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Activated K-*ras* and N-*ras* Oncogenes in Primary Renal Mesenchymal Tumors Induced in F344 Rats by Methyl(methoxymethyl)nitrosamine

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Administration of methyl(methoxymethyl)nitrosamine to newborn Fischer 344 rats results in the preferential induction of renal tumors arising from the mesenchymal component of the kidney. DNA from a significant proportion of these tumors was capable of transforming NIH/3T3 cells. This report describes the renal tumor model, the detection of two different *ras* transforming genes in the kidney tumors (the N-*ras* oncogene in 1 and K-*ras* oncogene in 10 kidney tumors) and the characterization of DNA sequences specifying the transformed phenotype.

It has been widely documented that a variety of human tumor cell lines as well as solid tumors contain transforming genes (3, 23). In the vast majority of these tumors, the transforming activity is attributed to the activation of members of the *ras* gene family: the Harvey *ras* (H-*ras*), Kirsten *ras* (K-*ras*), and the N-*ras* (4, 13, 16, 18, 19). As a result of screening of several representative samples of a wide variety of human tumors, it has become clear that only 10 to 15% of human tumors have oncogenes detectable by the NIH/3T3 assay (14, 15). The infrequency of detection of activated oncogenes in human tumors raised questions regarding the importance of this event in the multistep process of tumorigenesis in vivo (5). This has led, in the past 2 or 3 years, to the utilization of carcinogen-induced animal tumor models to provide some insight into the mode of activation of oncogenes and the significance of this event in the carcinogenic process. In animal models consisting of a single histologically defined neoplasm induced by a specific agent, activation of one specific oncogene has been demonstrated, usually by single-point mutations. Thus, transforming *ras* genes have been consistently detected in skin tumors induced by 7,12-dimethyl benz(a)anthracene (DMBA) in mice (1, 2), in mammary tumors induced by *N*-methyl-*N'*-nitrosourea (NMU) or DMBA in rats (21, 24), and more recently, in spontaneous liver tumors arising in aged B6C3F1 mice (8). However, activation of a specific member of the *ras* gene family does not seem to be associated with a specific kind of neoplasm, as a transforming N-*ras* gene has been detected in thymic lymphomas induced in mice by NMU, whereas an activated K-*ras* gene was present in similar neoplasms induced by gamma irradiation (9), possibly due to the target cells being at varying stages of differentiation. In this study, by using the NIH/3T3 transfection assay, a search was made for transforming genes in primary rat renal mesenchymal tumors induced by methyl(methoxymethyl) nitrosamine (DMN-OMe).

The carcinogenic compound DMN-OMe [$CH_3N(NO)CH_2OCH_3$] has been found by us (unpublished data) selectively to induce renal tumors when given to rats as a single intraperitoneal injection. Though the mechanism of action of this compound is still under study, from its structure DMN-OMe is predicted to be a methylating agent. DMN-OMe (Mw104) was synthesized by L. K. Keefer and P. P. Roller according to the method of Eiter et al. (6). Neonatal F344/NCr rats (39 males and 32 females) received a single intraperitoneal injection of DMN-OMe (4.0 mmol/kg of body weight) in phosphate-buffered saline (pH 7.0) within 48 h after birth. Tumors appeared in 32 of 54 surviving animals after carcinogen administration with a mean latency of 35 weeks. Rats with large palpable renal masses were killed by CO_2 inhalation. Samples of renal tumor tissue, contralateral kidney, liver, and brain were frozen at −70° until further analysis. Representative samples of each of these tissues were also fixed in Bouin fixative and processed for histology. Primary renal tumors were also transplanted to syngeneic recipients and maintained by serial passage.

Most renal tumors induced by this protocol were of mesenchymal origin, and were comparable morphologically to similar tumors induced by other agents (10). The tumors occurred in rats of both sexes, were rapidly growing, aggressively invaded adjacent renal parenchyma (Fig. 1A), and were composed of cells that varied from a small stellate cell to large spindle-shaped cells, both of which were highly basophilic. Some tumor contained foci of myoblastic differentiation. Mitoses were numerous (Fig. 1B). Although extension of tumor tissue into the renal vein was sometimes seen, and these tumors are known to be capable of metastasis to the lung, no metastases were detected in this study. Tumor tissues from 25 rats that were killed during the first 50 weeks after exposure to the carcinogen were included in this study. Tumors developed occasionally also at other sites, including lung, liver, and nasal cavities, and carcinogen-treated rats commonly had foci of cytoplasmic alteration in hepatic parenchyma that include precursors of hepatocellular neoplasia. Presence of transforming genes in these extrarenal sites is under investigation and will be reported elsewhere.

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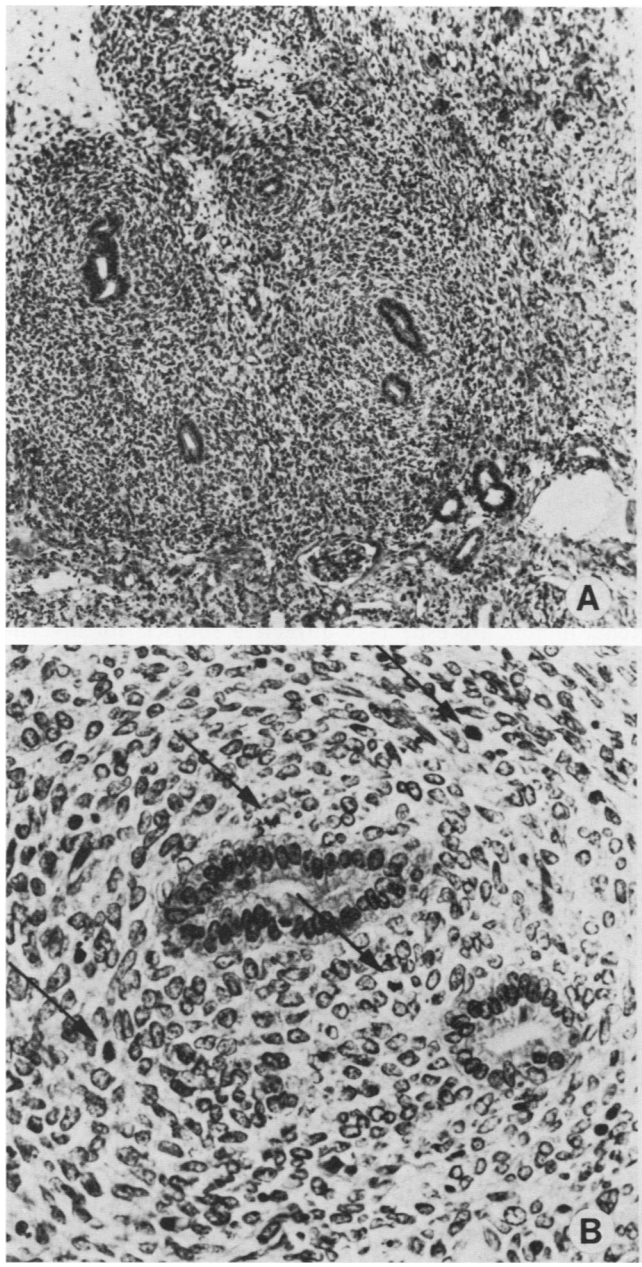

FIG. 1. (A) Renal mesenchymal tumor (top) surrounding hyperplastic renal cortical tubules as a result of diffuse infiltration of renal parenchyma (bottom). At higher magnification (B), frequent mitoses (arrows) are apparent in tumor cells, which resemble the undifferentiated mesenchymal cells of metanephrogenic blastema. This tumor yielded DNA that transformed NIH/3T3 cells and contained an activated K-*ras* oncogene.

To determine whether oncogene activation was involved in the induction of renal mesenchymal tumors induced by DMN-OMe, DNA extracted from tumors from each of 25 rats (9 females and 16 males) was tested in transfection assays for ability to transform NIH/3T3 cells (14). A total of 11 DNA preparations (from three females and eight males) of 25 tested induced morphologically transformed foci in NIH/3T3 recipient cells with a transformation activity ranging from 0.006 to 0.02 foci per μg of DNA, which increased to 0.025 to 0.54 foci per μg of DNA in the second cycle of transfection (Table 1). The transformed foci were picked, cells were cloned in soft agar, cultures were expanded, and DNA was isolated.

To ascertain that transformation of NIH/3T3 cells was caused by stable integration of a transforming gene of rat origin, first-cycle NIH/3T3 transformants were tested for the presence of rat DNA sequences. Each of the transformants derived from the 11 kidney tumors contained DNA sequences that hybridized with a probe specific for rat repetitive sequences (a *Pst*I insert in p3B5 kindly provided by A. Furano), thus suggesting that the transformed phenotype of the NIH/3T3 cells was mediated by an oncogene acquired from rat donor DNA (Fig. 2A).

Since *ras* genes seem to be most frequently detected in human and animal tumors, we next examined whether the rat sequences coding for the transformed phenotype were related to the *ras* gene family. Digestion of high-molecular-weight DNA from NIH/3T3 transformants was done using the indicated restriction enzymes, submitted to Southern blot analysis with probes specific for H-*ras* (2.9-kilobase [kb] *Sac*I/*Sac*I fragment of human c-H-*ras*), N-*ras* (1 kb composed of exons I and II of human N-*ras*), and K-*ras* (1-kb *Sst*/*Hin*dIII fragment of human c-K-*ras*) for detection of sequence homology (12, 16, 22).

The NIH/3T3 transformant DNAs derived from both first- and second-cycle transfection assays from 10 of 11 renal tumors on digestion with *Eco*RI yielded DNA fragments of 10.5, 3.6, and 1.8 kb, using the c-K-*ras*-specific probe described above. With DNA from first-cycle transformants, these fragments comigrated with rat K-*ras* sequences (Fig. 2B, lane a) and were quite distinct from endogenous mouse sequences of 12.5, 3.5, and 1.6 kb (Fig. 2B, lane b). Similar results were obtained using two other restriction enzymes, *Hin*dIII and *Bam*HI (data not shown). Amplification of rat-specific K-*ras* sequences was consistently observed; these were present at levels ranging from 5 to 10 times higher than endogenous NIH/3T3 sequences. DNA from the first-cycle transformants of tumor 11 were negative for rat K-*ras*-specific sequences, although rat repetitive sequences were readily demonstrated. Southern blot analysis, with the H-*ras* probe, was also negative. With the human N-*ras* probe, a rat-specific 8.8-kb *Eco*RI fragment was observed in the transformant DNA (Fig. 3), an observation that was reproducible with DNA from second-cycle NIH/3T3 transformants of this tumor (data not shown). This 8.8-kb *Eco*RI fragment present in primary transformant DNA (Fig. 3, lane e) corresponded to those present in normal rat kidney DNA (lane a) but not in NIH/3T3 DNA (lane b) or two other transformants previously shown to contain rat K-*ras* sequences (lane c and d). Thus, this tumor alone contains a N-*ras* transforming gene rather than a K-*ras* oncogene present in the other 10 tumors in this series. Histologically the tumors were indistinguishable, but this exception may reflect a cell at a different stage of differentiation having been a target of carcinogenic insult.

The presence of an activated oncogene in human as well as animal tumors has been shown to be the result of a somatic event and not related to germ line transmission of an aberrant gene carried in the tumor bearers (3, 21). DNA from grossly normal tissues from tumor bearers was tested in transfection assays using NIH/3T3 cells. These included the contralateral uninvolved kidney, liver, and brain of 14 tumor bearers. None of these DNA samples induced morphological transformation of NIH/3T3 cells (Table 1). Thus, by inference, the presence of an activated oncogene in the tumor

TABLE 1. Transforming genes in primary renal mesenchymal tumors in F344 rats given DMN-OMe at birth

Source of DNA	Transforming genes/no. of tissues tested	Transformation efficiency (foci/μg of DNA)		Oncogene[a]
		Cycle 1	Cycle 2	
Renal tumors	11/25	0.006–0.02 0.006	0.025–0.16 0.54	K-*ras* (10/11) N-*ras* (1/11)
Normal contralateral kidney, brain, and liver of 14 tumor-bearing rats	0/42			

[a] Numbers in parentheses represent number of transformant DNAs with rat-related restriction fragments that hybridized with the indicated oncogene per total number of transformants.

tissue is not due to a heritable trait in the F344 rat genome but to a change that occurred in a select population of cells after administration of DMN-OMe that ultimately gave rise to the tumor.

Malignant transformation of mammalian cells has been shown to occur by point mutations in the coding regions of *ras* oncogenes (2, 16, 18, 22, 23) resulting in the expression of an abnormal protein or by an amplification of the proto-oncogene resulting in an increase in the expression of the normal gene product (11, 17). The presence of an amplified (5 to 10 times) rat K-*ras* sequences in NIH/3T3 transformants compared with control NIH/3T3 cells presented the possibility that increased copy number of the normal rat K-*ras* gene was responsible for the transforming activity of tumor DNA. To test this premise, we performed quick-blot analysis of mRNA isolated from 5 transplanted, transfection positive kidney tumors. Hybridization of mRNA from cells derived from transplanted tumors and normal adult rat kidney with a viral K-*ras* specific probe (7) showed no significant differences in the levels of K-*ras* transcripts (data not shown). Thus, amplification of K-*ras* sequences did not occur in these renal tumors. Therefore, it is most likely that malignant activation of the K-*ras* oncogene in tumors induced by DMN-OMe proceeded via the commonly found mechanism of single-point mutation (3, 23).

This report is the first demonstration of activated K-*ras* and N-*ras* oncogenes in renal mesenchymal tumors in rats. Renal tumors are especially attractive subjects for investi-

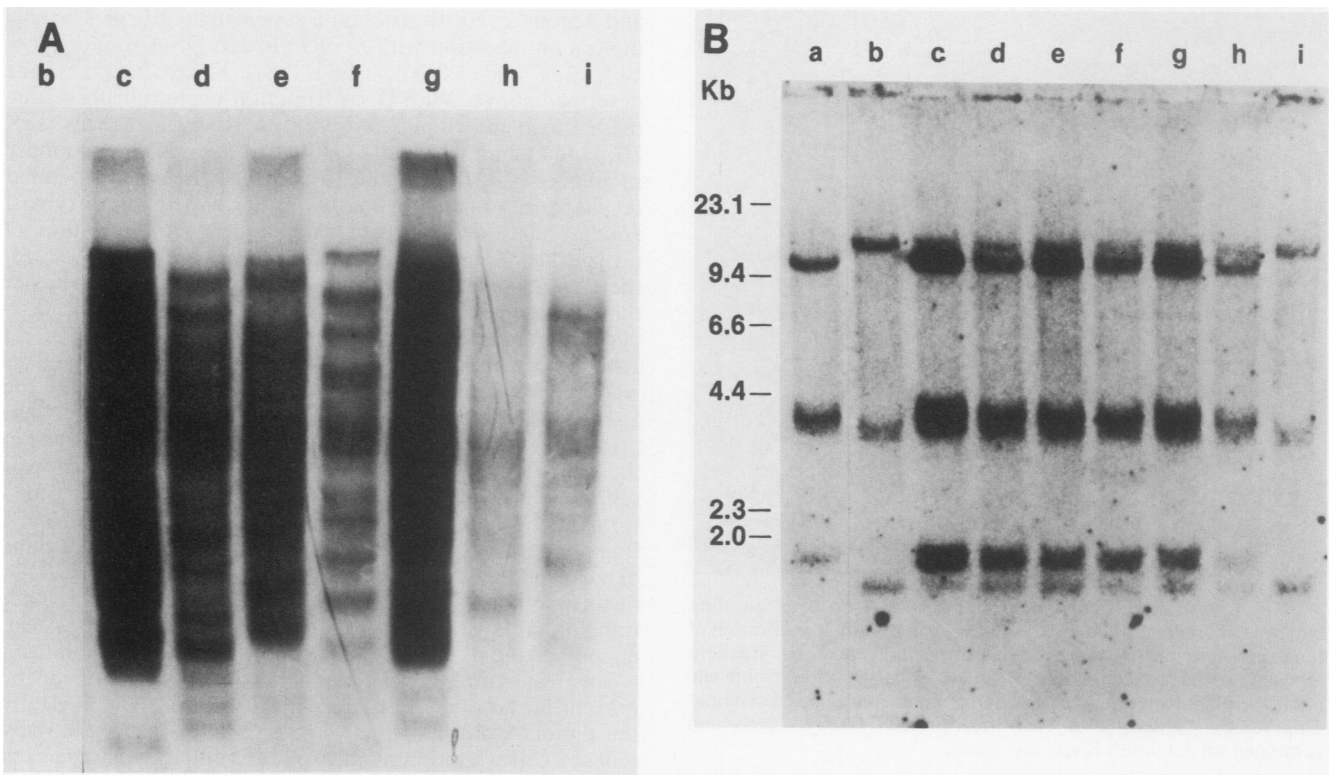

FIG. 2. (A) Detection of rat repetitive sequences in representative NIH/3T3 transformants derived from DMN-OMe-induced kidney tumors. High-molecular-weight DNA (20 μg) isolated from (lane b) control NIH/3T3 cells and from (lanes c, d, e, f, g, h, and i) first-cycle transformants derived from renal tumors was digested with *Hin*dIII, separated by gel electrophoresis in a 1% agarose gel, and blotted onto nitrocellulose filters as described by Southern (20). Filters were hybridized under stringent conditions with ^{32}P-labeled, nick-translated 225-bp *Pst*I insert of p3B5, a DNA fragment that contains rat repetitive sequences. DNA fragment sizes were determined from their migration relative to *Hin*dIII-digested fragments of λ *c*I857 DNA. (B) Southern blot analysis of K-*ras* sequences in NIH/3T3 cells transformed by DNA isolated from primary rat kidney tumors induced by DMN-OMe. Genomic DNA (30 μg) isolated from (lane a) normal rat kidney, (lane b) control NIH/3T3 cells, and (lanes c, d, e, f, g, h, and i) first-cycle transformants derived from renal mesenchymal tumors was digested with *Eco*RI for 1 h, subjected to 1% agarose gel electrophoresis, and blotted on nitrocellulose filters (20). Filters were hybridized under stringent conditions (50% formamide plus 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] at 42°C) for 48 h to 6 × 10^7 cpm ^{32}P-labeled, nick-translated 1-kb *Sst*I/*Hin*dIII fragment of c-K-*ras* (Lofstrand Laboratories Ltd., Gaithersburg, Md.) containing the exons I, II, and III of the human K-*ras*-2 gene. Size markers used were *Hin*dIII-digested fragments of λ *c*I857 DNA.

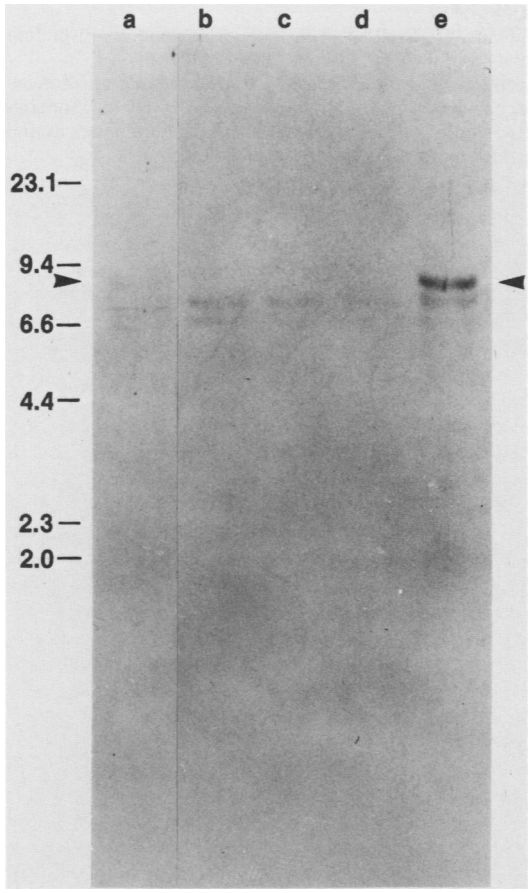

FIG. 3. Detection of N-*ras* sequences in NIH/3T3 cells transformed by renal tumor DNA. DNA from (lane a) normal rat kidney, (lane b) control NIH/3T3 cells, and (lanes c, d, and e) first-cycle transformants from renal tumors was digested with *Eco*RI and processed as described in the legend to Fig. 2. Two fragments of human N-*ras* gene (Lofstrand), a 0.3-kb *Hin*dIII/*Hin*dIII sequence containing exon I, and a 0.76-kb *Hin*dIII/*Eco*RI fragment containing exon II were labeled with ^{32}P and hybridized with blots under stringent conditions as described in the legend to Fig. 2. Rat-specific *Eco*RI 8.8-kb fragment indicated by arrowheads.

gation of the role of oncogene activation in chemical carcinogenesis. In rats, four major kinds of renal neoplasms are readily inducible: renal mesenchymal tumors and nephroblastomas (Wilms'-like tumors) in young animals, and epithelial tumors of the renal cortex and transitional cell tumors of the renal pelvis in older rats (10). Both categories of epithelial tumors, but not the others, are readily promoted in a two-stage (initiation/promotion) sequence, and the renal cortical, nephroblastic, and renal mesenchymal tumors originate from different target cells that share a common origin from embryonal metanephric mesenchyme. Accordingly, these neoplasms offer an opportunity to correlate activation of specific oncogenes with tumors that recapitulate different stages of differentiation of a common cell of origin, and to compare oncogene expression in tumors of a single cell type that arise from transient exposure to a genotoxic carcinogen, or from a defined initiation/promotion sequence.

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