Activation of the K-ras Protooncogene in Lung Tumors from Rats and Mice Chronically Exposed to Tetranitromethane


ABSTRACT

Dominant transforming genes were detected in lung tumors from Fischer 344 rats and C57BL/6 × C3H F1 mice chronically exposed by inhalation to tetranitromethane, a highly volatile compound used in several industrial processes. The rat lung neoplasms were classified as adenocarcinomas, squamous cell carcinomas (epidermoid carcinomas), or adenosquamous carcinomas. The mouse lung tumors were classified as papillary adenocarcinomas or adenomas. In both species, the tumors were morphologically similar to lung tumors in humans. The transfection assay using NIH/3T3 mouse fibroblasts detected transforming genes in 74% (14 of 19) of the rat lung tumors and in 100% (4 of 4) of the mouse lung tumors. Southern blot analysis indicated that transforming gene was an activated K-ras protooncogene in both species. The first exon of the K-ras gene in normal DNA and in DNA from two cell lines transformed by tumor DNA was compared by cloning and sequencing the gene. Experiments showed that there was a GC→AT transition in the second base of the 12th codon of the K-ras oncogene in the two transfected DNAs. Oligonucleotide hybridization indicated that all of the rat and mouse transfectedants had this activating lesion. Additional tumor DNA was then tested for the presence of a mutated allele with the GC→AT transition. All of the rat tumors tested and all of the mouse tumors tested had this mutation present. Hybridization using the normal oligonucleotide sequence around the 12th codon indicated that the normal allele was also present in the majority of the tumors, suggesting that the loss of normal allele is not necessary for the development of neoplasia. One rat lung tumor had no normal allele present, possibly suggesting that this tumor could have been in a more advanced stage than the other tumors. This is the first study to detect activated protooncogenes in rodent tumors induced under conditions which mimic human exposure to a chemical in the workplace. Tetranitromethane may exert its carcinogenic action by both activation of the K-ras oncogene and stimulation of cell proliferation by its irritant properties.

INTRODUCTION

Recent studies suggest that the activation of protooncogenes by genetic alterations may play a role in leading a cell to neoplastic development. These genetic alterations include gross chromosomal rearrangements, amplification of genes, and point mutations. Oncogenes that have been shown to acquire transforming activity by point mutation in their coding sequence include members of the ras oncogene family, the H-ras, K-ras, and the N-ras (1–13) and the neu oncogene (14). The activation of the ras family of genes usually occurs via a point mutation at the 12th, 13th, or 61st codons in human tumors and tumor cell lines (1–13). Studies in a variety of animal model systems have shown that specific activation of a protooncogene by point mutation can be caused by chemical or physical insult (1–8).

Animal model systems for carcinogenesis have provided a good means to study protooncogene activation in tumor development. The H-ras protooncogene has reproducibly been found activated in rat mammary carcinomas induced by a single injection of N-methyl-N-nitosourea given during sexual development (3). The H-ras protooncogene has also been found activated in mouse skin papillomas and carcinomas induced by DMBA2 followed by phorbol ester (12-O-tetradecanoylphorbol-13-acetate) promotion (1, 2, 7). In both models, the H-ras protooncogene was found to be activated in 90–100% of all of the tumors examined. Other studies have found K-ras and N-ras activation in X-ray- or N-methyl-N-nitosourea-induced mouse thymomas and in rat mesenchymal kidney tumors induced by treatment with methyl(methoxy-methyl)nitrosamine (4, 5, 15). One conclusion from these studies is that exposure to carcinogens either by relatively high single or multiple doses causes changes in the DNA resulting in activation of oncogenes. However, no studies have examined protooncogene activation in tumors that develop after long term, chronic exposure to chemicals. The identification of chemicals as potential human carcinogens is often made on the basis of long term rodent bioassays which are designed to consider route of human exposure and concentrations similar to those present in the environment, workplace or home.

In a recent bioassay conducted by the National Toxicology Program, chronic exposure to the industrial chemical TNM induced a high incidence of primary lung tumors in Fischer 344 rats and C57BL/6 × C3H F1 (hereafter called B6C3F1) mice.3 TNM is a highly volatile compound used as a reagent in industrial nitrosating processes, as an oxidant in rocket fuel, and as an explosive when mixed with tolune (tetranitrotoluene). Because of its irritant properties, TNM has also been proposed as a war gas. The threshold limit for occupational exposure to TNM based on its irritant properties has been set at 1 ppm. In the bioassay, groups of 50 male and 50 female Fischer 344 rats or B6C3F1 mice were exposed to TNM by inhalation for 6 h a day, 5 days a week for 2 years. The rats were exposed to 0, 2, and 5 ppm of TNM while the mice were exposed to 0, 0.5, and 1 ppm. Based on histopathological examination, the TNM-induced primary lung tumors were adenomas, adenocarcinomas, squamous cell carcinomas, and adenosquamous carcinomas in rats and papillary adenomas and adenocarcinomas in mice. These tumors were morphologically similar to primary lung tumors in humans. The purpose of this study was to identify and characterize any activated oncogenes that might be present in lung tumors from rats and mice after chronic exposure to TNM.

MATERIALS AND METHODS

Lung Tumor Generation. Two-year toxicity and carcinogenicity studies of TNM were performed under National Toxicology Program conditions. The data collected during the studies with TNM. Therefore, the apparent association of TNM exposure with lung tumors in rats or mice should be considered preliminary, pending approval of the National Toxicology Program Technical Report on TNM by the National Toxicology Program Board of Scientific Counselors Technical Reports Review Subcommittee.

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1 The abbreviations used are: DMBA, 7,12-dimethylbenzanthracene; TNM, tetranitromethane.

2 The National Toxicology Program has not yet completed its evaluation of the data collected during the studies with TNM. Therefore, the apparent association of TNM exposure with lung tumors in rats or mice should be considered preliminary, pending approval of the National Toxicology Program Technical Report on TNM by the National Toxicology Program Board of Scientific Counselors Technical Reports Review Subcommittee.
and a two-stage dilution system. Test atmospheres were monitored using a Miran II IR gas analyzer every 10-15 min during exposures. Groups of 50 male and 50 female Fischer 344 rats were exposed to 0, 2, or 5 ppm of TNM by inhalation for 6 h/day, 5 days/week for 2 years. Similar groups of B6C3F1 mice were exposed to 0, 0.5, and 1 ppm. All animals received a complete necropsy, and tissues were collected for microscopic evaluation which was performed by Pathology Associates Ijamsville, MD. At necropsy lungs were inflated to normal inspiratory volume with 10% neutral buffered formalin and immersed in the same fixative. Hematoxylin- and eosin-stained paraffin sections were prepared according to routine pathological procedures. During the terminal sacrifice, lung tumors and normal lung tissue were collected for this study. At this time representative portions of selected rat and mouse lung tumors were fixed in 3% glutaraldehyde and subsequently processed for transmission electron microscopic examination.

DNA Isolation. High molecular weight DNA was isolated from normal or tumor tissues by using Pronase-sodium dodecyl sulfate lysis. Following phenol-chloroform extraction and ethanol precipitation, the DNA samples were treated with RNase and additional phenol-chloroform extractions and ethanol precipitation (16). The size of the DNA was checked on a 0.7% agarose gel.

Transfection Assay. High molecular weight DNA from the rat or mouse lung tumors was transfected onto NIH/3T3 mouse fibroblasts (30 µg/plate, four plates/sample) by the calcium phosphate precipitation method described previously (16). The cells were maintained with Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 5% calf serum (Colorado Serum Co., Denver, CO) for 21 days until the foci were scored. Isolated foci were grown in 10% calf serum Dulbecco's modified Eagle's medium and stored as cell pellets until needed for DNA isolation and subsequent transfection or hybridizations.

Southern Blot Analysis. High molecular weight DNA was isolated, digested with HindIII (Boehringer-Mannheim, Indianapolis, IN), and electrophoresed on a 0.7% agarose gel (20 µg/lane). The DNA was then transferred to nitrocellulose (16). After baking and prehybridization, the blot was hybridized under stringent conditions (50% formamide/0.75 M NaCl/0.075 M sodium citrate; 42°C) to the StrlII-Xba1 fragment containing the first, second, and part of the third exons (HIIHI380) of the v-kis oncogene for rat DNA and the StrlII-HincII fragment (Oncor, Gaithersburg, MD) for the mouse DNA (17). The blot was washed to a final stringency of 0.2x sodium citrate solution-0.1% sodium dodecyl sulfate at 50°C. The blot was exposed to film overnight at -70°C with intensifying screens.

Cloning and Sequencing. Total normal rat DNA or transfectant DNA derived from an adnocarcinoma or a squamous cell carcinoma was digested with HindIII, ligated to phage λ Charon 28 HindIII arms, and packaged using the Promega Packagene System (Madison, WI). Positive plaques containing the first exon of the rat K-ras oncogene were identified by hybridization to the StrlII-Sau3A1 fragment (containing the first exon) of the v-kis oncogene (17). Phage from the positive plaques were grown and the size was checked by digestion with HindIII and electrophoresis on an agarose gel. Southern transfer and subsequent hybridization to the first exon probe confirmed the presence of the K-ras first exon 2.6-kilobase insert. This insert was subcloned into HindIII-cut pBR322. A restriction map was obtained by a combination of single and double digests of various enzymes. The first exon was localized to a 0.6-kilobase EcoRI-HindIII fragment. This fragment was then subcloned into M13mp19 for dyeoxy sequencing using the BRL Cloning and Sequencing Kit (Bethesda, MD) (18).

Oligonucleotide Hybridization. HindIII- or EcoRI-digested DNA was electrophoresed on a 0.7% agarose gel, hybridized, and washed according to the method of Bos et al. (19) with the following modifications: 50% formamide was used in the hybridization buffer; gels were hybridized at 42°C; and the gels were washed to a final stringency of two 15-min washes at 62°C in 2x sodium citrate solution. After being wrapped in plastic wrap, the gels were exposed to film for 1-3 days. The sequences of the oligonucleotide probes used in these experiments are the normal sequence 5' -TTGGAGCTGGTGGCGTAGG-3' from E. P. Reddy or the mutated sequence 5' -TTGGAGCTGATGGCGTAGG-3' (OCS Laboratories, Denton, TX).

RESULTS

Activated Oncogenes in Rat Lung Tumors

Tumor Generation. In contrast to the absence of primary lung tumors in controls, male and female rats exposed to TNM had a high incidence of benign and/or malignant lung tumors. There
Protooncogene Activation in Rat and Mouse Lung Tumors

Table 1  Transforming genes in TNM-induced lung tumors in rats

<table>
<thead>
<tr>
<th>DNA source</th>
<th>Samples tested</th>
<th>Transforming genes (% positive)</th>
<th>Transformation efficiency (foci/μg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary adenocarcinoma</td>
<td>12</td>
<td>9/12 (75)</td>
<td>0.003-0.009</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>4</td>
<td>3/4 (75)</td>
<td>0.003</td>
</tr>
<tr>
<td>Adenosquamous carcinoma</td>
<td>3</td>
<td>2/3 (67)</td>
<td>0.006</td>
</tr>
<tr>
<td>Normal tissue</td>
<td>8</td>
<td>0/8 (0)</td>
<td></td>
</tr>
</tbody>
</table>

DNA was isolated from rat lung tumors and transfected onto NIH/3T3 mouse fibroblasts by the calcium phosphate precipitation method described previously (6). The cells were maintained with Dulbecco’s modified Eagle’s medium (GIBCO) supplemented with 5% calf serum (Colorado Serum Co.) for 21 days until the foci were scored. Isolated foci were grown in 10% calf serum-Dulbecco’s modified Eagle’s medium for DNA isolation and subsequent transfection.

Fig. 2. High molecular weight DNA was isolated, digested with HindIII (Boehringer-Mannheim), and electrophoresed on a 0.7% agarose gel. Molecular weight standards from HindIII-digested wild-type λ DNA are noted at left. Bands at 7.4 and 2.6 kilobases (Kb) contain rat sequences that are homologous to the 5′-1′-3′ fragment of the v-kis oncogene (17). Lane 1, 20 μg of NIH/3T3 DNA; Lanes 2-5, 20 μg of secondary transfectant DNA generated initially from adenocarcinomas; Lanes 6-7, 20 μg of secondary transfectant DNA generated initially from squamous cell carcinomas; Lane 8, 20 μg of secondary transfectant DNA generated initially from an adenosquamous carcinoma; Lane 9, 20 μg normal rat DNA from the spleen.

was a dose-related increased incidence, increased multiplicity, and increased frequency of local invasion as well as distant metastases of the lung tumors in TNM-exposed rats. The earliest occurrence of lung tumors was observed in rats that died after 12 months of exposure to TNM. The benign lung tumors were solid bronchiolaralveolar adenomas (20, 21). The malignant tumors were adenocarcinomas usually with a significant amount of stromal proliferation (Fig. 1A), squamous cell carcinomas with abundant keratin formation (Fig. 1B), and adenosquamous carcinomas. Based on electron microscopic examination of some of the adenocarcinomas, the cells in some tumors were compatible with Clara cells and others with type II cells. Because of the small tumor size, no benign tumors were available for oncogene analysis from this bioassay.

Transfection Assay. Fourteen of 19 tumor DNA (74%) induced morphological transformation of the NIH/3T3 cells, indicating the presence of a dominant transforming gene (see Table 1). Individual tumor types had similar results: 75% of the primary adenocarcinoma DNA were positive; 75% of the squamous cell carcinoma DNA were positive; and 67% of the adenosquamous carcinoma DNA were positive. Eight samples of rat lung DNA obtained from the air-exposed controls were negative in this assay. The transforming frequency ranged from 0.012-0.036 foci/μg DNA for the first cycle of the transfection and was 10-fold higher for the second cycle. No histomorphological differences could be detected between those rat samples with or without transforming activity as detected by this assay.

Southern Analysis. Frequently, the transforming gene detected by the NIH/3T3 assay has been a mutated version of a member of the ras gene family (1-13, 19, 22-24). HindIII-digested rat transfected DNA was tested for the presence of novel or amplified restriction fragments that hybridized to H-ras-, K-ras-, or N-ras-specific oncogene probes using Southern blot analysis. An activated H-ras or N-ras could not be detected in any of the transfected DNA from the rat lung tumors induced by TNM (data not shown). As shown in Fig. 2, the K-ras probe hybridized to two HindIII fragments (at 7.4 and 2.6 kilobases) in each secondary transfectant DNA (Fig. 2, Lanes 2-8) in addition to the three NIH/3T3 mouse K-ras HindIII fragments (17.3, 3.6, and 1.6 kilobases in Lanes 1-8). These two novel bands appear to be amplified and comigrate with normal rat K-ras bands (Lane 9) suggesting that the transforming properties of the TNM lung tumor DNA were due to the transfer of an activated cellular homologue of the rat K-ras protooncogene into the NIH/3T3 cells.

Cloning and Sequencing. The most prominent lesion in activated K-ras protooncogenes to date has involved mutations in the 12th codon (4, 6, 9-11). Upon examination of TNM-activated K-ras protein products, it was found that these proteins comigrate with the normal ras proteins on a sodium dodecyl sulfate-polyacrylamide gel, which indicated no apparent mutation at the 12th or 61st codons (data not shown). To determine if there was a mutation present at the 12th codon of the K-ras oncogene in these rat lung tumors, the first exon of the normal rat K-ras protooncogene and the protooncogene activated in two TNM transfectant DNAs were cloned and the nucleotide sequences were determined. Total normal rat DNA or transfected DNA derived from an adenocarcinoma and a squamous cell carcinoma was digested with HindIII and cloned into Charon 28 vector. Restriction mapping of the 2.6-kilobase HindIII fragment after subcloning into the plasmid pBR322 localized the first exon of the K-ras gene in normal rat DNA and the two transfectant DNAs to a 0.6-kb EcoRI-HindIII fragment. This fragment was then subcloned into M13mp19 for dideoxy sequencing (18). Only a single base difference between the normal rat and both TNM transfected cloned sequences was found involving a GC—»AT transition in the second base of the triplet coding for amino acid 12, changing glycine to aspartic acid. This GC—»AT transition was seen in both of the cloned transfectants indicating that the activating lesions were the same regardless of the morphological appearance of the original tumors.

Oligonucleotide Hybridization. Normal and mutated radioactive oligonucleotide probes centered on the second base of the 12th codon were hybridized to the HindIII-digested transfectant DNA and cloned versions of the normal and mutated K-ras first exons to determine if more of the transfectants had this same activating lesions, GC—»AT (see Fig. 3). There was no hybridization of the mutated oligonucleotide probe to DNA from the normal clone (Fig. 3B, Lanes 10 and 12) under...
conditions where strong hybridization was observed with the normal oligonucleotide probe (Fig. 3A, Lanes 11 and 13). In contrast, strong hybridization to the mutated transfectant clone was observed with the mutated (Fig. 3B, Lanes 11 and 13) but not the normal oligonucleotide probe (Fig. 3A, Lanes 11 and 13). The mutated oligonucleotide probe also bound to each of the seven TNM rat transfectant first exons (Fig. 3B, Lanes 2–8) and not to normal rat DNA (Fig. 3B, Lane 9) or to NIH/3T3 DNA (Fig. 3B, Lane 1). The normal oligonucleotide probe bound only to the normal K-ras first exon in the rat genomic DNA (Fig. 3A, Lane 9) and not to the transfectant DNA (Fig. 3A, Lanes 2–8). Taken together these data indicate that the same activating lesion is present in each of the transfectants derived from tumors induced by chronic exposure to TNM.

To see if the mutation could be detected in the tumor directly, seven tumor DNA that were positive on the transfection assay and four tumor DNA that were negative on the assay were examined by oligonucleotide hybridization. Complete digestion of rat lung tumor DNA was not possible with the restriction enzyme HindIII used to digest the transfectant DNA probably...
PROTOONCOGENE ACTIVATION IN RAT AND MOUSE LUNG TUMORS

Fig. 5. Photomicrograph and electron micrograph of a papillary adenocarcinoma from a female mouse exposed to 1 ppm of TNM for 2 years. A, photomicrograph showing tumor composed of cuboidal to columnar epithelial cells forming irregular glands. H & E. × 150. B, electron micrograph of the same tumor showing cells forming a gland have microvilli on their luminal surface and containing developing and mature cytoplasmic lamellar bodies. Tubular myelin (surfactant protein) and lamellar bodies are present in the lumen of the gland, × 6000.

because of inhibitors present in the tumor tissue. Therefore, the rat lung DNA was digested with EcoRI for complete digestion. The mutated oligonucleotide allowed detection of a 3.7-kilobase band indicating that the mutated allele was in each of the tumor samples tested whether they were positive (Fig. 4B, Lanes 1–5, 10 and 11) or negative (Fig. 4B, Lanes 6–9) on the transfection assay. The mutated oligonucleotide did not hybridize to the normal rat DNA (Fig. 4B, Lane 12). Inconsistencies between the transfection data and the oligonucleotide hybridization data may be due to the fact that the K-ras is such a large gene and may be difficult to isolate and transflect into the mouse fibroblasts efficiently. The normal allele was also detected in 10 of 11 of the tumors tested (Fig. 4A, Lanes 1–11) and in normal DNA (Fig. 4A, Lane 12).

It appears that the tumor DNA examined in Fig. 4 hybridizes to the mutant probe with different levels resulting in variations in the intensities of the bands. This could be due to several reasons. One possibility could be that the mutant probe is hybridizing, although less efficiently, to other mutations in the 12th codon such as those coding for valine (GTT) or alanine (GCT). It must be pointed out, however, that one of those faint bands in Fig. 4B, Lane 11, is the tumor DNA corresponding to the transfected DNA in Fig. 3B, Lane 7. This transfected DNA was characterized as having a GC→AT transition in the 12th codon. Therefore, it is possible that the faint bands in the rest of the tumor DNA are the result of perfect hybridization of the mutant probe with tumor DNA having the same mutation. It is also unlikely that cross-hybridization occurs because all of these gels were washed above the critical temperature where mismatches should wash off. Another possibility could be differences in the amount of DNA loaded into each well. The most probable cause of the differences in the intensities of bands in the tumor DNA is the difference in the relative amounts of normal DNA compared to the mutant DNA present in a 20-μg sample of tumor DNA.

Activated Oncogenes in TNM-induced Mouse Lung Tumors

A low incidence of spontaneous benign and malignant lung tumors was observed in control mice while mice exposed to TNM had a dramatic dose-related increase of primary lung tumors. As in the rats, there was a dose-related increased incidence, multiplicity, and frequency of metastasis and invasion of the TNM-induced lung tumors in male and female mice. The earliest observation of a lung tumor was after 54 weeks of treatment in a high dose male. Lung tumors in treated mice were morphologically similar to but larger than those in controls. Morphological features of the tumors were compatible with solid papillary adenomas and adenocarcinomas (Fig. 5A) having minimal stromal proliferation. Several of the tumors in treated mice were composed of type II cells with lamellar bodies and, in some instances, tubular myelin was present in glands formed by these cells (Fig. 5B). Other tumors had ultrastructural cytological features compatible with Clara cells.

Four of four mouse lung tumor DNA tested induced morphological transformation of the NIH/3T3 mouse fibroblasts after transfection. The transforming frequency ranged from 0.067–0.233 foci/μg DNA. This slightly higher frequency compared to the rat tumor DNA-transforming frequency was probably due to a better quality DNA obtained from the mouse tumors than that obtained from the rat tumors.

The mouse transfectants were then examined for an activated K-ras protooncogene. The transfectant DNA and normal mouse lung DNA were digested with HindIII and probed with the SstII-HincII fragment of v-kis. Rearranged bands were detected in three of the transfectants (Fig. 6A, Lanes 1–3), and amplified signals were detected in one of the transfectants (Fig. 6A, Lane 4) in addition to the background NIH/3T3 DNA bands (Fig. 6A, Lanes 1–4) and the normal mouse lung DNA bands (Fig. 6A, Lane 5) at 17.3, 3.6, and 1.6 kilobases. The rearrangements and the amplification of these bands indicated that there was a transfer of the K-ras oncogene in these transfectants, similar to that observed in the rat transfectants.

Hybridization of the oligonucleotide probe containing the sequence around the 12th codon with the mutation seen in the

3216
Fig. 6. Activated K-ras oncogenes with 12th-codon mutations in TNM mouse transfectants. A, detection of rearranged or amplified v-kis sequences in HindIII-digested TNM mouse transfectants using the HIH3 v-kis probe. B, detection of the normal 12th codon of the K-ras oncogene in the HindIII-digested transfectant DNA (background), in normal lung DNA, and in NIH/3T3 DNA. C, detection of a mutated 12th codon in the K-ras oncogene of HindIII-digested mouse transfectant DNA using the mutated probe described in Fig. 3. In A, B, and C: Lanes 1-4, 10 μg four TNM mouse transfectant DNAs; Lane 5, 10 μg normal mouse lung DNA. In B and C: Lane 6, 10 μg NIH/3T3 DNA.

Fig. 7. Detection of a mutated 12th codon in TNM-induced mouse tumors by oligonucleotide hybridization. A, detection of the normal allele in the mouse tumor DNA. B, detection of the mutated sequence in the mouse tumor DNA using the mutated oligonucleotide probe described in Fig. 3. In A and B: Lane 1, 20 μg of normal mouse lung DNA; Lanes 2-4 and 8-10, 20 μg of HindIII-digested mouse adenocarcinoma DNA; Lane 5, 20 μg of HindIII-digested mouse adenoma DNA; and Lanes 6 and 7, 20 μg of HindIII-digested mouse adenocarcinoma/adenoma DNA.

rat DNA indicated that the same mutation was present in each of the mouse transfectants (Fig. 6C, Lanes 1-4). No hybridization of this probe could be seen with normal mouse lung DNA or NIH/3T3 DNA as expected (Fig. 6C, Lanes 5-6). The normal probe hybridized to all transfectants indicating the background NIH/3T3 DNA (Fig. 6B, Lanes 1-4) and to the normal and NIH/3T3 mouse DNA (Fig. 6B, Lanes 5 and 6).

Examination by oligonucleotide hybridization of nine mouse lung tumor DNA, including the four transfected into NIH/3T3 cells, showed that all of these tumors had the same GC→AT transition as that found in rat TNM-induced lung tumors (Fig. 7A, Lanes 2-10). These tumors range from benign adenomas (Fig. 7, Lane 5) to mixtures of adenomas and carcinomas (Fig. 7, Lanes 6 and 7) to carcinomas (Fig. 7, Lanes 2-4 and 8-10). Another adenoma (data not shown) also had this GC→AT transition. Each of these tumor DNA also had a normal allele
present that could be detected by oligonucleotide hybridization (Fig. 7B, Lanes 2–10). As with normal rat DNA, only the normal oligonucleotide would hybridize to normal mouse DNA (Fig. 7, Lane 1).

DISCUSSION

This is the first study to show ras protooncogene activation in a system where tumors can be induced under conditions similar to human occupational exposure to chemicals. Lung tumors were obtained from two species, the B6C3F1 mouse and the Fischer 344 rat, after long-term chronic exposure to TNM. Histomorphological and ultrastructural features of these tumors are similar to those described for human lung tumors. An activated K-ras protooncogene was detected in 100% of the mouse tumors tested and 74% of the rat tumors tested by the NIH/3T3 transfection assay. The detection of the activated K-ras gene in two benign mouse tumors suggests that the activation of this gene may be an early event in TNM-induced lung tumors.

The K-ras oncogene has been the only oncogene detected by the transfection assay with DNA from human lung tumors and tumor cell lines, with the exception of the HS242 and SW1271 lung tumor cell lines which have activated H-ras and N-ras oncogenes, respectively (9–13, 23–27). Amplification or increased expression of members of the myc oncogene family and the myb oncogene has been detected in a number of the human tumors as well (28–32). TNM-induced rodent tumors are the first rodent lung tumors that have been examined for activated oncogenes. Activation of the K-ras oncogene in these rat and mouse lung tumors is consistent with the published human lung data. Human and rodent data seem to suggest a tissue-specific activation of a particular protooncogene, at least in the case of the activation of the K-ras oncogene in the lung.

A variety of point mutations have been detected in activated ras genes from primary tumors and tumor cell lines. At present, K-ras protooncogene activation in vivo has involved mutations at the 12th codon except in two cases. In one case there is amplification of the normal gene and in another there is a AT→TA transversion in the 61st codon of K-ras (4, 6, 9–11, 23–27). The GC→AT transition observed in all of the TNM-induced lung tumors tested may be indicative of a specific lesion in DNA caused by TNM. Point mutations resulting in the activation of protooncogenes in several chemically induced rodent tumors have been consistent with the known alklation patterns of the carcinogen (1–3, 33–35). For example, mutations at the 12th codon of the H-ras detected in rat mammary tumors induced by methyl nitrosourea (3) are consistent with the formation of the O^6-methylguanine adduct, and the activating mutation found in DMBA-induced mammary and skin tumors is consistent with DMBA binding to adenosine residues (1, 2, 33, 34).

At present, no information concerning the possible interaction of TNM with DNA is available. However, TNM causes the mutant bacterial strains that detect base pair substitutions TA1535 and TA100 to revert to the wild type by the same GC→AT transition that is observed in the activated K-ras oncogene in TNM lung tumors. Since TNM is a known nitrating agent at physiological pH, it could possibly interact with DNA through this mechanism to damage DNA (36). It has also been suggested that nitro-containing compounds may deaminate a base such as cytosine to cause later base mispairing.

Several studies have shown that the loss of the normal allele of oncogenes such as c-H-ras and c-myc can be correlated with the aggressiveness and/or stage of development of human tumors (37, 38). In this study, we observed that one of 11 rat lung tumors and none of the mouse lung tumors examined had lost the normal allele of the K-ras oncogene. A similar loss of the normal N-ras allele was seen in a chemically induced thymic lymphoma (39). In that study, Guerrero et al. (39) found one tumor with a CG→TA transversion in the 61st codon of N-ras and not the normal N-ras allele. However, they also found that no tumors were heterozygous in their allelic composition. The presence of the mutated and normal allele in almost all of the TNM-induced lung tumors indicates that the loss of the normal allele is not a prerequisite for tumor formation in these rats and mice. However, this loss could be a sign of aggressiveness or progression as has been suggested by the human tumor data.

Reproducible detection of specific transforming genes in animal model systems strongly suggests that oncogenes play a significant role in the development of these tumors. This is the first study to show that long-term chronic exposure to a chemical is capable of reproducibly activating oncogenes similar to those observed in single dose and initiation-promotion studies. TNM may exert its carcinogenic action by both activation of the K-ras oncogene and stimulation of cell proliferation by its irritant properties.

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PROTOONCOGENE ACTIVATION IN RAT AND MOUSE LUNG TUMORS


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S. Jill Stowers, Paul L. Glover, Steven H. Reynolds, et al.


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