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Transforming c-Ki-*ras* Mutation Is a Preneoplastic Event in Mouse Mammary Carcinogenesis Induced In Vitro by *N*-Methyl-*N*-Nitrosourea

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Mouse mammary epithelial cells can be transformed in primary cultures to preneoplastic and neoplastic states when treated with *N*-methyl-*N*-nitrosourea (MNU). Mammary carcinomas arising from MNU-induced hyperplastic alveolar nodules (a type of mouse mammary preneoplastic lesion) contained transforming c-Ki-*ras* genes when examined by the NIH 3T3 focus assay. Hybridization of allele-specific oligonucleotides to c-Ki-*ras* sequences amplified by the polymerase chain reaction demonstrated the presence of a specific G-35→A-35 point mutation in codon 12 in each of the NIH 3T3 foci as well as the mammary carcinomas. This mutation resulted in the substitution of the normal glycine with an aspartic acid. Furthermore, this mutation in the c-Ki-*ras* proto-oncogenes was also detected in 9 of 10 hyperplastic alveolar nodules. These results demonstrate that the specific c-Ki-*ras* mutation is a preneoplastic event in MNU-induced mouse mammary carcinogenesis.

Cancer is recognized as a disease arising from a perturbation of gene function. Over 30 different cellular genes, called proto-oncogenes, have been investigated intensely because of their involvement in tumorigenesis. Of the proto-oncogenes identified in human and animal tumors, members of the *ras* gene family, c-Ha-*ras*, c-Ki-*ras*, and N-*ras*, are by far the most frequent (1, 2, 19). The *ras* genes are commonly activated by single point mutations in codons 12, 13, and 61 (1, 2). In rodent tumors induced by chemical or physical carcinogens, the nature of the mutational activation of *ras* proto-oncogenes has shown a remarkable correlation with the known mutagenic properties of the causative agents (1). For example, mammary tumors in rats induced by *N*-methyl-*N*-nitrosourea (MNU) contain c-Ha-*ras* proto-oncogenes activated by G→A transitions (26, 29). Mouse hepatic neoplasms induced by a variety of carcinogens contain c-Ha-*ras* proto-oncogenes activated by an alteration corresponding to the mutational specificity of each carcinogen (27). Therefore, the identification of the critical gene mutations directly involved in cancer is becoming feasible by use of animal model systems.

We have recently developed a transformation system in which mouse mammary epithelial cells in serum-free primary collagen gel cultures are transformed by treatment with chemical carcinogens (11, 18). Cells treated with carcinogens form mammary lesions when transplanted into a cleared mammary fat pad, an anatomically natural transplantation site from which mammary epithelial cells have been removed surgically (6). This in vitro-in vivo system facilitates a stepwise study of the transformation process at the molecular level as the transformants progress through morphologically well-defined preneoplastic stages (11, 17, 18). Furthermore, the molecular effects upon transformation by various factors that control growth and differentiation of mammary cells can be directly studied under well-defined conditions. In the present studies, in vitro MNU-induced mouse mammary preneoplasias and neoplasias were analyzed for the presence of activated proto-oncogenes. Such studies might provide an understanding of the molecular mechanisms involved in MNU-induced mouse mammary carcinogenesis.

MATERIALS AND METHODS

Mouse mammary transformants. The method for the induction of mouse mammary transformants has been described previously (18). Briefly, mammary epithelial cells were isolated from 3- to 4-month-old virgin BALB/cCrgl mice and grown in collagen gel cultures under serum-free growth conditions (a 1:1 mixture of Dulbecco modified Eagle medium and F-12 medium supplemented with insulin, progesterone, prolactin, linoleic acid, and bovine serum albumin). The cells were treated two or four times at weekly intervals with MNU at a dose of 100 μg/ml and further cultured for 2 weeks in the serum-free medium (total culture period of 3 or 5 weeks, respectively). MNU-exposed cells (5×10^5) were transplanted into cleared mammary fat pads of syngeneic female mice (6) and primary hyperplastic alveolar nodules (HANs) were recovered 2 months later. Three independent primary HANs were serially transplanted (2 to 4 months for each transplantation generation) and maintained as transplantation outgrowth lines. Seven primary HANs (~8 mm^3), HAN lines (one mammary fat pad filled with the outgrowth isolated at two to four transplantation generations), and mammary carcinomas (1 to 8 cm^3) derived from each of the HAN lines were removed from the host mice, immediately frozen on dry ice, and then kept at −70°C for later extraction of high-molecular-weight DNA. Whole mounts of mammary glands were prepared as described previously (18).

NIH 3T3 focus assay. The isolation of high-molecular-weight DNA and the transfection protocol were as described previously (26). Briefly, 40 μg of DNA isolated from each of the mouse mammary carcinomas and normal mammary glands was used for transfection by the calcium phosphate coprecipitation technique (9) of 1.5×10^5 NIH 3T3 cells seeded in 100-mm culture dishes. The transformed foci were counted at 2 to 3 weeks following transfection. DNA was

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also isolated from mass cultures of focus cells for secondary and tertiary transfection as described above.

Southern analysis of NIH 3T3 foci with oncogene probes. Southern hybridization was performed as described by Reed and Mann (22) with 10 μg of genomic DNA. Focus DNA was digested with *Eco*RI or *Bam*HI, electrophoresed in 0.8% agarose gels, transferred to Zeta-Probe membranes (Bio-Rad Laboratories, Richmond, Calif.) and screened with 14 oncogene probes. Of the 14 oncogene probes, 13 were isolated from plasmids purchased from the American Type Culture Collection (Rockville, Md.). They include pK5 (viral *abl* or v-*abl*), pAEPst0.45 (v-*erbA*), pAEBam0.50 (v-*erbB*), pSM3 (v-*fms*), pfps (v-*fps*), pv-mos (v-*mos*), pMC38 (v-*myc*), p171 (v-*raf*), HB-11 (v-Ha-*ras*), pKSma (v-Ki-*ras*), pNRSac (N-*ras*), pv-sis (v-*sis*), and pEcoRIB (v-*src*). The V101 plasmid (*int-1*) was the generous gift of Ann Tsukamoto.

Amplification of mouse c-Ki-*ras* sequences by PCR. The polymerase chain reaction (PCR) conditions were as described by Saiki et al. (24) with 1 μg of the genomic DNA and 1 U of *Taq* polymerase (The Perkin-Elmer Corp., Emeryville, Calif.) in a 100-μl reaction volume containing 2.5 mM $MgCl_2$ and 200 ng of each of the primers. The primers were 5′-GTATAAACTTGTGGTGGTT-3′ and 5′-GCTGTA TCGTCAAGGCGCTC-3′ for the 5′ and 3′ primers, respectively. The sequences of the primers were according to the report by George et al. (8). The reaction was performed for 45 cycles, each cycle consisting of sequential change of temperature and time duration as follows: 94°C for 35 s, 40°C for 15 s, and 72°C for 5 s, using the Programmable Cyclic Reactor (Ericomp, San Diego, Calif.).

Southern hybridization of PCR products. The PCR-amplified DNA (25 to 50 μl) was ethanol precipitated, electrophoresed in 4% (3.5% NuSieve plus 0.5% SeaKem) agarose gels (FMC Corp., Marine Colloids Div., Rockland, Maine), and blotted to Nytran membranes (Schleicher & Schuell, Inc., Keene, N.H.) as described previously (22). The blot was then prehybridized for 2 h at 65°C in 3 M tetramethylammonium chloride (Aldrich Chemical Co., Inc., Milwaukee, Wis.)–50 mM Tris hydrochloride (pH 7.5)–2 mM EDTA–0.3% sodium dodecyl sulfate–100 μg of single-stranded sonicated salmon testes DNA per ml–5× Denhardt solution (Sigma Chemical Co., St. Louis, Mo.). The allele-specific oligonucleotides were end labeled with ^{32}P (2×10^8 to 1×10^9 cpm/μg), added directly into the prehybridization buffer (3×10^6 to 1×10^7 cpm/ml), and hybridized for 2 h at 65°C. Blots were washed sequentially for 5 min each as follows: 6× SSC (0.9 M NaCl, 0.09 M sodium citrate, pH 7.0; room temperature); 6× SSC (74°C), 6× SSC–0.5% sodium dodecyl sulfate (50°C); and rinsed in 6× SSC (room temperature). The blots were exposed to X-ray film at −70°C with an intensifying screen.

RESULTS

Induction of HANs by in vitro MNU treatment of mouse mammary epithelial cells in primary collagen gel cultures. Mouse mammary epithelial cells isolated from adult virgin BALB/cCrgl mice were grown inside collagen gel matrix in a serum-free medium containing mammogenic hormones (prolactin and progesterone). Under this condition, the cells grew multifold (13) and formed three-dimensional colonies (28) with the apical and basal-lateral polarity maintained as in vivo (12). These cells were then exposed to a slightly cytotoxic dose of MNU (100 μg/ml [11]) two or four times at weekly intervals and cultured for two more weeks. Transplantation of these in vitro MNU-treated cells into cleared mammary fat pads (6) resulted in efficient induction of HANs as reported previously (11, 18). In this system, the control untreated cells consistently formed normal mammary ductal outgrowths in the cleared mammary fat pads within 2 months following transplantation (Fig. 1A). However, if the MNU-treated cells contained a mixture of transformed and untransformed cells, both types of cells gave rise to their respective outgrowths (Fig. 1B). The HANs detected in this way (referred to as primary HANs) were relatively small structures, typically 1 to 3 mm in diameter. They were immortal in vivo (5) as judged by their capacity to produce alveolar outgrowth lines (referred to as HAN lines) when small pieces (~1 mm^3) surgically removed from each of HANs were transplanted into cleared mammary fat pads (Fig. 1C and D). In the present studies, three such HAN lines, designated as HAN-1, -2, and -3, were maintained in vivo by serial transplantations. Many of the transplanted HAN pieces progressed to mammary adenocarcinomas (Fig. 1C, asterisk) with variable latencies of 2 to 12 months.

Transforming genes in mouse mammary carcinomas derived from in vitro MNU-induced HANs. To determine the presence of transforming genes in mouse mammary carcinomas developed as above, we performed gene transfer assays using NIH 3T3 mouse fibroblasts as hosts (25). High-molecular-weight DNA was isolated from each of the mouse mammary tumors derived from the three HAN lines and transfected into NIH 3T3 cells by the calcium phosphate coprecipitation technique (9). Tumors that developed from all three lines showed the presence of transforming genes as judged by their NIH 3T3 focus-forming capacity. The focus-forming efficiency of these DNAs (0.006 to 0.06 focus per μg of DNA) was much lower than that of the rat mammary tumor DNA containing activated c-Ha-*ras* proto-oncogenes (0.038 to 0.75 focus per μg of DNA), a positive control used for the focus assay (26). DNA isolated from 10 normal mouse mammary glands was incapable of inducing any foci. DNAs isolated from representative NIH 3T3 foci were screened with 14 different oncogene probes (see Materials and Methods). Only the c-Ki-*ras* proto-oncogenes were detected as the transforming genes in the foci induced by DNA from HAN-1- and HAN-2-derived tumors (Fig. 2, lanes 1 to 4), as judged by the presence of extra copies of the c-Ki-*ras* sequences above the endogenous levels (Fig. 2, lane 6). In contrast, the transforming genes in the HAN-3-derived tumors (five of seven) were neither c-Ki-*ras* nor any of the other oncogenes examined (Fig. 2, lane 5; others not shown). These transforming genes detected in HAN-3-derived tumors were capable of inducing NIH 3T3 foci upon secondary and tertiary transfection, and the foci induced were capable of forming colonies in soft agar.

Point mutations in c-Ki-*ras* proto-oncogenes. We next examined the mode of activation of the transforming c-Ki-*ras* proto-oncogenes detected by the NIH 3T3 focus assay. Since *ras* proto-oncogenes are commonly activated by a point mutation (1, 2) and mouse T-cell lymphomas induced by MNU contain c-Ki-*ras* proto-oncogenes activated by G-35→A-35 point mutations in codon 12 (10), the transforming c-Ki-*ras* genes detected in the present studies were analyzed for the presence of the same point mutations in codon 12. To assay for this specific point mutation, hybridization to allele-specific oligonucleotide (23) was performed with PCR-amplified c-Ki-*ras* sequences (a 59-base-pair sequence between codons 3 and 23 of mouse c-Ki-*ras*). Using this technique, all the NIH 3T3 foci having the transforming c-Ki-*ras* genes demonstrated the presence of the G-35→A-35 mutation (data not shown). To confirm the presence of the

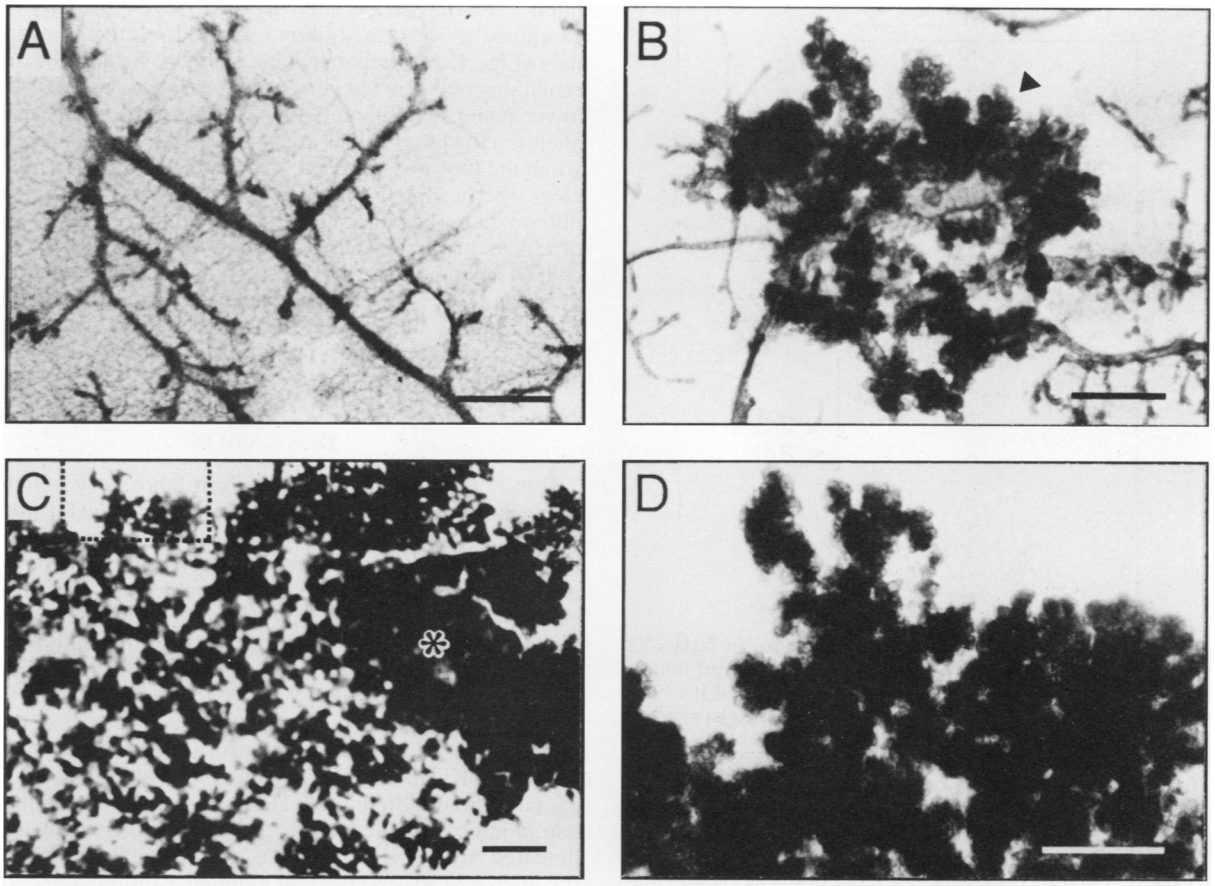

FIG. 1. Whole mounts of in vitro MNU-induced mouse mammary lesions. Mouse mammary epithelial cells isolated from virgin BALB/cCrgl mice were grown under serum-free conditions inside collagen gels and treated with MNU, and then 5×10^5 cells were transplanted into cleared inguinal mammary fat pads of syngeneic female virgin mice. The recombinant mammary glands were removed from the animals and processed as described previously (18) for preparation of whole mounts after 2 months of transplantation. The blood vessels and lymph nodes were removed for better visibility of mammary outgrowths. The bars in each panel represent 0.5 mm (A, B, and D) and 1.0 mm (C). (A) Untreated control cells giving rise only to normal ductal outgrowths, typical of virgin glands. (B) MNU-treated cells showing a transformed preneoplastic HAN (arrowhead). Notice the presence of normal ductal outgrowths in the same fat pad. (C) Secondary transplant of a HAN piece (~1 mm^3) giving rise to HAN structures filling the whole mammary fat pad. Notice the maintenance of the lobuloalveolar structures in a virgin host. The asterisk shows a tumor originating from this HAN. (D) The same HAN as in panel C (the boxed area) at a higher magnification.

mutated c-Ki-*ras* proto-oncogenes in the MNU-induced mouse mammary tumors themselves, we analyzed the tumor DNA as above. The same mutation was detected in the DNA isolated from mammary tumors derived from HAN-1 (Fig. 3, lanes 1 to 5) and HAN-2 (Fig. 3, lanes 9 to 11). The comparison of the signal intensities between mutated and normal alleles, while taking into account the possible normal stromal cell contamination, suggests that the majority of the tumor cells are heterozygous for the c-Ki-*ras* mutation. Some tumors (Fig. 3, lanes 1, 4, 5, and 10) had more mutated alleles than normal alleles, suggesting either homozygosity or hemizygosity for this mutation in these tumors. HAN-3-derived tumors (Fig. 3, lanes 6 to 8), on the other hand, did not show the presence of the c-Ki-*ras* mutation, as expected from the results of NIH 3T3 focus assay (Fig. 2, lane 5). The c-Ki-*ras* mutation was also undetectable by this method in the DNA isolated from cultured normal mammary epithelial cells (Fig. 3, lanes 14 and 15) as well as the cells that were treated twice with MNU and cultured for 2 weeks (lanes 12 and 13). We could not detect less than 1 mutated allele in approximately 10^3 normal alleles using this assay (data not shown). The DNAs isolated from untransfected NIH 3T3 cells (lane 18) and NIH 3T3 focus cells (lane 17) were also processed as above for negative and positive controls for allele-specific oligonucleotide hybridization, respectively. One PCR reaction was done without any exogenous genomic DNA (lane 16) to demonstrate the absence of any DNA contamination during PCR amplification. More tumors derived from all the HAN lines were examined for the presence of the transforming c-Ki-*ras* mutation, and the results are shown in Table 1. These results demonstrate the presence of a G-35→A-35 point mutation in the c-Ki-*ras* genes in all the tumors derived from HAN-1 and HAN-2 preneoplastic outgrowth lines and its absence in all the HAN-3-derived tumors.

c-Ki-*ras* mutations in MNU-induced HANs. We next examined whether the activation of c-Ki-*ras* genes was a preneoplastic event in MNU-induced in vitro mouse mammary transformation. It was possible to analyze this hypothesis because transformants induced in this system went through a well-recognizable preneoplastic stage. Therefore, we analyzed the preneoplastic HANs for the presence of the c-Ki-*ras* proto-oncogenes to test this hypothesis. The HAN-1 and HAN-2 lines were examined for the presence of the

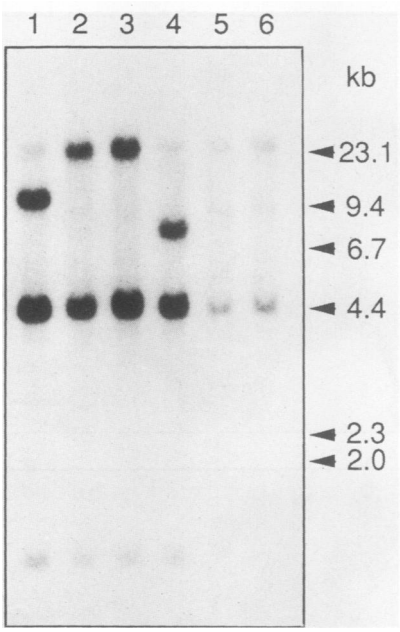

FIG. 2. Detection of the activated c-Ki-*ras* genes in NIH 3T3 foci induced by DNA isolated from in vitro MNU-induced mouse mammary carcinomas. DNA (10 μg) isolated from each of the NIH 3T3 foci was digested with *Eco*RI and electrophoresed in an agarose gel. The DNA was then transferred to a Zeta-Probe membrane, hybridized to ^{32}P-radiolabeled v-Ki-*ras* oncogene probe (0.8 kilobase [kb] *Pvu*II-*Xba*I fragment of pKSma), and exposed to X-ray film with an intensifying screen at −70°C for 10 h. The foci were induced by DNA isolated from a HAN-1-derived tumor (lanes 1 and 2), a HAN-2-derived tumor (lanes 3 and 4), and a HAN-3-derived tumor (lane 5). Lane 6, Untransfected NIH 3T3 cells. Notice the presence and absence of extra copies of the c-Ki-*ras* genes in the foci 1 to 4 and focus 5, respectively, when compared with the untransfected NIH 3T3 cells.

G-35→A-35 mutations in the c-Ki-*ras* genes, because the tumors derived from these preneoplastic lesions contained the mutated c-Ki-*ras* genes (Fig. 3; Table 1). Since the NIH 3T3 focus assay requires a large amount of high-molecular-weight DNA and extraction of such amounts of DNA requires pooling of many HANs, the DNA isolated from each HAN was analyzed by hybridization to allele-specific oligonucleotide after PCR amplification as above. Both HAN-1 and HAN-2 showed the presence of the mutated c-Ki-*ras* proto-oncogenes (Fig. 4, lanes 1 and 2). The intensity of the mutated alleles compared with normal ones suggested that either not all cells in the HAN cell populations contained mutated alleles or there was variation in the amount of contaminating stromal cells in these samples. In either case, this result clearly demonstrates the occurrence of the activated c-Ki-*ras* proto-oncogenes in the MNU-induced preneoplastic mouse mammary lesions, a novel observation for any mammary system. In contrast, the HAN-3 line, which produced tumors lacking the c-Ki-*ras* oncogenes, did not contain this specific mutation (Fig. 3, lane 3).

Since the HAN lines were maintained in the host mice by serial transplantation for months, it could be argued that the activation of the c-Ki-*ras* genes was the result of the serial transplantation and not due to initial MNU treatments in culture. To rule out this possibility, we examined seven independent in vitro MNU-induced primary HANs that had not been serially transplanted for the presence of the mutated c-Ki-*ras* genes. Five of the seven primary HANs examined as described above showed relatively strong signals (Fig. 4, lower panel, lanes 4 to 6, 8, and 9), and the remaining two showed very weak but positive signals (Fig. 4, lower panel, lanes 7 and 10). The presence of the mutated alleles in the latter two primary HANs was more pronounced when the blot was exposed longer (data not shown). It is not clear whether the difference in signal intensities is due to the difference in the percentage of the mutated cells within each of HANs or the difference in contaminating normal stromal cells. The comparison of the signal intensities suggested that most of the HANs were heterozygous for this mutation. These results demonstrate the presence of the specific c-Ki-*ras* mutation in MNU-induced primary HANs before serial transplantation.

DISCUSSION

It has been a long-term goal of our laboratory to delineate the cellular and molecular mechanisms of transformation of mammary epithelial cells. Toward this end, we have previously developed an in vitro transformation system for primary mouse mammary epithelial cells in which such studies can be performed under well-defined conditions (11, 18). In the present studies, we analyzed the presence of dominantly transforming gene mutations in two types of MNU-induced mammary transformants, preneoplastic HANs and tumors subsequently developed from them by progression.

We identified a frequent occurrence of dominantly transforming c-Ki-*ras* proto-oncogenes activated by a G-35→A-35 point mutation in the HAN-derived mouse mammary carcinomas. No activated c-Ha-*ras* proto-oncogene was detected by the NIH 3T3 focus assay, unlike the majority of the in vivo MNU-induced rat mammary tumors (26, 29). This difference of proto-oncogene activation in these systems may be due to the species difference of the mouse and rat systems. The presence of activated c-Ki-*ras* genes in in vivo MNU-induced mouse mammary tumors cannot be directly examined, because MNU induction of mammary tumors in mice has not been established. However, this seems to be a logical explanation for the difference in the type of activated *ras* observed in MNU-induced mouse and rat mammary tumors, because when a different chemical carcinogen, 7,12-dimethylbenz[*a*]anthracene, was used, both the in vitro- and in vivo-induced mouse mammary tumors yielded c-Ha-*ras* activation (4; our preliminary observation) suggesting that our in vitro system mimics in vivo phenomena. These results suggest that MNU-induction of mammary carcinomas in mice involves a specific c-Ki-*ras* mutation, unlike the rat system.

Since mouse mammary transformants induced in our system go through morphologically well-defined preneoplastic stages, it was possible to examine for the presence of the activated c-Ki-*ras* proto-oncogenes in transformants before tumor development. Our present studies identified the presence of a G-35→A-35 point mutation in c-Ki-*ras* genes in 90% of HANs, the first demonstration of a transforming gene mutation in a precancerous mammary lesion. Other studies, including those of mouse skin tumors (1) and human leukemia (16) and colon cancers (3, 7), have also identified mutated *ras* genes in precancerous states. Our results, together with the above studies, strongly suggest a causative role for *ras* oncogenes in carcinogenesis.

Since MNU is known to cause predominantly G→A transitional mutations (20) and the majority of HANs contained such point mutations, it is likely that MNU has

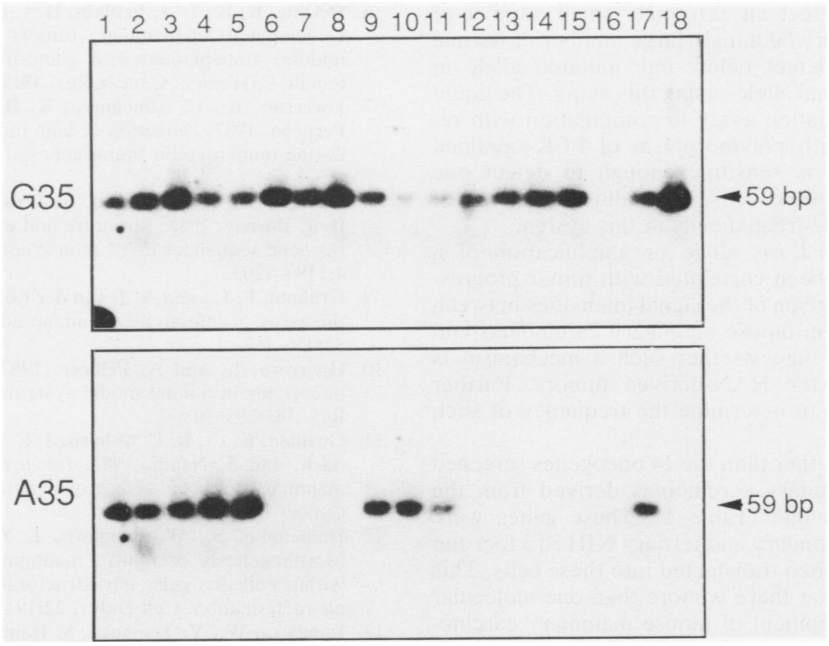

FIG. 3. Hybridization of allele-specific oligonucleotides to determine the presence of a G-35→A-35 mutation in the c-Ki-*ras* genes in mouse mammary adenocarcinomas derived from in vitro MNU-induced HANs. Genomic DNA (1 μg) was used to amplify a 59-base-pair (bp) sequence containing the c-Ki-*ras* codon 12 (codons 3 to 23) by the PCR technique in a total reaction volume of 100 μl. One-fourth of the reaction mix (25 μl) was electrophoresed in a 4% (3.5% NuSieve plus 0.5% SeaKem) agarose gel, transferred to a Nytran membrane, and hybridized to ^{32}P-radiolabeled normal G-35 allele-specific oligonucleotide (5′-GGAGCTGGTGGCGTAGGCA-3′). After exposure to X-ray film for 16 h at −70°C, the probe was completely removed from the blot and A-35 allele-specific probe was hybridized. The exposure time was 14 h at −70°C. The samples were as follows: HAN-1-derived tumors (lanes 1 to 5); HAN-3-derived tumors (lanes 6 to 8); HAN-2-derived tumors (lanes 9 to 11); MNU-treated mouse mammary epithelial cells (lanes 12 and 13); untreated mouse mammary cells (lanes 14 and 15); no exogenous DNA (lane 16); NIH 3T3 focus cells containing an approximately 1:1 ratio of G-35/A-35 alleles (lane 17); and untransfected NIH 3T3 cells (lane 18). Notice the presence of mutated alleles in lanes 1 to 5, 9 to 11, and 17.

directly interacted with c-Ki-*ras* genes during cell culture and induced this mutation. Furthermore, the c-Ki-*ras* mutation must have occurred during the initiation of transformation as hypothesized previously (29) because the half-life of MNU in culture is extremely short (as short as 8 min [14]). We directly examined the presence of the mutation in the c-Ki-*ras* genes in MNU-treated cells by the PCR-allele-specific oligonucleotide assay. We were not able to detect this mutation in mammary cells treated with MNU twice and cultured for 2 weeks. However, this possibility still remains to be resolved, because this assay might have not been

TABLE 1. G-35→A-35 transforming mutations in c-Ki-*ras* genes detected in mouse mammary lesions induced in vitro by MNU

Type of lesion	Preneoplastic outgrowth line[a]	Total no. of samples	NIH 3T3 foci	c-Ki-*ras* (G-35→A-35)	
				NIH 3T3 foci	PCR analysis[b]
Carcinoma	HAN-1	4	1/1	1/1	4/4
	HAN-2	15	1/1	1/1	15/15
	HAN-3	7	5/7	0/5	0/2
HANs[c]		10	NT[d]	NT	9/10

[a] These MNU-induced preneoplastic mouse mammary lesions were maintained by transplanting a small piece (~1 mm^3) of each lesion into cleared mammary fat pads of syngeneic BALB/cCrgl mice.

[b] All samples were analyzed by hybridization to allele-specific oligonucleotides.

[c] HANs include both transplantation lines and primary lesions.

[d] NT, Not tested.

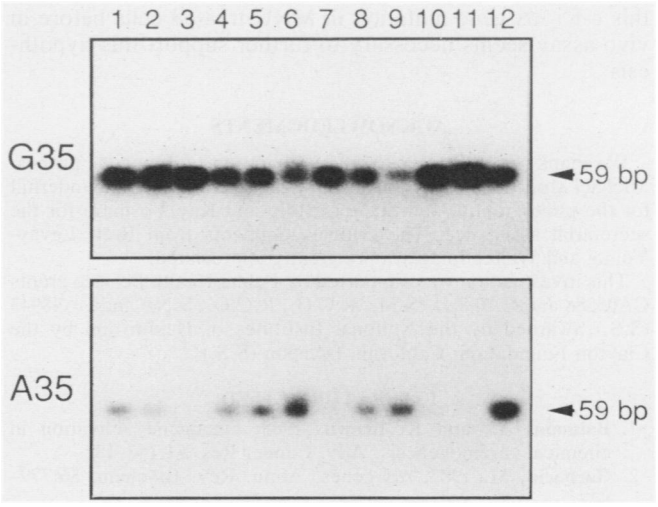

FIG. 4. Hybridization of allele-specific oligonucleotides to determine the presence of a G-35→A-35 mutation in HANs induced in vitro by MNU. c-Ki-*ras* was amplified by using 1 μg of DNA isolated from each of the MNU-induced preneoplastic HANs, and 50 μl of the reaction mix was used to detect the two alleles as described in the legend to Fig. 3. The exposure time for the G-35-specific probe was 1 h and that of the A-35-specific probe was 20 min. The samples were as follows: HAN-1 (lane 1); HAN-2 (lane 2); HAN-3 (lane 3); primary HANs (lanes 4 to 10); normal mouse mammary gland (lane 11); and HAN-1-derived tumor (lane 12) (same as lane 3 in Fig. 3). Notice the presence of the c-Ki-*ras* mutation in lanes 1, 2, 4 to 10, and 12. bp, Base pairs.

sensitive enough to detect an extremely small number of mutated cells in an overwhelmingly large number of normal cells. We could not detect below one mutated allele in approximately 10^3 normal alleles using this assay. The liquid hybridization-gel retardation assay in combination with restriction fragment length polymorphism of PCR-amplified fragments (15), which is sensitive enough to detect one mutated cell in 10^5 normal cells, may allow detection of mutated alleles in MNU-treated cells in this system.

The loss of a normal *ras* allele or amplification of a mutated *ras* allele has been correlated with tumor progression (1, 21). The comparison of the signal intensities between G-35 and A-35 probes in mouse mammary carcinomas and HANs (Fig. 3 and 4) suggests that such a mechanism is operating in some of the HAN-derived tumors. Further studies are in progress to determine the frequency of such events in this system.

Transforming genes other than the 14 oncogenes screened were detected in mammary carcinomas derived from the MNU-induced HAN-3 line (Table 1). These genes were capable of inducing secondary and tertiary NIH 3T3 foci and colonies on soft agar when transfected into these cells. This observation suggests that there is more than one molecular pathway for the development of mouse mammary carcinomas going through the preneoplastic HAN stage, although the incidence is much lower than the activated c-Ki-*ras* pathway. Identification of these genes may provide tools to further analyze mouse mammary carcinogenesis at the molecular level.

In summary, our present studies demonstrate a novel finding—that the transforming c-Ki-*ras* mutation is a preneoplastic event involved in MNU-induced HAN-type mouse mammary carcinogenesis. This mutational event is most likely involved in the initiation phase of mammary carcinogenesis as judged by the short half-life of MNU in culture and the MNU-specific mutations observed. Identification of this c-Ki-*ras* gene mutation in MNU-treated cells before in vivo assay seems necessary to further support this hypothesis.

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