Ha-ras oncogene activation in mammary glands of N-methyl-N-nitrosourea-treated rats genetically resistant to mammary adenocarcinogenesis

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ABSTRACT A single dose of N-methyl-N-nitrosourea given to sexually immature female Buf/N rats produces a high incidence of mammary adenocarcinomas. A large percentage of these tumors contain the Ha-ras oncogene, activated by a G → A transition at the second nucleotide of codon 12. Copenhagen rats, on the other hand, are completely resistant to mammary tumor induction by a number of carcinogens, including N-methyl-N-nitrosourea. Here we show, using a sensitive method involving PCR, that codon 12 Ha-ras mutations occur in the mammary glands of both Buf/N and Copenhagen rats 30 days after N-methyl-N-nitrosourea treatment. These mutations were evenly distributed among individual mammary glands and were present in purified mammary epithelial cells. In Buf/N rats, the fraction of cells containing a mutated Ha-ras allele increased by a factor of 10--100 between 30 and 60 days, whereas in Copenhagen rats, there was no such increase during this time period. We conclude that the resistance of the Copenhagen rat to mammary carcinogenesis is not due to a defect in initiation but rather appears to be due to the inability of cells containing a mutated ras allele to undergo sustained clonal expansion.

N-Methyl-N-nitrosourea (MNU) induces a high incidence of mammary adenocarcinomas in a number of strains of rats (1). Even a single dose given to sexually immature (50-day-old) animals is sufficient to induce these tumors (2, 3). The Ha-ras oncogene, activated by a G → A transition mutation at the second nucleotide of codon 12, is found in >85% of the tumors (3, 4). MNU is known to form O6-methylguanine in DNA, leading to G → A transitions during DNA replication (5, 6). Since MNU has a half life of <1 h under physiological conditions (7), its mutagenic effects must occur within a short time after its administration. It has been suggested, therefore, that the Ha-ras gene is activated during the initiation of carcinogenesis (3, 4). In support of this notion, Kumar et al. (8) have shown the presence of an activated Ha-ras gene in mammary gland DNA as early as 2 weeks after administration of MNU to neonatal rats, which is at least 2 months before the onset of neoplasia. Furthermore, Wang et al. (9) recently demonstrated carcinoma induction after direct infusion into the central mammary duct of a replication-defective retrovirus containing v-Ha-ras.

Susceptibility to mammary gland carcinogenesis varies considerably, however, in different strains of rat. Buf/N rats, for example, are highly susceptible, developing multiple mammary tumors after a single injection of MNU at 50 days of age (3). Copenhagen rats, on the other hand, are completely resistant to mammary adenocarcinoma induction by a number of carcinogens, including MNU (10, 11). Promotion of carcinogenesis in susceptible animals is thought to be caused by the proliferation of initiated cells stimulated by estrogen-induced mammary gland development (12–14). Ovariectomy prior to or after carcinogen administration reduces mammary tumor development to negligible levels (1, 8), whereas administration of estrogens to the ovariectomized animals leads to tumor formation (8). Therefore, one possible factor leading to resistance in the Copenhagen rat might be estrogen deficiency. Isaacs (11) has demonstrated, however, that there is no difference between susceptible and resistant rats in a series of factors, including host and mammary gland growth rates, between 20 and 200 days of age. Isaacs also demonstrated that at 50 days of age, both susceptible and resistant rats are continuously proceeding through the estrous cycle; there are no differences in 17β-estradiol, progesterone, or prolactin levels at any point in their cycles (11). Breeding experiments in which resistant Copenhagen rats were crossed with highly susceptible inbred strains suggest that a single autosomal dominant tumor suppressor gene in the mammary parenchyma of Copenhagen rats confers resistance to MNU-induced mammary carcinogenesis in the hybrids (10, 11, 15, 16). However, the molecular basis of the resistance of Copenhagen rats, including the stage at which the putative tumor suppressor gene blocks mammary carcinogenesis, is not known.

To understand the molecular basis of the resistance of the Copenhagen rat to mammary carcinogenesis, we began our studies by investigating DNA methylation in rats treated with MNU. We showed that the kinetics of formation and repair of O6-methylguanine in DNA from mammary gland epithelial cells is essentially identical in the Buf/N and Copenhagen rat (unpublished data). Furthermore, we showed that the extent of methylation by MNU of a restriction fragment containing exons 1–4 of the Ha-ras gene in mammary gland DNA and the level of Ha-ras expression in mammary tissue are not different for the two strains. These studies suggested that the Ha-ras gene in Copenhagen mammary glands is not protected from carcinogen modification and should be susceptible, therefore, to mutational activation. Here we show, using a sensitive method involving PCR, that Ha-ras mutations by G → A transitions at codon 12 occur in the mammary glands of MNU-treated Copenhagen rats, and we have characterized the frequency and distribution of these mutations at 30 and 60 days after MNU treatment in both Buf/N and Copenhagen rats.

MATERIALS AND METHODS

Animal Treatment and DNA Isolation. Fifty-day-old inbred Buf/N or Copenhagen female rats (Harlan–Sprague–Dawley) were given a single injection into the tail vein of MNU (30 or 50 mg/kg of body weight, Sigma) that had been dissolved in 0.05% acetic acid in normal saline and used immediately. Control animals received vehicle only. Animals were killed 30 or 60 days after treatment, and DNA was isolated from individual mammary glands of this article.

Abbreviation: MNU, N-methyl-N-nitrosourea.

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glands or mammary epithelial cells by the method of Miller et al. (17). Briefly, the excised mammary glands were pulverized in liquid nitrogen and then digested with Pronase in the presence of 2% sodium dodecyl sulfate. After proteins were precipitated by salt, DNA was obtained by precipitation with ethanol. Mammary epithelial cells were prepared by digestion with collagenase as described by Fong et al. (18).

**Analysis of Ha-ras Mutations.** A G → A transition mutation at the second guanosine of codon 12 of the Ha-ras gene eliminates an Mnl I recognition sequence (4). Therefore, DNA samples (≏1 μg) from individual mammary glands were first digested with Mnl I (2 units; New England Biolabs) to enrich the preparation in the mutant Ha-ras allele. Mutant Ha-ras sequences were then detected by the PCR/lipid hybridization and gel retardation assay described by Kumar and Barbacid (19) with some modifications. We used Vent DNA polymerase (New England Biolabs) instead of Taq polymerase. Before the addition of the template DNA and polymerase, the mixtures containing 100 μl of 20 mM Tris, pH 8.8/10 mM KCl/10 mM (NH₄)2SO₄/0.1% Triton X-100/100 μM of bovine serum albumin per ml/6 mM MgSO₄/0.2 mM of each dNTP/0.25 μM of each primer were irradiated with 254-nm and 300-nm UV light for 20 min to eliminate any contaminating DNA (20). Thirty cycles of polymerization were carried out in a Perkin-Elmer/Cetus thermal cycler machine. The times for denaturation, annealing, and polymerization were 1, 2, and 3 min at 94°C, 62°C, and 72°C, respectively. The polymerization time increased by 5 sec per cycle. The primers were 5'-ACCCCTGTTAGAGCGAT-GAC-3' and 5'-AGGGACTCTTTCGAAAGGC-3' (8). The 3' primer contained the two mismatched bases indicated in bold, which together with a G → A mutation at codon 12, create an Xmn I restriction site in the mutant allele. The PCR product of both the normal and mutant alleles is a 71-base-pair (bp) fragment encompassing the codon 12 region of the Ha-ras gene. However, only the mutant allele is digested by Xmn I to generate 53- and 18-bp fragments.

Samples (5 μl) of PCR-amplified DNA were digested with 12 units of Xmn I in a volume of 20 μl. Reproducibility of the Xmn I digestion was confirmed for every sample. The probe, 5'-CAAGCTTGTTGTGTTGCCC-3' (8), was labeled with [γ-32P]ATP (NEN) by using T4 polynucleotide kinase (BRL) followed by precipitation with ethanol. Five microliters of the probe (5–10 × 10⁵ cpm) were added to the Xmn I digest, and the NaCl concentration was adjusted to 0.75 M in a volume of 30 μl. The mixture was incubated under mineral oil at >95°C for 10 min and then at 55°C for 2–3 hr. The samples were chilled on ice and then electrophoresed through 0.8 mm of 10% polyacrylamide gels (19:1 acrylamide/methylene-bisacrylamide) in 0.089 M Tris borate/0.025 M EDTA, pH 8.3 (TBE). Gels were run at 10 V/cm for 4–5 hr at room temperature, dried, and exposed to Kodak XAR film with an intensifying screen at ~70°C.

DNA isolated from a rat mammary adenocarcinoma cell line that is known to contain a heterozygous G → A mutation at codon 12 of the Ha-ras gene was generously supplied by S. Sukumar (Salk Institute, San Diego). The tumor DNA was mixed in various ratios prior to Mnl I digestion and PCR with DNA isolated from the mammary glands of control rats. Mixtures containing tumor/normal DNA in ratios of 10⁻⁵, 10⁻⁴, and 10⁻³ were run on each gel for comparative purposes. Negative controls were mammary gland DNA samples isolated from control rats.

**RESULTS**

To detect and quantify G → A transitions at the second nucleotide of codon 12 of the Ha-ras gene in the DNA of rat mammary glands a short time after MNU treatment, we required a method to measure small amounts of the mutated allele in the presence of a large excess of the normal allele. Our method was based on the method of Kumar and Barbacid (19) that utilized PCR amplification with a liquid hybridization and gel retardation assay. We found, however, that Taq DNA polymerase lacked the required fidelity, creating false positives in control DNA samples. Other researchers have also described this problem (21, 22). Therefore, we used Vent DNA polymerase that contains a 3'-to-5' exonuclease activity, thereby increasing the fidelity of DNA replication compared with Taq polymerase (23). Furthermore, since a G → A transition in codon 12 of the Ha-ras gene eliminates the GAGG sequence recognized by the restriction endonuclease Mnl I (4), we enriched our samples in the mutant allele by an Mnl I digestion prior to PCR amplification. This step increased the detection limit of our method by about an order of magnitude (data not shown). Finally, our PCR mixtures were irradiated with UV light (254 and 300 nm) for 20 min prior to adding the genomic DNA and polymerase. Such UV treatment has been shown to eliminate most sources of contamination (20).

The calibration gel shown in Fig. 1 illustrates our method using known dilutions in DNA from normal mammary glands of DNA from a rat mammary adenocarcinoma cell line that contains a mutated Ha-ras gene. In addition to detecting simply the presence of low levels of Ha-ras mutations, we were able to estimate the fraction of cells containing a mutant allele in individual glands (hereafter referred to as the mutant cell fraction). These fractions were estimated visually to the nearest order of magnitude with the aid of standards of tumor cell line DNA diluted in normal DNA run on each gel. Fig. 1 illustrates that the detection limit of the method was a mutant cell fraction of about 10⁻³, while fractions higher than about 10⁻³ gave a nonlinear response. When the entire method was replicated with DNA from the same glands, reproducible band intensities were obtained. Gels loaded with smaller amounts of PCR product than those illustrated in Figs. 2–4 confirmed the presence of approximately equal amounts of the normal Ha-ras allele in each lane over the range of observed mutant cell fractions (10⁻⁸–10⁻³).

Fifty-day-old female BUF/N and Copenhagen rats were injected intravenously with a single dose of either 30 or 50 mg of MNU per kg—regimens known to produce a high yield of mammary tumors in BUF/N and other susceptible rats (2, 3) but no such tumors in Copenhagen animals (10, 11). Individual mammary glands were excised and analyzed for Ha-ras mutations 30 or 60 days after treatment. At these times, the glands were macroscopically normal. Fig. 2A illustrates that

![Fig. 1. Calibration gel with known dilutions of tumor cell line DNA containing a mutated Ha-ras gene in normal mammary gland DNA. (A) PCR products (5 μl) were analyzed as described with an exposure time of 16 hr. (B) PCR products (1 μl) were analyzed as described with an exposure time of 3 hr. Lanes: 1–7, dilutions of 1 part of tumor cell line DNA in 0, 10, 10², 10³, 10⁴, and 10⁵ parts of normal DNA respectively; 8, normal DNA.](image-url)
a number of mammary glands from Buf/N rats contained a mutant cell fraction of somewhat less than \(10^{-5}\) 30 days after an MNU dose of 50 mg/kg. Sixty days after treatment, several mammary glands contained one mutant cell in \(10^2-10^4\) normal cells (Fig. 2B). Table 1 summarizes the data from a large number of such analyses. None of 24 glands from control Buf/N rats showed a codon 12 mutation at 30 days and none of 16 at 60 days after treatment. Only one rat treated with 30 mg of MNU per kg and analyzed 30 days later had no positive glands; in all other cases, individual rats had at least one positive gland. A larger fraction of the glands was positive at the higher MNU dose. Furthermore, there were no glands after 30 days, even at an MNU dose of 50 mg/kg, in which the mutant cell fraction was >\(10^{-3}\), whereas after 60 days, 5 of 32 glands had fractions of \(10^{-5}-10^{-4}\), 1 gland had a fraction of \(10^{-4}-10^{-3}\), and 1 had a fraction of >\(10^{-3}\). The distribution of mammary glands containing a mutant Ha-ras gene is shown in Table 2. It is clear that the Ha-ras mutations were rather uniformly distributed amongst the three thoracic and three inguinal pairs of glands. Within these pairs, the distribution was also uniform (data not shown).

Fig. 3 and Tables 1 and 2 present the data for Copenhagen rats. It is clear from Fig. 3 and Table 1 that a number of mammary glands from Copenhagen rats 30 or 60 days after MNU treatment contained mutated Ha-ras alleles, with mutant cell fractions of about \(10^{-5}\). Four glands taken 30 days after an MNU dose of 50 mg/kg contained mutant cell fractions of \(10^{-5}-10^{-4}\), whereas none of the glands taken at 60 days contained this high a fraction. None of 24 glands from control Copenhagen rats showed a codon 12 mutation at 30 days, and none of 16 showed it at 60 days. All MNU-treated rats at both 30 and 60 days had at least one mammary gland containing mutant cells. Furthermore, like the Buf/N rats, a larger fraction of glands from Copenhagen rats were positive at the higher MNU dose and the longer time period (Table 1). The distribution of the mutated gene among the Copenhagen glands was uniform (Table 2). However, a major difference between the two strains was that in the Copenhagen rats, the mutant cell fraction showed no increase between 30 and 60 days (highest value at either time was \(10^{-5}-10^{-4}\)), whereas in the Buf/N rats, there was a clear increase in the mutant cell fraction during this time period in some of the glands.

Mammary epithelial cells were purified from both Buf/N and Copenhagen mammary glands 60 days after treatment with 50 mg of MNU per kg. Ha-ras mutations were present in DNA from cells of both strains (Fig. 4). The incidence of mutations was similar in DNA isolated from the epithelial cells and whole glands (14/24 vs. 25/32 and 9/24 vs. 19/32 for Buf/N and Copenhagen rats, respectively). The mutant cell fractions were also similar, although one epithelial cell preparation from a Copenhagen rat contained a higher fraction \(10^{-5}-10^{-4}\) than we observed in any of the whole-gland preparations.

**DISCUSSION**

In this study we have shown that the Ha-ras gene, mutated at codon 12 by a G-C \(\rightarrow\) A-T transition, is present in DNA...
isolated from the mammary glands of Buf/N rats 30 days after treatment of pubescent animals with a single carcinogenic dose of MNU. These results are in agreement with those of Ronai et al. (24) using pubescent F344 rats, who were also able to detect Ha-ras mutations 3 weeks after MNU treatment in DNA isolated from whole mammary glands. However, our results extend these observations in three areas.

First, we have shown that the fraction of cells in several glands containing a mutated Ha-ras allele increases by a factor of 10–100 between 30 and 60 days, undoubtedly because of clonal expansion of the initiated cell population stimulated by gland development (12–14). This is in accord with the high susceptibility of the Buf/N rat to mammary carcinogenesis.

Second, the sensitivity of our method for analysis of Ha-ras mutations allowed us to examine individual mammary glands. Although tumor formation predominates in the thoracic glands (pairs 1–3) (1), we saw no evidence for the preferential formation of Ha-ras mutations in these glands. Sixty days after treatment, however, the incidence of glands having mutant cell fractions >10⁻⁵ was higher in the thoracic glands than in the inguinal glands (pairs 4–6). This suggests that the expansion of the initiated cell population is stimulated to a greater extent in the target glands for tumor formation.

Third, we have shown that Ha-ras mutations are present in epithelial cells purified from individual mammary glands. The incidence of mutations and the magnitude of the mutant cell fractions in DNA from the purified cells were similar to those in DNA from whole glands. These results are consistent with the cellular origin of mammary tumors in this model. Adenocarcinomas of the alveolar or ductal epithelial cells predominate; fibrosarcomas also occur but to a minor extent (1).

Our analysis of DNA from the mammary glands of Copenhagen rats clearly shows the presence of mutated Ha-ras alleles 30 and 60 days after treatment. Indeed, the incidence and distribution of Ha-ras mutations in the resistant Copenhagen rat are comparable in many respects to those in the susceptible Buf/N rat. Also, like the Buf/N rat, the ras mutations are present in purified mammary epithelial cells. A strong case has been made that Ha-ras genes are directly activated by MNU during the initiation of neoplasia (3, 4, 8). Since the Copenhagen rat is totally resistant to MNU-induced mammary adenocarcinoma formation (10, 11), our results show that the resistance is not caused by a defect in initiation. Furthermore, our results provide a molecular basis for the notion suggested by the breeding and transplantation experiments of Isaacs (10, 11, 15) and Zhang et al. (16) that the resistance of the Copenhagen rat does not involve a gene that inhibits the initial interaction of the carcinogen with the mammary epithelial cells. Resistance, these authors suggest, is due to the presence of a dominant autosomal suppressor gene within the mammary parenchyma that acts at some stage after initiation. Our results suggest that the product of this putative suppressor gene acts early in carcinogenesis. Thirty days after an MNU dose of 50 mg/kg, several glands in the Copenhagen rats contained larger cell fractions with mutated Ha-ras than those in the Buf/N rats (10⁻⁵–10⁻⁴ compared to <10⁻⁵), suggesting, perhaps, that there is an initial rapid burst of proliferation of initiated cells in the Copenhagen animals. Between 30 and 60 days, the fraction increased by several orders of magnitude in a number of

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dose, mg/kg</th>
<th>Days after treatment</th>
<th>Positive/total ratio</th>
<th>Glands with mutant cell fraction</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Rats tested</td>
<td>Glands tested</td>
</tr>
<tr>
<td>Buf/N</td>
<td>0</td>
<td>30</td>
<td>0/3</td>
<td>0/24</td>
</tr>
<tr>
<td></td>
<td>30</td>
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<td>3/4</td>
<td>8/34</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>30</td>
<td>3/3</td>
<td>13/24</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>60</td>
<td>0/2</td>
<td>0/16</td>
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<td></td>
<td>50</td>
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<td></td>
<td>30</td>
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<td>3/3</td>
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<tr>
<td></td>
<td>50</td>
<td>60</td>
<td>4/4</td>
<td>19/32</td>
</tr>
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Cop, Copenhagen.
### Table 2. Distribution of Ha-ras mutations among mammary glands

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dose, mg/kg</th>
<th>Days after treatment</th>
<th>Positive glands, no.</th>
<th>Glands with mutant cell fraction $&gt;10^{-5}$, no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buf/N</td>
<td>30</td>
<td>30</td>
<td>Thor. 5, Ing. 3</td>
<td>Thor. 0, Ing. 0</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>30</td>
<td>Thor. 6, Ing. 7</td>
<td>Thor. 0, Ing. 0</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>60</td>
<td>Thor. 13, Ing. 12</td>
<td>Thor. 5, Ing. 2</td>
</tr>
<tr>
<td>Cop</td>
<td>30</td>
<td>30</td>
<td>Thor. 2, Ing. 4</td>
<td>Thor. 0, Ing. 0</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>30</td>
<td>Thor. 6, Ing. 7</td>
<td>Thor. 3, Ing. 1</td>
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<tr>
<td></td>
<td>50</td>
<td>60</td>
<td>Thor. 10, Ing. 9</td>
<td>Thor. 0, Ing. 0</td>
</tr>
</tbody>
</table>

Cop, Copenhagen; Thor., Thoracic; Ing., Inguinal.

*Thoracic = gland pairs 1-3, inguinal = gland pairs 4-6.

glands from the Buf/N rats, indicating that the expansion of initiated cells is being promoted. During this period in the Copenhagen rats, however, the growth of initiated cells appears to be suppressed, since all glands containing a mutated Ha-ras gene had a mutant cell fraction of $<10^{-5}$. The drop in the mutant cell fraction in these animals during the 30- to 60-day period may be due to the loss of cells from a terminally differentiating population.

A number of genetic studies have led to the prediction that the mammary tumor suppressor gene that is active in the resistant strains is functionally inactivated in the germ lines of highly susceptible strains (10, 11, 15, 16). In addition, there is evidence that susceptible animals carry several independently segregating, dominant, autosomal susceptibility genes, while strains of intermediate susceptibility carry neither suppressor nor susceptibility genes (16, 25, 26). It is not clear how the suppressor gene in the Copenhagen rat is protected from inactivation by carcinogen exposure, especially since it has been shown that even multiple treatments with MNU do not lead to tumors (10). Indeed, our finding that these animals are initiated by MNU treatment suggests that there may be treatment regimes that result in the inactivation of the suppressor gene with subsequent tumor growth.

In summary, we have shown the presence of Ha-ras codon 12 mutations in the mammary glands of both susceptible Buf/N and resistant Copenhagen rats 30–60 days after MNU treatment. Therefore, the resistance of the Copenhagen rat to mammary carcinogenesis is not due to a defect in initiation but rather appears to be due to the inability of cells containing a mutated ras allele to undergo sustained clonal expansion. While estrogens are promoters, they are present at similar levels in both resistant and susceptible animals. Therefore, uncharacterized suppressor and/or susceptibility genes appear to control strain-dependent mammary tumor development.

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