in the text, starting approximately 2 weeks after NMU treatment. *Columns*, means; *bars*, SE. *B*, average number of mammary tumors per rat (□) and average number of tumors with activated H-ras per rat (▨), developing in both hyperprolactin (+*PRL*, $n = 24$) and intact (−*PRL*, $n = 25$) female WF rats following 50 mg/kg NMU administration. *Columns*, means; *bars*, SE.

response study. As suggested above, NMU-initiated cells are produced by either *ras* or non-*ras* events. Most of the *ras*-initiated cells then progress to form tumors without additional hormonal treatment, while the non-*ras* initiated cells may require additional promotion/progression events such as hyperphysiological levels of prolactin. It is hypothesized that promotion events similar to that provided by prolactin are provided here by NMU-induced cellular changes. Thus, mammary cells exposed to NMU, a complete mammary carcinogen with both initiation and promotion activities, are modified in a dose-responsive manner that promotes initiated cells to progress to form carcinomas. A possible cellular promotional activity of NMU is the stable induction of hypomethylation (12). This epigenetic change which increases gene expression in mammary cells may, like prolactin treatment, drive non-*ras* initiated cells to carcinomas. The data in Fig. 4 are compatible with this model. Here the number of tumors constantly increases as NMU doses increase from 20 to 50 mg/kg. However, the absolute yield of NMU-induced tumors with activated *ras* only increases slightly at first when compared to the increase in tumor yield and then plateaus between NMU doses of 30 and 50 mg/kg. Most of the increase in total tumor yield as a function of NMU dose between 20 and 50 mg/kg (Fig. 4) is thus hypothesized to be independent of additional *ras* activation and most likely due to NMU-induced epigenetic changes that promote tumor development from initiated cells.

We speculate that the shape of the curve that presents the average number of tumors with *ras* activation per rat (Fig. 4) *versus* NMU dose is controlled by the frequency of *ras* mutations in NMU-exposed mammary cells. It is suggested that cells with activated *ras* are maximally promoted by *ras* in combination with the minimal NMU dose (20 mg/kg) examined. Thus, the rise in the curve between 20 and 30 mg/kg is due to a NMU-induced increase in the frequency of mammary cells with *ras* activation. The plateau seen at higher doses may be due to a functional saturation of mutations at this locus. This phenomenon of saturation is often seen for chemical mutagenesis of mammalian somatic cell even after correction for cell killing (13). We are currently testing the possibility of saturation of *ras* activation in mammary cells *in vivo*. The frequency of mammary cells with mutated *ras* following NMU exposure *in situ* is being measured with a modified PCR procedure (8).

In conclusion, we have shown that increasing the exposure of mammary cells to NMU increases the average number of mammary carcinomas per rat. However, the percentage of these tumors with activated *ras* decreases with increasing NMU dose. Likewise, increasing prolactin exposure of mammary cells after

NMU treatment increases the yield of carcinomas and decreases the percentage of tumors with activated *ras* but does not affect the absolute yield of tumors per rat with activated *ras*. We interpret these observations to suggest that NMU initiates mammary cells via more than one mechanism. If initiation of a given cell is by *ras* activation, then this cell may progress without additional external stimulation to form a carcinoma. Mammary cells initiated by alternative non-*ras* routes usually require additional epigenetic stimuli to progress to carcinomas. This stimulation may be provided by NMU-induced epigenetic cellular changes such as gene activation by hypomethylation, or by promoters such as prolactin, which drive initiated cells to progress to a malignant phenotype. We are currently examining the effects of prolactin and NMU promotion on mammary cells that have been infected *in situ* with v-Ha-*ras*-containing replication-incompetent retroviral vectors. In addition, we are quantitating the ratio of NMU-initiated cells and cells containing activated *ras* shortly after NMU exposure. Such data and those presented here should help define the role of *ras* activation in NMU-induced rat mammary carcinogenesis.

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