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Specific Activation of the Cellular Harvey-*ras* Oncogene in Dimethylbenzanthracene-Induced Mouse Mammary Tumors

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Genomic DNAs from dimethylbenzanthracene-induced BALB/c mouse mammary tumors arising from the transplantable hyperplastic outgrowth (HPO) line designated DI/UCD transformed NIH 3T3 cells upon transfection. Transforming activity was attributed to the presence of activated Harvey *ras*-1 oncogenes containing an A → T transversion at the middle adenosine nucleotide in codon 61. DNAs from untreated DI/UCD HPO cells and radiation-induced and spontaneous mammary tumors from the DI/UCD HPO line failed to transform NIH 3T3 cells. The results indicated that the mutation activation of Harvey *ras*-1 oncogenes was specific to dimethylbenzanthracene treatment in the mouse mammary tumor system.

The carcinogenic activity of dimethylbenzanthracene (DMBA) and other chemical carcinogens has been attributed to their ability to bind to DNA and thus to cause somatic mutations (5, 28). Attempts to identify molecular targets of carcinogens have led to isolation of activated oncogenes from chemically induced tumors and cell lines by NIH 3T3 transfection-transformation assay (3, 14, 17, 18, 25, 31, 34). *N*-Nitroso-*N*-methylurea-induced rat mammary tumors contained activated Harvey *ras*-1 (H-*ras*-1) oncogenes with a mutated codon 12, whereas in DMBA-induced tumors, mutation was localized to codon 61 of H-*ras*-1 oncogenes (34). DMBA-induced mouse skin carcinomas also contained activated H-*ras*-1 oncogenes (3). However, the role of activated oncogenes in chemical carcinogenesis is still unclear.

To study molecular mechanisms underlying carcinogen-induced mammary neoplasia, we used the mouse mammary tumor system because it provides a reproducible biological experimental model. Mouse mammary neoplasias progress through well-characterized stages of nodulogenesis (immortalization) and tumorigenesis (transformation) (6, 7, 22, 23). During nodulogenesis, a morphologically distinct premalignant lesion called the hyperplastic alveolar nodule arises in a normal mammary gland. Hyperplastic alveolar nodules can be isolated and established as hyperplastic outgrowth (HPO) lines by serial transplantation in gland-cleared fat pads of syngeneic mice (9, 23). Mammary tumors arise from these transplants.

The present study involved mammary tumors derived from the DI/UCD HPO line. This line was developed from a hyperplastic alveolar nodule that arose after hormonal stimulation of the BALB/c host (1, 24). It has been maintained by serial transplantation in BALB/c mice. Since the DI/UCD line does not carry an exogenous mouse mammary tumor virus, it has been used as a mouse mammary tumor virus-free model to study the effects of several different carcinogens (2). The spontaneous tumor incidence in this line is 43% at 22 weeks posttransplantation. In contrast, the tumor incidence increased to 81% at 22 weeks after exposure to DMBA (2). Exposure of DI/UCD HPOs to X-irradiation does not increase tumor incidence so dramatically but does decrease the latency period for tumor development (10, 15).

We analyzed DNAs from DMBA-induced, radiation-induced, and spontaneous mammary tumors derived from the DI/UCD HPO line for transforming activity in the NIH 3T3 assay. Activated H-*ras*-1 with a mutated codon 61 was found only in DMBA-induced tumor DNAs.

Search for activated oncogenes from DNAs of HPO and HPO-derived mammary tumors by NIH 3T3 assay. To test for transforming activity, we transfected genomic DNA from DMBA-induced, radiation-induced, and spontaneous mammary tumors from the DI/UCD HPO line and from untreated DI/UCD HPO cells into NIH 3T3 cells by using the calcium phosphate precipitation method (16, 33). DNAs from untreated DI/UCD HPO cells and from five spontaneous and nine radiation-induced tumors were negative in the assay. In contrast, DNAs from each of the four DMBA-induced mammary tumors induced morphological transformation of NIH 3T3 cells at 0.05 foci per μg of DNA. Secondary Rat-2 transformants were obtained at an efficiency of 0.06 foci per μg upon transfection with DNAs from primary NIH 3T3 transformants. The data suggested that transforming genes were limited to DMBA-induced tumors.

Detection of an activated H-*ras*-1 oncogene from transformants. Southern blot analysis (29) of *Eco*RI- or *Bam*HI-digested DNA samples from NIH 3T3 and Rat-2 transformants with the H-*ras*-specific DNA probe BS-9 (13) revealed additional copies of H-*ras*-1 sequences (Fig. 1). *Eco*RI digestion of NIH 3T3 transformant DNA samples yielded a 23-kilobase (kb) endogenous NIH 3T3 H-*ras*-1 fragment and at least one additional H-*ras*-1-related fragment ranging from 8 to 20 kb (Fig. 1A, lanes a to g). *Bam*HI digestion of DNA samples from NIH 3T3 transformants resulted in a 3.4-kb endogenous H-*ras*-1 fragment of NIH 3T3 and additional H-*ras*-1 fragments ranging from 4 to 6 kb (Fig. 1B, lanes a to g). The data indicated that transfection with DMBA-induced tumor DNA resulted in transformation of NIH 3T3 cells and was associated with acquisition of H-*ras*-1 DNA.

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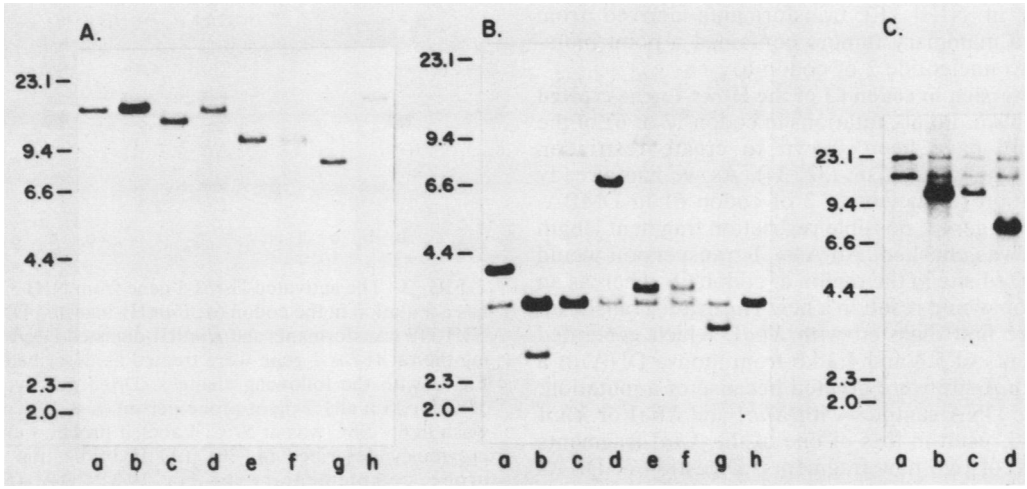

FIG. 1. Activated H-*ras*-1 sequences were detected in primary NIH 3T3 and secondary Rat-2 transformants derived from DMBA-induced D1/UCD mouse mammary tumors. Genomic DNAs from NIH 3T3 transformants were digested with *Eco*RI (panel A) or *Bam*HI (panel B), and DNAs from Rat-2 transformants were digested with *Eco*RI (panel C). DNA samples were separated in 0.8% agarose gels and transferred to nitrocellulose paper (29). Southern blots were hybridized with nick-translated viral H-*ras*-specific BS-9 probe (14, 19) in 50% formamide–3× SSC (1× SSC = 0.15 M sodium chloride plus 0.015 M sodium citrate)–3× Denhardt solution–100 μg of yeast RNA per ml–50 μg of sheared salmon sperm DNA per ml at 37°C. Blots were sequentially washed with 2× SSC in 0.1% sodium dodecyl sulfate–0.2× SSC in 0.1% sodium dodecyl sulfate at 25°C and 1× SSC in 0.1% sodium dodecyl sulfate for 1 h at 65°C, dried, and exposed to Kodak XAR-5 film. Panels A and B show DNAs extracted from NIH 3T3 transformants obtained after transfection of DNAs from T 13265 (lanes a to d), T 13271 (lanes e and f), and T 13273 (lane g) mammary tumor cells and untransfected control NIH 3T3 cells (lane h). Panel C shows *Eco*RI-digested DNAs from secondary Rat-2 transformants transfected with DNAs from T 13265 (lanes b and c) and T 13273 (lane d) tumor cells and untransfected Rat-2 cells (lane a). *Hin*dIII-cut λ c1857 DNA fragments were coelectrophoresed as size markers. Note that the bands associated with the activated H-*ras* genes are darker than the endogenous NIH 3T3 H-*ras* fragments, suggesting a higher copy number. This copy number varied from one transformant to another.

Southern blot analysis of *Eco*RI-digested DNA samples from secondary Rat-2 transformants also showed additional H-*ras*-1 fragments ranging from 8 to 13 kb (Fig. 1C, lanes b to d). They were smaller than the 16- and 23-kb endogenous rat H-*ras*-1 fragments (Fig. 1C, lane a).

Activated H-*ras*-1 oncogene has a normal codon 12. Carcinogen-induced rat mammary tumors have been shown to contain point mutations in codons 12 and 61 of the H-*ras*-1 gene (34). Therefore, we investigated the possibility that the transforming activity of the H-*ras*-1 gene from DMBA-induced tumors was a result of a mutation in codon 12 or 61. A set of synthetic nucleotide probes was used to search for point mutations in codons 12 and 61 of the H-*ras*-1 gene.

A synthetic nonadecamer, Ha 19-GG^{35}A (5′-TGGG CGCTGGAGGCGTGGG-3′), recognized nucleotides +26 to +44 of the normal rat H-*ras*-1 locus (34). We assumed that rat and mouse H-*ras*-1 sequences are identical and that hybridization with oligonucleotide probes under defined conditions would allow detection of a difference in a single base. Agarose gels containing *Bam*HI-digested genomic DNA samples from primary NIH 3T3 transformants were hybridized to ^{32}P-end-labeled Ha 19-GG^{35}A (8, 19, 34). The labeled nonadecamer hybridized to the additional H-*ras*-1 fragments in each of the primary NIH 3T3 transformants. The difference in the intensities of activated H-*ras*-1 fragments was due to unequal amounts of DNA samples in the lanes (Fig. 2). The single-copy normal H-*ras*-1 fragment from NIH 3T3 DNA was faint compared with multiple copies of the transfected H-*ras*-1 gene. The results indicated that the activated H-*ras*-1 gene in the NIH 3T3 transformants has a normal codon 12.

Activated H-*ras*-1 gene has a mutated codon 61. DNAs from primary NIH 3T3 transformants were next analyzed for possible mutations in codon 61. We synthesized a nonadecamer, Ha 19-C^{181}AA (5′-CAGCAGGTCAAGAAG AGTA-3′) to detect a normal codon 61 in the H-*ras*-1 gene. It represented the rat H-*ras*-1 sequence with a normal codon 61, CAA. ^{32}P-labeled probe complementary to Ha 19-C^{181}AA was synthesized with a primer, 5′-TACTCTTC-3′ (4, 30). The probe hybridized to the H-*ras*-1 fragment from the NIH 3T3 cell line containing multiple copies of the rat H-*ras*-1 sequence with a normal codon 61 (Fig. 3A, lane a). However, this probe failed to hybridize with the additional H-*ras*-1 sequences in transformants derived from each of the three DMBA-induced mouse mammary tumors (Fig. 3B, lanes c to g) or in the NIH 3T3 cell line containing multiple copies of rat H-*ras*-1 sequences with a mutated codon 61 (lane b) (34). The data indicated that the activated H-*ras*-1 sequences in NIH 3T3 transformants may have a mutated codon 61.

The position of these mutations was determined with a series of mixed-sequence oligomers similar to those previously described (4, 34). These probes had sequences similar to Ha 19-C^{181}AA with the following modifications. (i) Ha 19-N^{181}AA was capable of detecting a mutation in nucleotide 1 of codon 61 (where N is A or G). (ii) Ha 19-CN^{182}A was capable of detecting a mutation at nucleotide 2 (where N is T, C, or G). (iii) Ha 19-CAN^{183} was capable of detecting a mutation in nucleotide 3 (where N is T or C). Nonadecamers Ha 19-N^{181}AA and Ha 19-CAN^{183} failed to hybridize to the additional H-*ras*-1 sequences in NIH 3T3 transformants (data not shown). In contrast, the Ha 19-CN^{182}A probe readily hybridized with the additional H-*ras*-1 sequences present in NIH 3T3 transformants derived from each of the three DMBA-induced mouse mammary tumors (Fig. 3B, lanes d to g). These results indicated that the activated

H-*ras*-1 genes in NIH 3T3 transformants derived from DMBA-induced mammary tumors contained a point mutation localized to nucleotide 2 of codon 61.

A → T transversion in codon 61 of the H-*ras*-1 gene created *Xba*I polymorphism. Point mutations in codon 12 or 61 of the *ras* gene family have been shown to create restriction fragment length polymorphism (32, 34). As we had already localized mutation to nucleotide 2 of codon 61 in DMBA-activated H-*ras*-1 genes, possible restriction fragment length polymorphism was checked. An A → T transversion would induce a new *Xba*I site in the region of codon 61, whereas an A → G transition would result in a new *Taq*I site. Transformant DNAs were first digested with *Xho*I, which generated H-*ras*-1 fragments of 5.4 and 4.3 kb from mouse DNA. If a new *Xba*I or *Taq*I site were created because of a mutation, digestion of the DNA samples with *Xho*I and *Xba*I or *Xho*I and *Taq*I would result in loss of one of the *Xho*I fragments and appearance of two new fragments. Digestion of DNAs from NIH 3T3 cells, untreated D1/UCD HPO cells, and spontaneous D1/UCD mammary tumors with *Xho*I and *Xba*I resulted in two fragments of 5.4 and 4.3 kb (Fig. 4C, lane 1; A, lanes 1 and 2; B, lane 1). This indicated that the *Xho*I fragments of the mouse H-*ras*-1 gene contained no *Xba*I sites. In contrast, when DNAs from NIH 3T3 transformants and DMBA-induced tumors were double digested with *Xho*I and *Xba*I, two new fragments appeared, suggesting induction of new *Xba*I sites (Fig. 4B, lanes 2 to 4). The sizes of additional fragments from transformant DNAs were different, which probably was due to loss of *Xba*I sites in flanking DNA during the transfection process (Fig. 4C). However, additional fragments were all the same size, 2.8 and 1.7 kb, in DMBA-induced tumors (Fig. 4B). No evidence of a new *Taq*I site was found in transformant or tumor DNA. The new *Xba*I site was found only in the three DMBA-induced tumors and not in the five independent, untreated HPO tissues or the seven spontaneous D1/UCD tumors. We were also able to detect *Xba*I polymorphism in these tumors by digesting DNA samples with *Xba*I alone. (Fig. 4D, lanes 2 to 4).

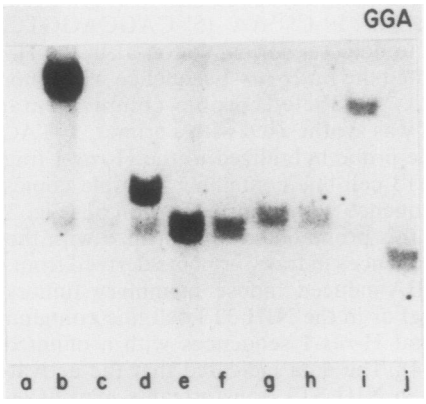

FIG. 2. The activated H-*ras*-1 gene from NIH 3T3 transformants has a normal codon 12. *Bam*HI-digested DNA samples from NIH 3T3 transformants were separated in 0.7% agarose gels and prepared for hybridization as previously described (8, 19, 34). Dried gels were hybridized to ^{32}P-end-labeled oligomer Ha 19-GG^{35}A for 18 h at 64°C at 5 ng/ml of probe with 2×10^9 to 5×10^9 cpm of activity per μg (34). Gels were washed five times with 6× SSC for 15 min each time and finally washed by rinsing with 6× SSC at 66°C and then cooled to room temperature. The gels were dried and autoradiographed. Ha 19-GG^{35}A (5′-TGGGCGCTGGAGGCGTGGG-3′) represents the +26 to +64 nucleotides of the normal rat H-*ras*-1 locus. DNA was isolated from (lanes): a, NIH 3T3 cells transfected with the rat H-*ras*-1 gene with a mutated codon 12 (GAA); b, NIH 3T3 cells cotransfected with the normal rat H-*ras*-1 gene and pSV-neo; c, NIH 3T3 cells; d to j, NIH 3T3 transformants derived from T 13265 (lanes d, e, f, and i are also represented in Fig. 1B, lanes a, b, c, and d), T 13271 (lanes g and h are also represented in Fig. 1B, lanes e and f), and T 13273 (lane j is also represented in Fig. 1B, lane g) tumor cells.

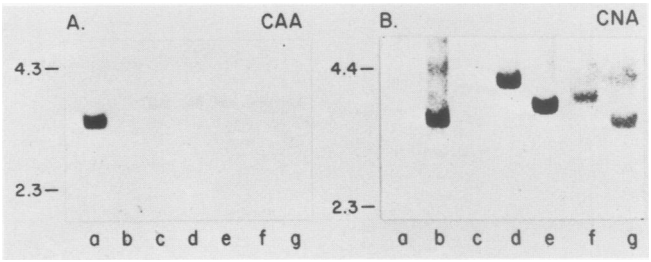

FIG. 3. The activated H-*ras*-1 gene from NIH 3T3 transformants has a mutation in the codon 61. *Bam*HI-ingested DNA samples from NIH 3T3 transformants and *Hin*dIII-digested DNA samples containing the rat H-*ras*-1 gene were treated as described in the legend to Fig. 2 with the following changes. Dried gels were hybridized at 55°C for 18 h at 1.7 ng of probe per ml (6×10^9 cpm/μg). The final wash in 6× SSC was at 57°C. Labeled probes were synthesized as previously described (4, 30). (A) Hybridization with ^{32}P-labeled probe complementary to Ha 19-C^{181}AA (5′-CAGCAGGT CAAGAAGAGTA-3′), representing the +173 to +191 nucleotides of the normal rat H-*ras*-1 locus. (B) Hybridization with ^{32}P-labeled probe complementary to Ha 19-CNA^{182}A (5′-GAGCAGG TCNAGAAGAGTA-3′), where N is C, G, and A. DNAs were isolated from (lanes): a, An NIH 3T3 transformant containing the rat H-*ras*-1 locus with a mutated codon 12 (GGA) but a normal codon 61 (CAA); b, NIH 3T3 transformant containing the rat H-*ras*-1 locus with a normal codon 12 (GAA) but a mutated codon 61; c, NIH 3T3 cells; d through g, NIH 3T3 transformants derived from mouse mammary tumors. The transformant in lanes d and e represents lanes d and e of Fig. 2 derived from T 13265 cells; lane f represents one g of Fig. 2 derived from T 13271 cells; lane g represents lane j of Fig. 2 derived from T 13273 cells.

These experiments demonstrated that DMBA induction of mouse mammary tumors is associated with activation of the H-*ras*-1 gene. This activation is associated with A → T transversion-mutation at the middle adenosine nucleotide of codon 61 that occurred in the DNA of the DMBA-induced D1/UCD tumor. It was interesting that Balmain's group (26) has found the same A → T transversion in 90% of DMBA-induced skin carcinomas and premalignant papillomas.

The action of DMBA may differ from that of other carcinogens such as benzo[a]pyrene or *N*-nitroso-*N*-methylurea (28). Both benzo[a]pyrene and *N*-nitroso-*N*-methylurea bound preferentially to deoxyguanosine residues and induced mutations in codon 12 of the H-*ras*-1 gene (21, 34). In contrast, DMBA formed adducts mainly with deoxyadenosine in mouse skin and embryo cultures (11, 12). Mutation at the adenosine of codon 61 in the H-*ras*-1 gene in DMBA-induced mouse and rat tumors (our results; 3) and in skin carcinomas (26) and mammary tumors supports the notion that a carcinogen could induce mutations by interacting directly with DNA. Consistent detection of the activated H-*ras*-1 gene from mouse, rat, and human mammary tumors suggests that this oncogene could be crucial for mammary epithelial cell proliferation and transformation (our results; 20, 34). Detection of chemically activated oncogenes from tumors is not sufficient to understand their role in the initiation and development of chemical carcinogenesis. A transplantable mouse mammary system with defined

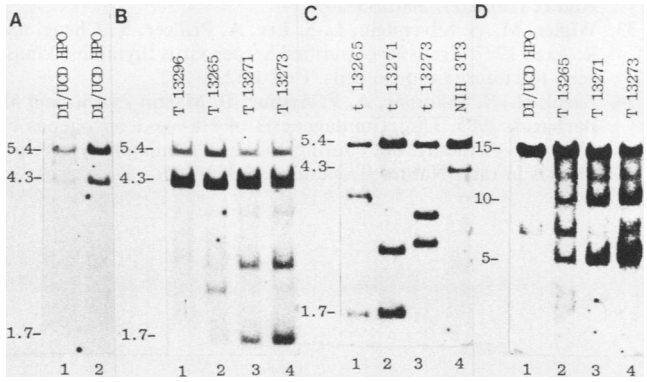

FIG. 4. An A → T transversion in codon 61 generated *Xba*I polymorphism. Southern blots containing *Xho*I and *Xba*I double-digested DNA samples (panels A, B, and C) or *Xba*I-digested DNA samples (panel D) were hybridized with labeled H-*ras* probe BS-9 as described in the legend to Fig. 1. DNA samples were obtained from (panel A, lanes 1 and 2) untreated D1/UCD HPO; (panel B, lane 1) spontaneous mammary tumor cells derived from D1/UCD HPO; (panel B, lanes 2 to 4) DMBA-induced mammary tumor cells from D1/UCD HPO; (panel C, lanes 1 to 3) NIH 3T3 transformants, t, derived from DMBA-induced T 13265, T 13271, and T 13273 tumor cells; (panel C, lane 4) NIH 3T3 cells; (panel D, lane 1) untreated D1/UCD HPO; or (panel D, lanes 2 to 4) DMBA-induced mammary tumors. The *Xba*I fragment of the normal H-*ras*-1 fragment was about 13 to 15 kb. In tumors, the additional *Xba*I fragments were about 8 to 10 and 5 kb long.

premalignant and malignant stages, together with DMBA, could be used to understand mammary carcinogenesis.

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