Effect of promoter and intron 2 polymorphisms on murine lung K-ras gene expression

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Differences in tumor formation among inbred mouse strains with high (A/J) and low (C3H) susceptibility for lung cancer have been linked to a repetitive element within the second intron of the K-ras gene. The purpose of this investigation was to determine whether differences within the K-ras gene promoter region or the intron 2 polymorphism affect K-ras gene expression in lung tumors and target alveolar type II cells isolated from A/J and C3H mice. Ribonuclease protection assays were performed using RNA isolated from 4-(methylamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumors from each mouse strain and alveolar type II cells isolated from A/J and C3H mice. An 838 bp fragment of the murine K-ras gene promoter region was amplified by PCR, cloned and sequenced from both mouse strains. Promoter regions from both mouse strains were inserted into a luciferase reporter gene vector, with and without the second intron polymorphism, and transfected into sensitive, intermediate and resistant lung tumor cell lines. No significant differences in K-ras gene promoter activity were found between the two strains using these specific reporter gene constructs. Consistent with these results, levels of K-ras expression did not differ between alveolar type II cells, whole lung or tumors induced by NNK in A/J or C3H mice. Moreover, in lung tumor cell lines derived from mice with differing susceptibility for lung cancer, K-ras expression did not correlate with the growth rate of tumors induced in nude mice from these cell lines. These results indicate that factors involved in modulating the rapid clonal expansion of the mutated K-ras allele from A/J mice are not directly linked to expression of this gene. Other genetic changes or losses in conjunction with hypothesized modifier loci, such as the Par1 locus, must play a significant role in establishing the phenotypic strain differences for lung tumor formation.

Introduction

Inbred strains of mice have long been recognized for forming spontaneous and chemically induced lung tumors at different rates (1). For example, the C3H mouse, a lung tumor-resistant strain, has decreased size, longer latency and lower multiplicity of 4-(methylamino)-1-(3-pyridyl)-1-butanone (NNK*)-induced lung tumors compared with the lung tumor-sensitive A/J strain (2). The genetics underlying this difference in susceptibility were examined in recombinant inbred strains generated by crossing the A/J mouse with another resistant strain, the C57BL/6J mouse. Tumor incidence and multiplicity following exposure of 21 different inbred mouse strains to the carcinogen urethane supported a three-locus model and genes associated with these loci were designated ‘Pas’, for pulmonary adenoma susceptibility (3). Subsequent linkage analysis strongly implicated the K-ras gene, a gene frequently mutated in spontaneous and chemically induced murine lung tumors, as one of thePas genes. The involvement of the K-ras gene in murine lung tumor susceptibility was further substantiated by identification of an EcoRI restriction fragment length polymorphism that correlated with the inherited susceptibility of 14 inbred strains of mice to lung cancer induced by urethane (4).

This polymorphic difference that predicted lung tumor susceptibility was subsequently linked to a repetitive element polymorphism within the second intron of the K-ras gene (5). Lung tumor-resistant strains have a 37 bp repetitive element polymorphism within the second intron of the K-ras gene, whereas lung tumor-sensitive strains lack one 37 bp repetitive element. This polymorphism was used to determine which K-ras allele would contain the codon 12 mutation in NNK-induced lung tumors from the C3A mouse (F1 hybrid cross of the C3H and A/J mice). The A/J allele was mutated and overexpressed in 90% of lung tumors from the C3A mouse (5).

The predominance of mutations within the A/J allele from hybrid mice could stem from higher rates of transcription of the A/J allele compared with the C3H allele. Although the K-ras gene is mutated in both inbred strains following chronic exposure to NNK, in vitro studies indicate that mutation alone is not enough to cause cellular transformation (6). Some other event is required, such as overexpression of the affected gene (6). Thus, the underlying differences in lung tumor susceptibility may involve effects on regulation of the K-ras gene. Sequence differences within the promoter or the second intron regions could alter K-ras gene expression. High affinity protein binding to the repetitive regions within the second intron of the K-ras gene was found for all strains, suggesting that this may be a regulatory region to which transacting factors bind (7).

The purpose of this study was to determine whether the transcription rate and expression of the K-ras allele differ between A/J and C3H mice. The promoter region of the K-ras gene from both mice was cloned in order to compare the sequence between the two strains and to determine whether differences in the promoter region along with the repetitive element in the second intron affect the transcription rate of the K-ras gene. Levels of K-ras gene expression in target cells (alveolar type II), whole lung and lung tumors from A/J and C3H mice were also quantified.

Materials and methods

Cloning and sequencing

An 838 bp fragment containing exon 0 of the K-ras gene was generated from normal lung DNA by PCR amplification using primers based on the published

*Abbreviations: FBS, fetal bovine serum; NNK, 4-(methylamino)-1-(3-pyridyl)-1-butanone; RPA, ribonuclease protection assay.
mouse promoter region sequence (8). These primers were designed to incorporate the 688 bp Smal fragment that has been shown to contain the most active promoter region (8). Due to the presence of a CpG island (9) within the amplified region, the PCR was modified by using a two-step cycle, high annealing temperature reaction with 10% DMSO (10). The 838 bp fragment was then ligated into the TA cloning vector (Invitrogen, San Diego, CA) and five clones from each mouse strain were sequenced using an automated ABI sequencing system (Perkin-Elmer, Foster City, CA). Single-strand template was sequenced from the five different clones from each mouse strain following subcloning into a pBluescript vector (Promega, Madison, WI). Clones were selected that were inserted in both directions into the pBluescript vector so that both strands were isolated and sequenced. Single-strand template was recovered using a standard single-strand phage recapture protocol (Stratagene, La Jolla, CA).

Animal treatment, cell isolation and tumor induction

Twenty A/J and 20 C3H mice (6–8 weeks old; Jackson Laboratory, Bar Harbor, ME) were killed and their lungs collected. Alveolar type II cells were obtained by centrifugal elutriation following protease digestion of the pooled lungs (11). Purity of the type II cells, assessed by a modification of Papaincleaved staining, was 71%. The isolated cells were divided into two aliquots, snap frozen in liquid nitrogen as cell pellets and stored at –80°C. Total RNA was then isolated from the cell pellets using the Trizol reagent (Life Technologies, Gaithersburg, MD).

Lung tumors were induced in A/J and C3H mice by treatment of mice three times a week for 7 weeks (50 mg/kg by i.p. with NNK (Chesmyn Science Laboratories, Lenexa, KS). Tumors were collected from mice sacrificed 44–68 weeks after initiation of treatment. Rat lungs from tumor prone NIH 3T3 cells were established in these mouse strains (12,13) and the size of the tumors harvested 68 weeks after initiation of treatment. Based on the size of the tumors harvested (60–150 mg), these neoplasms were most likely adenocarcinomas. RNA was isolated from 12 different NNK-induced lung tumors from each mouse strain using the Trizol method (Life Technologies) and was stored as 10 µg aliquots for ribonuclease protection assay (RPA).

Tissue culture and RNA isolation

Five cell lines which were derived from mice with differing susceptibility (sensitive, intermediate or resistant) for lung tumor development were utilized in this investigation: (i) the C10 cell line derived from normal lung tissue isolated from the intermediate Balb/C mouse strain; (ii) the SPON4 cell line derived from an adenocarcinoma taken from the sensitive A/J mouse strain; (iii) the CMT64 cell line derived from an adenocarcinoma taken from the resistant C57 mouse strain; (iv) the IO33 cell line derived from an adenocarcinoma taken from the sensitive A/J mouse strain; (v) the MC7pp cell line derived from an adenocarcinoma taken from the resistant B6C3F1 mouse strain. Two additional cell lines used were the MNKN cell line, which is an NIH 3T3 transformant generated by transfection of lung tumor DNA containing a mutated K-ras gene (14), and normal NIH 3T3 cell lines. The C10, SPON4, CMT64 and MC7pp cell lines were kindly provided by Dr Fred Tyson (National Institute of Environmental Health Sciences, Research Triangle Park, NC). The IO33 cell line was grown in CMRL medium with 10% FBS, L-glutamine and gentamycin added (Gibco BRL, Gaithersburg, MD). The SPON4 and CMT64 cell lines were grown in DMEM medium with 10% FBS, L-glutamine and gentamycin added (Gibco BRL). The IO33, MC7pp, MNKN and NIH 3T3 cell lines were grown in RPMI (Gibco BRL) with 10% FBS, L-glutamine and gentamycin added. All cell lines were grown to 80% confluence, harvested and total RNA isolated using TrisReagent (Molecular Research Center, Cincinnati, OH). The SPON4, IO33, C10, CMT64 and MC7pp cell lines (1×106 cells) were isolated s.c. on the left and right flanks of nude mice (2 mice/cell line). Tumor growth rates were determined through biweekly measurement of tumor size. Tumor tissue was harvested, snap frozen in liquid nitrogen and stored at –80°C. Total RNA was also isolated from nude mouse tumors using the Trizol method (Life Technologies).

Ribonuclease protection assay

Ten micrograms of RNA were used in the RPA. Antisense pTRI-β-actin-mouse and pTRI-c-Ki-ras-mouse linearized plasmid cDNA probes (Ambion, Austin, TX) were radiolabeled with [γ-32P]UTP using an Sp6 or T7 RNA polymerase following the Maxscript™ kit instructions (Ambion). Hybridization and digestion conditions were carried out as per the RPA TM™ kit instructions (Ambion). The products were ethanol precipitated, redissolved in a 95% formamide loading buffer and separated on 5% polyacrylamide–8 M urea gels. Levels of activity for both actin and K-ras gene expression were quantified using a Collage image analysis system (Fotodyne, Hartland, WI). K-ras gene expression levels in lung tumors, whole lung, murine lung cell lines and C3H type II cells were normalized to actin levels and then compared with normalized levels in A/J type II cells. K-ras gene expression for MNKN and NIH 3T3 cell lines were normalized to actin levels from the MNKN cell line and compared with each other. The mouse cyclophilin gene was also used as a probe in some assays. The size of the cyclophilin gene probe was 103 bp and, therefore, RNA samples could be simultaneously probed with this gene and the K-ras gene in order to ensure sample-to-sample RNA integrity. Assay of expression levels in cell lines and isolated cells were repeated at least three times.

Reporter gene constructs and analyses

The relative activity of the A/J and C3H K-ras promoter regions was compared using the luciferase reporter gene system pGL3 (Promega). The 388 bp fragment from both mouse strains was inserted upstream of the luciferase gene in the pGL3-β-galasticase vector by blunt end ligation at the Smal site. DNA fragments containing the murine K-ras second intron repetitive element sequence with varying lengths of flanking sequence were also inserted into the pGL3-β-galasticase vector at the BamHI site. The various intronic regions were as follows (5): short intron (bp +241 to +395, downstream of the translational start site), medium intron (bp +241 to +583, downstream of the translational start site) and long intron (bp –131 to +583, containing exon 1 plus 583 bp downstream of the translational start site). All these intron regions contain the 37 bp repetitive element sequence from either the A/J or the C3H mouse and one or more of these constructs include other polymorphisms identified 5′ and 3′ of the repetitive element (7). Vector combinations generated with and without the intronic inserts are shown in Figure 1. The resulting constructs were transfected into three different cell lines, C10, SPON4 and MC7pp, using Lipofectamine (Gibco BRL). Co-transfection with a β-galactosidase vector (pSVβ-gal; Promega) was done to normalize the transfection efficiency for all reporter gene construct transfection experiments. β-Galactosidase activity was measured by spectrophotometric quantification of o-nitrophenyl-β-D-galactopyranoside hydrolysis. Luciferase activity was measured in a Turner Designs Model 20e luminometer (Turner, Sunnyvale, CA) using the luciferase assay system (Promega). All reporter gene constructs were transfected on replicate plates and each transfection experiment was done in duplicate. All transfections were normalized by dividing the luciferase activity of the experimental constructs by the activity of the β-galactosidase reporter gene. Luciferase activity was then expressed as the ratio of the normalized activity of the sample construct to the normalized activity of the control pGL3-β-galasticase vector, which contains the SV40 promoter without an enhancer. This allows for comparison of the transfections from one experimental construct with the transfections of the other constructs.

Results

K-ras promoter region sequence comparison

To determine if the promoter region sequence of the K-ras gene differed between the two mouse strains, an 838 bp fragment containing exon 0 of the K-ras gene was amplified by PCR, cloned and sequenced. Only minor base pair differences were seen in the K-ras gene promoter region sequence between the two strains (Figure 2). A computer search for consensus sequence transcription factor binding sites in and around these base pair changes revealed four additional putative Sp1 binding sites for the C3H mouse compared with the A/J mouse. Sp1 is a ubiquitous transcription factor and has been shown to induce an asymmetric bend in DNA that is thought to enhance activity of Sp1-dependent promoters by bringing necessary transcriptional elements into closer proximity to each other (16). Ribonuclease protection assays and reporter gene con-
Fig. 2. Sequence differences and effect on putative transcription factor (TF) binding sites in the promoter region of the A/J and C3H mouse. Sequence differences are indicated in bold. The first base difference in the sequence does not fall within a binding site but is immediately adjacent to the binding sites of the transcription factors shown. (A) Putative binding sites adjacent to the C→G base difference (A/J to C3H respectively) for BGP-RS1, Sp1-CS2, Sp1-IE (GGCGG, consensus binding site), Sp1-hsp70 and hsp70.2 (GGCGG, consensus binding site). (B and C) Putative additional TF binding sites for the C3H promoter region including Sp1-IE, Sp1-hsp70, hsp70.2 (GGCGGG or CTTTGY, consensus binding sites).

Fig. 3. Representative RPA data. Analysis of K-ras gene expression in alveolar type II cells and lung tumors from A/J and C3H mice. Total RNA (10 μg) was hybridized to riboprobes for murine actin (245 bp) and murine K-ras (130 bp). Lane 1, MNNK (NIH 3T3 cell line transformant); lane 2, normal NIH 3T3 cells; lane 3, A/J sham type II cells; lane 4, C3H sham type II cells; lanes 5, 7, 9, 11, A/J NNK-induced lung tumors; lanes 6, 8, 10, 12, C3H NNK-induced lung tumors.

Ribonuclease protection assays
K-ras gene expression levels were determined by RPA because it provided significantly greater sensitivity and reproducibility for detection of K-ras gene transcripts than did nuclear run-on or northern analysis (data not shown). The expression levels in the MNNK cell line and the NIH 3T3 cell line were used to optimize the RPA assay. The MNNK cell line was found to have a 3-fold higher K-ras gene expression than the NIH 3T3 cell line in repeated RPA assays, but this degree of difference could not be distinguished when using northern or nuclear run-on analysis (data not shown). The difference seen in the RPA comparison of K-ras gene expression levels of the MNNK to the 3T3 cell line is consistent with data from Southern analysis showing amplification of the K-ras gene in the MNNK cell line (14). No significant differences in K-ras gene expression were observed in endogenous alveolar type II cells (the progenitor cell for lung tumors) or whole lung between the A/J and C3H mice (Figure 3 and Table I).

Expression levels were higher in whole lung than in alveolar type II cells for both strains. Expression levels varied for the A/J-derived lung tumors from 38 to 494% and from 37 to 417% for the C3H-derived lung tumors as compared with alveolar type II cells isolated from each strain. Levels of K-ras gene expression were 1.8- and 1.3-fold higher in lung tumors from A/J and C3H mice respectively when compared with expression in A/J alveolar type II cells (see Table I). Similar results were also observed using the cyclophilin gene to normalize expression levels (data not shown).

K-ras gene expression in murine lung

<table>
<thead>
<tr>
<th>Sample</th>
<th>Exposure</th>
<th>Relative expression</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>A/J strain</td>
</tr>
<tr>
<td>Type II</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Lung</td>
<td>None</td>
<td>298 ± 47</td>
</tr>
<tr>
<td>ras</td>
<td>NNK</td>
<td>180 ± 32</td>
</tr>
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*Lung tumors were induced by treatment of mice for 7 weeks (50 mg/kg NNK, i.p., 3 doses/week).

Values for type II cells and whole lung are mean ± SEM from three to four different RPAs. All data were normalized to actin levels of the A/J type II cell, no exposure and expressed as a percentage of K-ras expression of A/J mice.

Twelve different NNK-induced lung tumors from each mouse strain were analyzed. Duplicate RPAs were performed on five different tumors from each mouse strain and values differed by <5%.

Table I. K-ras gene expression in alveolar type II cells, whole lung and tumors from A/J and C3H mice

Lung tumor cell lines
K-ras gene expression levels were also determined in the SPON4, IO33, CMT64 and MC7pp cell lines, which are tumorigenic in nude mice and have a mutated K-ras gene. The C10 cell line has a wild-type K-ras gene and is non-tumorigenic in nude mice. K-ras gene expression levels varied considerably between cell lines (Table II). The C10 cell line had the lowest level of K-ras gene expression of all cell lines and was even significantly lower than expression levels seen in A/J alveolar type II cells (Table II). Expression levels varied by ~70% between the SPON4, IO33, C10, CMT64 and MC7pp cell lines. These results parallel similar studies where K-ras gene expression levels in the non-tumorigenic C10 cell line decreased when the cells approached confluence, while expression levels in tumorigenic cell lines remained constant (17).

K-ras gene expression did not correlate with the growth rates of tumors induced in nude mice from the respective cell lines (Figure 4). As anticipated, the C10 cell line produced no tumor when injected into nude mice. Levels of K-ras gene expression were compared using RNA from cell lines harvested in culture and from nude mouse tumor tissue. No significant differences in K-ras gene expression were seen between the in vitro and in vivo conditions (data not shown).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin*</th>
<th>K-ras expression (normalized)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPON 4</td>
<td>A/J</td>
<td>28 ± 6</td>
</tr>
<tr>
<td>IO33</td>
<td>A/J</td>
<td>71 ± 38</td>
</tr>
<tr>
<td>C10</td>
<td>Balb C</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>CMT64</td>
<td>C57</td>
<td>78 ± 22</td>
</tr>
<tr>
<td>MC7pp</td>
<td>B63CF1</td>
<td>64 ± 11</td>
</tr>
</tbody>
</table>

*Lung cells from which the lung cell line was derived. The A/J is a lung tumor-sensitive, the Balb C is a lung tumor-intermediate and the C57 and B63CF1 are lung tumor-resistant mouse strains. All cell lines were derived from lung tumor cells except C10, which was derived from whole lung cells.

**Values are mean ± SEM from three different RPAs. All data were normalized to actin levels of the A/J sham type II cell and expressed as a percentage of K-ras expression of A/J mice.

Table II. Normalized K-ras gene expression in murine cell lines derived from mouse strains with different susceptibilities for lung cancer

Reporter gene expression assays
The transcriptional activity of the 838 bp K-ras gene promoter region from the A/J and C3H mouse was compared in a...
Fig. 4. Growth rates of murine cell lines as xenografts in nude mice. The tumor areas ($\pi r^2$) from four nude mice were determined and averaged for each cell line. The SPON4 cell line grew three times faster than the other cell lines. Statistical significance was seen at days 10 and 13 for the SPON4 cell line compared with the other cell lines by Student’s $t$-test.

 transient expression assay in order to corroborate or refute our expression studies, which contrasted with previous studies in hybrid mice (7). The promoter fragments were cloned into a luciferase reporter gene vector together with or without various segments of the second intron of the K-ras gene inserted immediately downstream of the luciferase gene. These constructs were transfected into three different lung tumor cell lines (C10, SPON4 and MC7pp) from mice with different lung tumor susceptibilities. This was done to determine whether transacting factors differed between the cell lines and whether these factors differentially modulate K-ras gene expression. In order to compare results from different transfections, luciferase activity was expressed as the ratio of the normalized activity of the experimental construct to the normalized activity of the control pGL3-Promoter, which contains the SV40 promoter without an enhancer.

There was a small but statistically significant trend for higher transcriptional activity of constructs containing the C3H promoter relative to those containing the A/J promoter (Figure 5) in all three transfected cell lines ($P = 0.028–0.032$). In addition, both A/J and C3H promoter activity was significantly greater in SPON4 cells compared with C10 and MC7pp cells. However, these differences are relatively small (<1-fold) and thus unlikely to be biologically significant. Similar differences were seen for the constructs containing both the K-ras promoter and various segments of the second intron. However, none of the intronic regions had any differential effect on luciferase activity driven by either promoter in any of the three cell lines (Figure 5). Similar results were observed when the long intron from the A/J or C3H mouse was inserted into the luciferase reporter gene vector containing the SV40 promoter (not shown). Moreover, inserting the long intronic segment immediately upstream of the SV40 promoter instead of downstream of the luciferase gene did not affect reporter gene activity (not shown). All constructs with the long intronic segment inserted in the reverse orientation consistently gave slightly lower activities than corresponding constructs with the same inserts in the forward orientation (not shown).

Discussion

The studies described in this investigation indicate that the selective clonal expansion of cells containing A/J alleles harboring a mutated K-ras gene did not result from greater
endogenous transcriptional activity of this gene in sensitive compared with resistant strains. This conclusion is based on a lack of difference in endogenous K-ras gene expression in alveolar type II cells, the progenitor cell for murine lung tumors, between the two strains. Moreover, consistent with these findings, there was no affect from differences within the K-ras gene promoter or intron 2 sequences on transcriptional activity of the K-ras gene. Chronic exposure of the resistant C3H mouse to a carcinogen induces lung tumors containing K-ras gene mutations (2). In the present study, expression levels of the K-ras gene in lung tumors from C3H mice were similar to levels seen in A/J-derived lung tumors. Together, these studies suggest that factors involved in modulating the rapid clonal expansion of the mutated K-ras allele from A/J mice are not directly linked to expression of this gene. This conclusion is supported by our findings that levels of K-ras gene expression in cell lines did not correlate with growth rates of xenograft tumors in nude mice.

Studies by Chen et al. (7) have reported that expression levels of the A/J K-ras allele were 2 to 5 times greater than the C3H allele in normal lung tissue from A/J×C3H F1 hybrid mice. These expression levels were determined using a reverse transcription–PCR single-strand conformation polymorphism assay. In contrast, using RPA in the present study, endogenous K-ras expression in the target alveolar type II cells, whole lung and lung tumors were similar between strains. The RPA was highly reproducible, consistently detecting a 3-fold difference in expression of the K-ras gene between NIH 3T3 cells and the MNNK cell line. Furthermore, the following studies corroborate some of our findings using the RPA. First, we found whole lung K-ras gene expression to be 1.5 to 3 times higher than alveolar type II cell expression levels in both strains. This result is consistent with immunohistochemical studies demonstrating more abundant protein in ciliated bronchial epithelial cells than alveolar type II cells (18), indicative of heterogeneity for ras expression within the lung. Second, the considerable variation seen in ras expression in murine lung tumors from both strains has also been observed in human lung cancers (19).

One suspected modifier of murine lung tumor induction is the repetitive element polymorphism present within the second intron of the K-ras gene, which has been shown to segregate with susceptibility (5). However, several studies cast doubt on the role of this repetitive element in conferring cancer susceptibility. Our data revealed no effect of the repetitive element on K-ras gene expression. Previous studies with the M.spretus strain, which has the same 37 bp repetitive element as the A/J strain but behaves as a lung tumor-resistant strain, support this conclusion (20). In those studies, lung tumors were induced in F1 hybrid mice generated by crossing the M.spretus with the A/J mouse. Only the A/J allele was mutated in the lung tumors (20). If the repetitive element polymorphism, either the full 74 bp or the 37 bp sequence, influenced the mutation frequency or clonal expansion of ras-initiated cells, then the K-ras alleles from the M.spretus strain should have been mutated. Finally, if the repetitive element played a role in modifying K-ras gene expression, it should be conserved across species. This hypothesis was tested by performing Southern analysis on normal human lung DNAs digested with different restriction enzymes and on human genomic DNA clones that contain sequences spanning all but 1000 bp toward the 3′-end of the second intron. Low stringency hybridization using the repetitive element as the probe did not detect any signal on the blot (S.E.Jones and S.A.Belinsky, unpublished data). In addition, sequencing of 2300 bp of intron 2 immediately downstream of exon 1 did not reveal any sequence homology to the repetitive element nor was there any evidence for the existence of any other type of repetitive element within the 2300 bp region.

Transient luciferase assays of the 838 bp K-ras gene promoter with or without the intronic sequence polymorphism were performed using cell lines derived from mouse strains with varying susceptibility for lung tumor formation to determine if trans-acting factors influence K-ras gene transcription rates. Promoter activity was similar in all three cell lines, suggesting that the levels or types of trans-acting factors that may influence gene transcription rates do not differ between strains. Furthermore, no differences in promoter activity were observed between the A/J and C3H K-ras alleles. Modifier loci present within the C3H or A/J genome could either facilitate the mutation or expansion of cells harboring a mutated A/J allele or play a protective role in the C3H mouse. The existence of a pulmonary adenoma resistance locus (Par1) on chromosome 11, derived from the M.spretus mouse, has been shown to decrease expression of the Par1 allele but did not affect lung tumor susceptibility in mice that do not carry the susceptible Par1 allele (21). Recently, more Par loci have been identified on murine chromosomes 6, 12 and 18 (22,23). SMXA recombinant inbred mouse strains containing all four known susceptibility genes were highly resistant to pulmonary adenoma formation because of Par on chromosomes 11 and 12, designated Par1 and Par3 (23). In addition, in the Min mouse, a model for multiple intestinal neoplasia (24), an unlinked modifier locus, Mom1, has been shown to modulate adenoma multiplicity and growth rate. This finding parallels the proposed existence of a locus on murine chromosome 4 which affects lung tumor size and has been designated as pulmonary adenoma progression 1 (Papgl) (22). One gene associated with the murine chromosome 18 locus is Smad4, a mouse mad-related gene which is the homolog of the human DPC4 (deleted in pancreatic cancer) gene (25). The Smad4 gene, and another gene Smad2, were evaluated as possible candidates for the Par2 (chromosome 18) resistance locus, but no evidence was found to support their involvement as lung tumor resistance or suppressor genes in a study of lung tumors and normal lung from B6C F1 mice (25). Evidence for an unlinked modifier for murine lung cancer susceptibility has also been suggested by studies performed in congenic strains of mice (26). Significant differences in lung tumor load and burden were seen in congenic strains with identical MHC haplotypes and identical K-ras alleles. Together these studies implicate a myriad of genes affecting lung tumor susceptibility.

In summary, while mutation of the K-ras gene is clearly an important event in murine lung cancer, the transcriptional regulation of this gene does not appear to be the dominant factor distinguishing susceptible strains from resistant strains. Other genetic changes or losses along with inherent modifiers, such as genes associated with the Par1 locus, are necessary to establish one strain as more or less susceptible than another.

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S.E. Jones-Bolin et al.