Mutational Activation of the *MAP3K8* Protooncogene in Lung Cancer

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The *MAP3K8* protooncogene (*Cot/Tpl-2*) activates the MAP kinase, SAP kinase, and NF-κB signaling pathways. *MAP3K8* mutations occur in the rat homologue, but activating mutations have yet to be identified in primary human tumors. We have identified *MAP3K8* as a transforming gene from a human lung adenocarcinoma and characterized a 3′ end mutation in the cDNA. In addition, we confirmed that the mutation occurs in the original lung tumor, and we screened a series of lung cancer cell lines to determine whether the *MAP3K8* mutation is a common occurrence in lung tumorigenesis. The oncogene was isolated and identified with the NIH3T3 nude mouse tumorigenicity assay and cDNA library screening. The gene was analyzed by polymerase chain reaction (PCR), single-strand conformational polymorphism (SSCP), and 3′RACE for mutations. The mutation was localized to *MAP3K8* exon 8 and confirmed in the primary tumor DNA. Both wild-type and mutant *MAP3K8* cDNAs transformed NIH3T3 cells, but the transforming activity of the mutant was much greater than that of the wild type. PCR-SSCP screening of cell line cDNAs identified one silent polymorphism in cell line SK-LU-1. Although we were unable to find additional activating mutations, these data support a role for *MAP3K8* activity in cellular transformation, but suggest that mutational activation of the gene is a rare event in lung cancer.

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths in the United States for both men and women. The initiation and promotion of a normal lung cell into a cancer cell is a multistep process involving alterations of genes regulating cellular growth, proliferation, and survival (Sekido et al., 1998). MAP kinase signaling cascades and NF-κB signalsomes are key participants in these processes. MAP kinases transfer signals received at the cell surface from assorted receptors and G proteins through an intricate network leading to the nucleus, where a variety of cellular responses are initiated. Likewise, the NF-κB signalsome is composed of a multiprotein network that induces the translocation of NF-κB transcription factors into the nucleus, influencing cytokine production, antiapoptotic pathways, cellular differentiation, and production of cellular adhesion molecules (Ghosh et al., 1998). In lung cells, activation of these pathways plays a role in tumorigenesis, metastasis, and resistance to chemotherapy (Jones et al., 2000; Brognard and Dennis, 2002).

The *MAP3K8* protooncogene (also known as *Cot* and *Tpl-2*) is a serine/threonine kinase that participates in the MEK-1, MKK6, SAPK, NFAT, and NF-κB signaling pathways (Aoki et al., 1991; Patriotis et al., 1994; Salmeron et al., 1996; Tsatsanis et al., 1998; Belich et al., 1999; Hagemann et al., 1999; Chiariello et al., 2000). Transcriptional overexpression, mutational activation, and extracellular stimulation through LPS (lipopolysaccharide), LMP1 (latent membrane protein 1), and CD3/CD28 influence *MAP3K8*-specific regulation of these downstream cascades (Lin et al., 1999; Dumitru et al., 2000; Eliopoulis et al., 2002). Overexpression and mutation of *MAP3K8* are implicated in a variety of carcinomas including thymoma, lymphoma, breast cancer, Hodgkin's disease, and nasopharyngeal carcinoma, suggesting that altered activation of the gene is significant in tumorigenesis (Patriotis et al., 1993; Ceci et al., 1997; Sourvinos et al., 1999; Eliopoulos et al., 2002).

The *MAP3K8* gene was originally identified in human thyroid tumor DNA as a gene capable of transforming SHOK (Syrian Hamster Osaka Ka-

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nazawa) cells (Miyoshi et al., 1991). It was later discovered that the transforming gene contained a carboxy-terminal mutation of the *MAP3K8* gene (Chan et al., 1993). However, this mutation was not found in the original thyroid tumor DNA, suggesting that it occurred as an artifact during the transfection procedure (Miyoshi et al., 1991). In contrast, the rat *MAP3K8* homologue, *Tpl-2*, is frequently mutated in rat T-cell lymphomas by integration of Moloney murine leukemia virus (MMLV) into the last intron of the gene. This proviral integration alters the 3′ end of the transcript and the subsequent carboxy terminus by linking the rat *Map3k8* exon 7 and intron 7 sequences. This insertion alters the expressed transcript by prematurely terminating the mRNA sequence in the 5′ proviral LTR (Makris et al., 1993; Patriotis et al., 1993).

Regardless of the means by which the gene is mutated, the alterations of the carboxy terminus found in both the human thyroid tumor and rat *Map3k8* oncogenes resulted in a higher transforming ability of the mutated gene when compared to the wild type (Aoki et al., 1993; Chan et al., 1993). In addition, transgenic mice expressing mutated but not wild-type *MAP3K8* had an increased incidence of lymphoblastic lymphomas by 9 months of age (Ceci et al., 1997). Loss of the MAP3K8 carboxy terminus is associated with increased activation of several pathways associated with tumorigenesis, including the MAP kinase and SAP kinase signal transduction cascades, nuclear factor of activated T-cells (NFAT), AKT, and NF-κB, suggesting that *MAP3K8* has pleiotropic roles in cellular activity (Salmeron et al., 1996; Ceci et al., 1997; Tsatsanis et al., 1998; Belich et al., 1999; Hagemann et al., 1999; Chariello et al., 2000; Kane et al., 2002).

Here, we report the first known mutation of the human *MAP3K8* gene occurring in a human primary tumor. We originally isolated the *MAP3K8* oncogene as one of several unknown transforming genes from a series of primary lung tumors (Reynolds et al., 1991). In this study, DNA sequencing revealed that this unknown transforming gene was *MAP3K8* and that the gene encoded an altered 3′ end of the *MAP3K8* transcript. PCR amplification of the original tumor DNA and subsequent sequencing of the amplicon confirmed that the mutation resided in the primary tumor. Transfection of the *MAP3K8* wild-type and mutant cDNAs into NIH3T3 cells induced tumorigenic growth in nude mice and demonstrated that *MAP3K8* was the transforming gene isolated from the original transfection, paralleling the research by other groups that identified *MAP3K8* as an oncogene (Miyoshi et al., 1991; Chan et al., 1993; Ceci et al., 1997). To search for additional mutations, we screened a variety of lung cancer cell lines by polymerase chain reaction–single-strand conformational polymorphism (PCR-SSCP) analysis for point mutations and examined alterations occurring in the 3′ end of the transcript using 3′RACE (rapid amplification of cDNA ends). In the cell lines examined, no additional 3′ alterations were identified, but the SK-LU-1 cell line harbored a mutation/polymorphism in exon 7 of the coding region. Although activation of *MAP3K8* plays a role in NIH3T3 transformation, mutational activation of the gene appears to be a rare event in lung cancer.

MATERIALS AND METHODS

Identification of the Transforming Gene

The nude-mouse tumorigenicity assay was performed as described previously (Fasano et al., 1984). In brief, high-molecular-weight DNA was isolated from a human lung adenocarcinoma, transfected into NIH3T3 cells selected for positive transfection and colony formation, and subcutaneously injected into nude mice. The resulting tumors were probed for human Alu-positive sequences by Southern blot and cloned into the λgt10 vector, and Alu-negative fragments from these clones were hybridized to a zoo blot. Of the Alu-positive tumors, λgt11 cDNA libraries were made, screened for the human sequence, sequenced, and BLAST-searched, identifying the *MAP3K8* transcript. To ensure that *MAP3K8* was the gene responsible for inducing cellular transformation, NIH3T3 cells were retransfected with the cDNA transcripts of the *MAP3K8* gene and assayed for tumor formation in nude mice.

Cell Lines

The following lung cancer cell lines were grown in RPMI 1640 with 10% fetal bovine serum: H28, H69, H125, H157, H187, H324, H345, H372, H378, H446, H460, H526, H661, H719, H720, H726, H727, H740, H865, H1048, H1062, H1334, H1581, H1607, H2122, N417, A549, SHP77, NE-18, Calu1, Calu3, H322, CHAGO-K-1, Calu6, SK-LU-1, H441, NU-6-1, SW900, SK-MES-1, and H290. In addition, the 9HTE cell line was grown in DMEM with 10% FBS.

PCR Amplification

Products were amplified from lung cancer cell line cDNAs according to standard procedures. The mutation from lung tumor 41 was amplified from genomic tumor DNA with one *MAP3K8* gene–specific primer (3PF: 5′-CCCTGGAGAGAAACCCCAATCACC-3′) and one mutation-specific primer (MUT-UX5: 5′-TGACAGGAGTAGACCAAATTGAA-3′).

Single-Strand Conformational Polymorphism Analysis

The primers for the PCR-SSCP produced overlapping fragments spanning the open reading frame of the *MAP3K8* (Table 2). cDNA was amplified by PCR, run in 0.5× MDE polyacrylamide sequencing gels (Cambrex Biosciences, Rockland, ME) with and without 5% glycerol, and silver-stained for viewing of the bands (Promega, Madison, WI). Bands with altered migration were sequenced to confirm polymorphic DNA sequences.

3′RACE

SMART RACE cDNAs (BD Biosciences Clontech, Palo Alto, CA) were made according to the manufacturer's instructions. Nested primers were used to increase sensitivity and specificity. The first round of amplification was performed with the MAP3K8-2ACF-RACE (5′-CGATGAGCGTTCTAAGTCTCTGCTGC-3′) primer, followed by a second round of amplifications with the MAP3K8-5ACF (5′-CAAAGCAGACATCTACAGCC-3′) primer and the RACE primers.

Computer Analysis

BLAST analysis was performed through the National Institute of Health program at http://www.ncbi.nlm.nih.gov/BLAST/ and the Ensembl program at http://www.ensembl.org. Amino acid analysis was performed with the Scansite program at http://scansite.mit.edu.

RESULTS

Identification of a Novel *MAP3K8* Transforming Mutation from a Lung Adenocarcinoma

The NIH3T3 nude mouse tumorigenicity assay was designed to identify transforming genes in genomic DNA from tumors (Fasano et al., 1984). NIH3T3 cells were transfected with genomic DNA isolated from the human lung adenocarcinoma L-41 that had been subcutaneously injected into nude mice, and were then assayed for tumor formation. Southern blots probed with human Alu repeats confirmed the presence of human DNA in the mouse tumors. DNA from the nude mouse tumors was reassayed to enhance selection of the transforming gene (Fig. 1A).

TABLE 1. Tumor Formation in Nude Mice

Sample	Frequency	Tumor appearance
pRc/CMV	1/8	70 days
pRc/WT	3/8	46–82 days
pRc/MUT	21/24	19–31 days

Genomic DNA from tumors in nude mice was digested with *Eco*RI and cloned into λgt10. The human Alu element was used to screen plaques, and three positive λgt10 clones were identified. These were subcloned into the plasmid vector pBluescript (data not shown). Alu-positive plasmid clones were digested with *Bam*HI, and Alu-negative fragments were identified by Southern blot and subcloned. The Alu-negative clones were hybridized to a zoo blot in order to identify cloned sequences conserved across species that potentially contained exons (Fig. 1B). A zoo blot–positive clone was used for the screening of λgt11 cDNA libraries, which were prepared from the nude mouse tumors and a normal human cDNA library, in order to isolate the transforming and wild-type genes. Sequence analysis and BLAST searches confirmed that the transforming gene was human *MAP3K8*. However, the cDNA isolated from the tumor library displayed a novel mutation in the transcript near the 3′ end of the open reading frame.

Human *MAP3K8* is located on chromosome 10 at 10p11.2. Figure 2A illustrates the nucleotide and amino acid sequences of mutant *MAP3K8*. A BLAST homology search with the mutant sequence against the Ensembl database confirmed that the mutation occurred as a genetic rearrangement/recombination of the penultimate exon of *MAP3K8* with a sequence from chromosome 9. The wild-type sequence localized with high homology only to chromosome 10, whereas the mutant mapped to two regions of high homology, the chromosome 10/*MAP3K8* locus and a chromosome 9–specific region from the 3′ end of the sequence (Fig. 2B). The mutant sequence from the lung tumor differed from that of both the thyroid tumor and the rat MMLV-induced mutant sequence [Fig. 2(C and D)].

Wild-type *MAP3K8* produced a protein of 467 amino acids, and the human thyroid tumor muta-

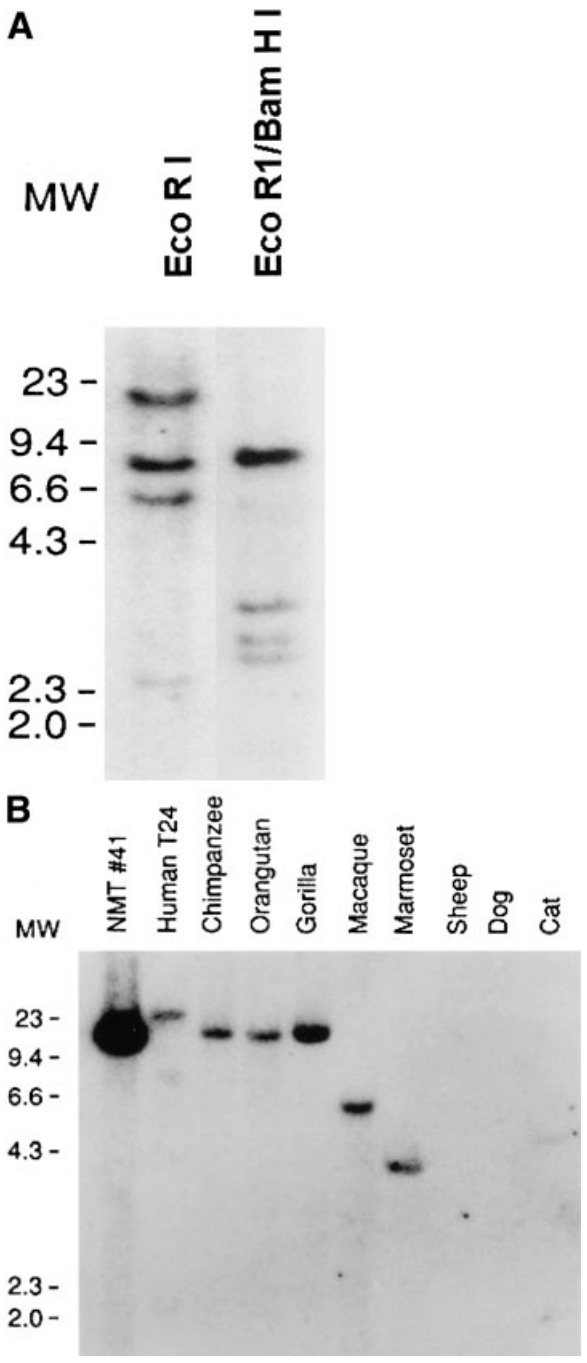

Figure 1. Southern blot analysis of the transforming gene. (A) Nude mouse tumor DNA was digested with *Eco*RI (left lane) or *Eco*RI and *Bam*HI (right lane) and probed at high stringency with the human Alu sequence confirming the presence of human tumor DNA in the mouse tumor. (B) Zoo blot probed with the human transforming sequence cloned into the pBluescript vector. Nude mouse tumor DNA (NMT #41) was used as a positive control. The probe hybridized at a molecular weight identical to that of the human DNA in lane 2 (Human T24) and of the primate DNA in the subsequent lanes, demonstrating that the sequence is conserved and suggesting that the probe contains an exon.

tion resulted in a protein of 415 amino acids and an altered carboxy terminus (Miyoshi et al., 1991). The lung tumor mutation we identified resulted in a putative protein of 429 amino acids with the first 421 identical to the wild-type and the last 8 novel to this mutation or alteration (Fig. 2D). The wild-type *MAP3K8*, lung mutation and thyroid mutation amino acid sequences were analyzed with the Scansite program under medium stringency conditions in order to characterize differences in the sequences (Fig. 3). The program identified three putative serine/threonine phosphorylation sites within the carboxy terminus of the wild-type sequence. The serines at 400 and 413 are known phosphorylation sites of AKT; serine 443 is not a known phosphorylation site but has been recognized as a putative CAMKII site (Kane et al., 2002; Scansite CAMKII data not shown). The lung mutation retained serines 400 and 413, but the thyroid mutation did not, and both mutations lost serine 443. Interestingly, the lung mutation sequence acquired a putative PDZ domain, a motif utilized in protein–protein interaction and localization of molecular signaling complexes (Harris and Lim, 2001).

To demonstrate that the mutation occurred in the original lung adenocarcinoma DNA, we designed a *MAP3K8*-specific sense primer upstream of the mutation site (5ACF) and a mutation-specific antisense primer downstream of the recombination site (MUT-UX5). This primer combination would amplify only the *MAP3K8*/chromosome 9 mutation. Genomic DNA from the lung adenocarcinoma (L-41), an immortalized lung cell line (9HTE), and several other lung cancer cell lines were amplified with this primer combination. After amplification and DNA sequencing, L-41 was the only DNA to amplify a product and to contain the mutant sequence, confirming that the mutation occurred in the primary lung tumor DNA [Fig. 4(A and B)].

To confirm the transforming potential of the mutated *MAP3K8* gene, we cloned cDNAs of both the wild-type and the mutated genes into the eukaryotic expression vector pRc/CMV, transfected the construct into NIH3T3 cells, and injected it subcutaneously into nude mice. Transfections of the wild-type gene showed a moderate level of activity-inducing tumors in 3 of 8 mice. However, the mutated *MAP3K8* strongly induced tumors with a shorter latency and a higher frequency than the wild-type gene (Table 1).

MAP3K8 Mutations Were a Rare Occurrence in Lung Cancer

Because previously characterized mutations of the *MAP3K8* gene occurred in the 3′ end of the

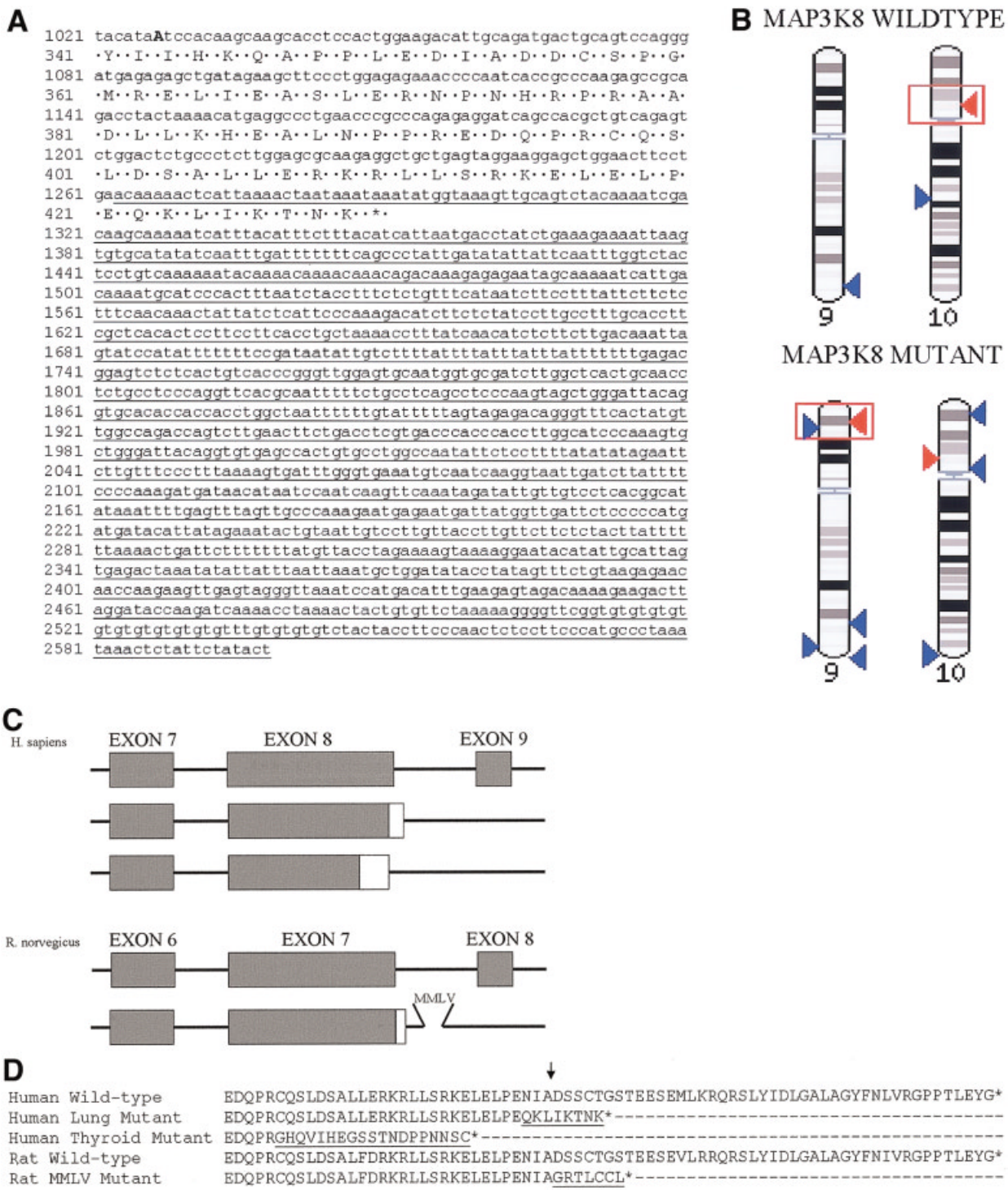

Figure 2. The *MAP3K8* lung cancer mutant sequence. (A) Partial cDNA sequence of the *MAP3K8* lung cancer mutation with the deduced amino acid sequence below. The bold capital A denotes the start of the penultimate exon of *MAP3K8*. The underlined sequence corresponds to the mutation sequence from the lung tumor. (B) Ensembl BLAST analysis demonstrates that the wild-type *MAP3K8* sequence (top) localizes only to chromosome 10, whereas the mutant sequence (bottom) localizes to chromosome 9 and chromosome 10. The red boxes indicate the region of highest homology to the genomic DNA. The red arrowhead indicates a high-scoring region, and the blue arrowhead indicates a low-scoring region. The *MAP3K8* mutant sequence has two red arrows because of the *MAP3K8* wild-type sequence homology and the chromosome 9 homology contained in the mutation site. (C) Illustration of the intron/exon structure of the 3′ end of the wild-type and mutant MAP3K8 genes. The shaded boxes represent the wild-type exon sequence, and the clear boxes represent an altered exonic sequence. Note that the altered rat sequence arises from integration of MMLV into the last intron of the gene. (D) Carboxy terminal amino acid sequences of the wild-type and mutant MAP3K8 proteins. An asterisk denotes a stop codon. The arrow indicates the boundary between human exons 8 and 9 and between rat exons 7 and 8.

TABLE 2. PCR-SSCP Primer Pairs

Sense primer	Antisense primer	Product size (bp)
5PF: 5′-CTCTCCAGAAAGAGDAACAGTAA-3′	5PR: 5′-CCTCCACAGTTCCATATCTG-3′	268
2ACF: 5′-GATGAGCGTTCTAAGTCTCTG-3′	2ACR: 5′-TACACGCCATTCTTTTCTTCG-3′	330
3ACF: 5′-TATTGGTTCTGATTTTATTCC-3′	3ACR: 5′-GAGTGTAGAAAATCAAGTCC-3′	323
4ACF: 5′-GGACTTGATTTTCTACACTC-3′	4ACR: 5′-ACAGGTAGGAGGGATAGGCTG-3′	303
5ACF: 5′-CAAAGCAGACATCTACAGCC-3′	5ACR: 5′-TCCAAGAGGGCAGAGTCCAGA-3′	302
3PF: 5′-CCCTGGAGAGAAACCCCAATCACC-3′	3PR: 5′-CTTAATTTAGAGCAAACATGGCA-3′	328

open reading frame, we examined whether this was a common occurrence in lung cancers. Alterations of the 3′ end of the gene made standard RT-PCR impossible because one end of the sequence was unknown, but the 3′RACE technique overcame this obstacle. cDNA was prepared from 22 of the lung cancer cell lines for the RACE experiment, and the immortalized 9HTE cell line was used as a positive control. Amplification was performed with a *MAP3K8*-specific primer (5ACF) designed to anneal prior to any of the known mutation sites and the 3′ primer supplied with the kit. The amplified product from all the cell lines tested was consistent with the expected molecular size of the product, approximately 1,500 bases (Fig. 5).

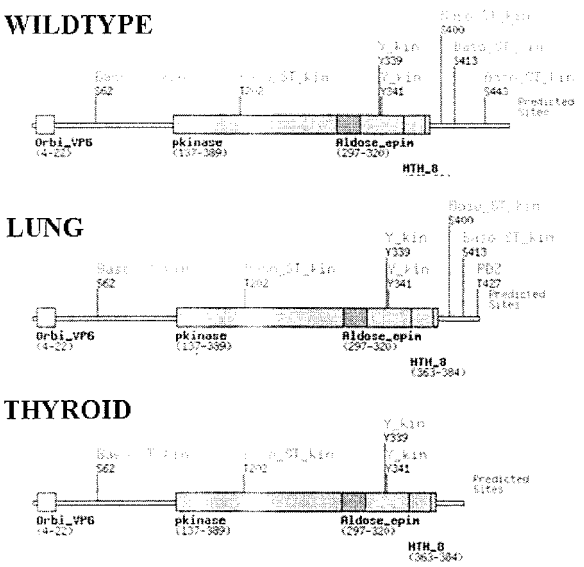

Figure 3. Scansite analysis of the wild-type and mutant MAP3K8 amino acid sequences. The illustrations aligned from top to bottom are wild-type MAP3K8, lung mutant MAP3K8, and thyroid mutant MAP3K8. The sequences were analyzed under medium stringency. The program predicts the protein kinase domain from amino acids 137–389 in all sequences and three predicted serine phosphorylation sites in the carboxy terminus. Serine 443 lies within a phosphorylation motif that is lost in both mutants, whereas the lung mutant retains serines 400 and 413 and acquires a PDZ domain.

To identify additional mutations occurring within the open reading frame of *MAP3K8*, we designed six sets of primers for the coding region of the gene to amplify overlapping fragments approximately 300 bases in length (Table 2). Amplification with primer sets 4ACF and 4ACR demonstrated results characteristic of the various primer sets, displaying a wide range of *MAP3K8* expression in the different lung cancer cell lines (Fig. 6A). The PCR fragments from the different primer sets were denatured and run on SSCP polyacrylamide sequencing gels. Only one polymorphism, from cell line SK-LU-1, was identified in the 40 cell lines examined (Fig. 6B). DNA sequencing revealed that this was an A/T heterozygous polymorphism residing in exon 7 of the *MAP3K8* and did not alter the amino acid sequence of the protein [Fig. 6(C and D)].

DISCUSSION

Several groups independently identified the *MAP3K8* gene as a transforming oncogene (Miyoshi et al., 1991; Chan et al., 1993; Patriotis et al., 1993). In the current study, we identified an activating mutation of human *MAP3K8* occurring in a primary human tumor. In addition, our results support the finding that overexpression of the wild-type gene as a result of a strong promoter induces transforming potential in nude mouse tumorigenicity assays but that alterations of the carboxy terminus of MAP3K8 increase this potential to a much greater degree.

MAP3K8 is one of several serine/threonine MAP kinase kinase kinases identified in mammals. MAP3K8 transduces MAP kinase signals in response to stimulation from agonists such as LPS and CD40 (Dumitru et al., 2000; Eliopoulos et al., 2003). In addition, the protein participates in several non–MAP kinase signaling cascades such as AKT and NF-κB (Tsatsanis et al., 1998; Belich et al., 1999; Lin et al., 1999; Kane et al., 2002; Waterfield et al., 2003). Although the exact mechanisms

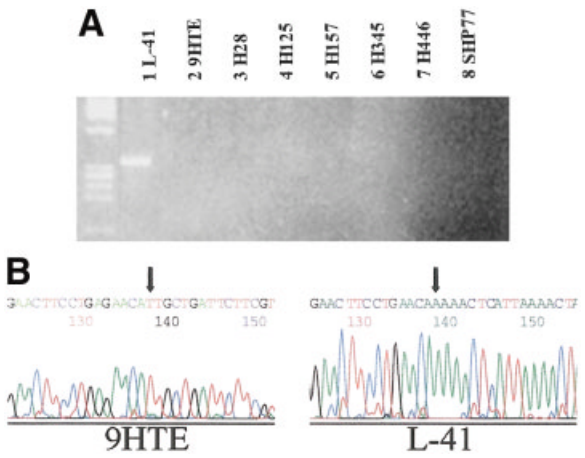

Figure 4. The *MAP3K8* mutation exists in primary lung adenocarcinoma L-41. (A) DNA isolated from L-41 and several cell lines was amplified with a *MAP3K8* specific primer and a mutation specific primer. Only L-41 amplified a product demonstrating that the tumor contained the mutation. (B) To confirm the presence of the mutation, 9HTE DNA (left) was sequenced with the sense primer and compared to the product amplified in L-41. Arrows indicate the mutation site.

of MAP3K8 regulation are not fully understood, the carboxy terminus plays an important role in governing the activity of the protein. When coexpressed with a truncated mutant MAP3K8, the wild-type carboxy terminal tail can physically interact with the mutant MAP3K8 protein and suppress its kinase activity (Ceci et al., 1997). Loss of the tail region causes an increase in the overall kinase activity of the protein while also stabilizing it (Gandara et al., 2003). In addition, the tail associates with the NF-κB/p105 protein, and the serines 400 and 413 in the tail are substrates for AKT-induced phosphorylation (Belich et al., 1999; Kane et al., 2002; Beinke et al., 2003). Thus, loss of the tail region has biological consequences that affect multiple pathways influential in cellular tumorigenesis.

We performed the NIH3T3 nude mouse tumorigenicity assay to identify transforming oncogenes from primary lung tumor DNA (Reynolds et al., 1991). DNA from a lung adenocarcinoma, L-41, was transfected into NIH3T3 cells, and the cells were injected subcutaneously into nude mice. Human Alu probes confirmed the presence of human DNA in the developing tumors, and hybridization of cloned DNA to a zoo blot suggested that the human DNA sequence contained exonic sequences conserved across primate species. The zoo blot–positive clone was used for screening various cDNA libraries from the mouse tumors. From these screens, this clone identified *MAP3K8* as the transforming gene. The *MAP3K8* cDNA was sequenced to reveal an alteration at the 3′ end of the open reading frame. To confirm that the mutated *MAP3K8* induced the tumors, the cDNA was cloned into a pRc/CMV eukaryotic expression vector, transfected into NIH3T3 cells, and injected subcutaneously into nude mice. Wild-type *MAP3K8* overexpressed in NIH3T3 cells demonstrated a moderate level of tumorigenicity, causing tumor formation in 3 of 8 mice between 46 and 82 days post injection. The mutated *MAP3K8* gene demonstrated much greater transforming ability, inducing tumors in 21 of the 24 mice within 31 days post injection. These data are consistent with those reported in previous studies demonstrating higher transforming potential in mutated *MAP3K8* (Aoki et al., 1993; Ceci et al., 1997).

We performed a BLAST analysis of the *MAP3K8* mutation sequence to help characterize the mutant gene isolated from the lung tumor. We identified the mutation site as a region involving a genetic rearrangement or recombination with DNA from human chromosome 9. Compared to the size of the wild-type protein, which contained 467 amino acids, this alteration resulted in a shortened protein of only 429 amino acids and a different carboxy terminus. The mutation occurred in the penultimate exon of the *MAP3K8* mRNA, producing a novel 3′ end and an altered carboxy terminus. Interestingly, the *MAP3K8* mutation isolated from the thyroid tumor also demonstrated the mutation

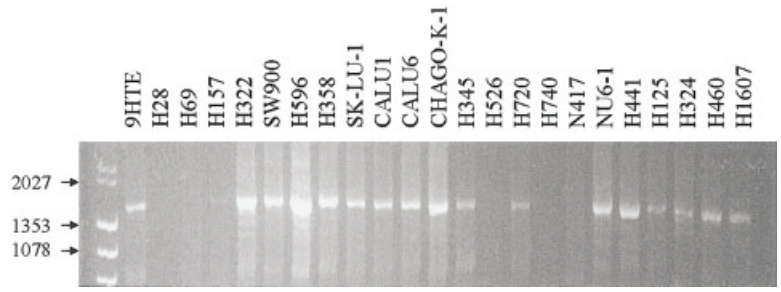

Figure 5. RACE analysis of the *MAP3K8* gene in lung cancer cell lines. RNAs were reverse-transcribed from a variety of lung cancer cell lines with the SMART RACE kit. A primer was designed to anneal to *MAP3K8* exon 7. Molecular weight markers are noted on the left. Lane 1 contains the immortalized lung cell line 9HTE as a positive control. None of the cell lines amplified a product that differed from the 9HTE wild-type gene.

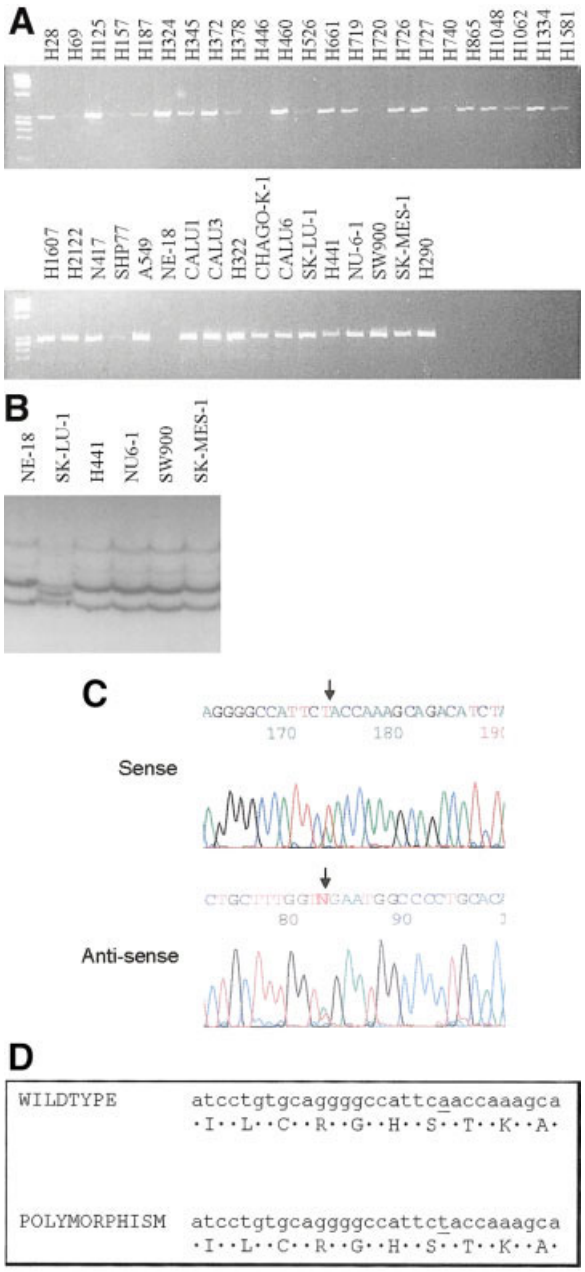

Figure 6. SSCP analysis of the open reading frame of *MAP3K8*. Primers amplified overlapping fragments of the *MAP3K8* gene in 40 lung cancer cell lines. (A) Amplification with *MAP3K8*-4AC-F and *MAP3K8*-4AC-R primers. Several of the cell lines did not amplify product, suggesting that the gene is down-regulated or absent in these cells. (B) SSCP analysis of the *MAP3K8*-4AC-F/4AC-R product reveals a heterozygous polymorphism in the SK-LU-1 cell line. (C) Sequence analysis of the SK-LU-1 sense (top) and anti-sense (bottom) sequences. Arrows denote the polymorphism. (D) Alignment of the wild-type sequence (top) and the polymorphic sequence (bottom) reveals no change in the amino acid sequence of the SK-LU-1 MAP3K8 protein. The polymorphic nucleotide is underlined, and the predicted amino acids are centered below the codon.

in the penultimate exon but at a site upstream from the mutation we report (Miyoshi et al., 1991). The rat *Map3k8* mutation results from a provirus insertion into the last intron, again resulting in an altered 3′ end and a changed carboxy terminus (Patriotis et al., 1993). Although all three mutations are different, all confer transformation potential to the gene through an alteration in the carboxy terminus.

Activation of the classic MAP kinase pathway, but not the JNK pathway, and the subsequently elevated ERK activity elicit the transformed phenotype in NIH3T3 cells (Janulis et al., 1999). In cotransfection experiments, increased MAP kinase activity through oncogenic *MAP3K8* was negatively regulated when coexpressed with the wild-type MAP3K8 carboxy terminal tail (Ceci et al., 1997). Thus, it is probable that the mutated *MAP3K8*-induced transformation of NIH3T3 cells results from increased activation of the classic MAP kinase pathway. However, the carboxy terminus of *MAP3K8* is also required for binding to the p50 precursor NF-κB1 (Belich et al., 1999). Likewise, serines 400 and 413 in MAP3K8 are phosphorylation sites for Akt-induced nuclear translocation of NF-κB (Kane et al., 2002). Interestingly, the lung, thyroid, and MMLV-induced mutant forms all also lose serine 443, a potential CAMKII phosphorylation site according to the Scansite program. However, to date no functional relationship has been reported between MAP3K8 and CAMKII. Taken together, these data suggest that there may be other pathways affected by loss of the carboxy terminus, such as altered NF-κB signaling activation, in addition to the classic MAP kinase pathways.

The *MAP3K8* oncogene was first isolated by a procedure to transform SHOK cells with human thyroid tumor DNA (Miyoshi et al., 1991). However, the researchers were unable to detect the mutation in the original primary tumor DNA, suggesting that the mutation occurred during the transfection procedure. To confirm that the mutation in adenocarcinoma L-41 was not an experimental artifact, we designed primers to amplify the *MAP3K8* mutation specifically. Through standard PCR with a wild-type *MAP3K8*– and a mutated *MAP3K8*–specific primer pair, the DNA from L-41 amplified a product, whereas the immortalized lung cell line 9HTE and the other lung cancer cell lines did not, demonstrating that the mutation occurred in the original primary tumor DNA and was not an artifact of the transfection procedure.

Next, we wanted to determine whether 3′ alterations of *MAP3K8* are a common occurrence in human lung cancer. We performed 3′RACE to identify the major genetic alterations that would occur in the 3′ end of the open reading frame. Of the cell lines we examined, none showed addi-

tional alterations, suggesting that major genetic mutations occurring at the 3′ end of the human *MAP3K8* were not common in lung cancer. Unlike the lung and thyroid mutations, the *Map3k8* mutation found in rats occurs from an MMLV provirus insertion into the last intron of the gene. A comparison of the human and rat sequences of this intron showed little homology, even though the open reading frame of the gene is highly conserved (data not shown). Therefore, a similar method of proviral integration may not exist to activate human *MAP3K8*.

The open reading frame of *MAP3K8* was examined by overlapping fragments for PCR-SSCP to identify point mutations. One polymorphism was identified in the human lung cancer cell line SK-LU-1 as an A/T heterozygous polymorphism in exon 7 of the *MAP3K8* transcript. However, the polymorphism did not result in a change in the amino acid sequence. The SSCP data taken together with the 3′RACE data suggest that activating mutations of the *MAP3K8* transcript are a rare event in lung cancer.

Certain genes frequently show mutations in cancer. Mutation of the *RAS* gene is a common occurrence in lung cancer, as are mutations in the genes that encode PI3 and Akt kinases in other cancers (Sekido et al., 1998; Blume-Jensen et al., 2001). Mutation of the *RAS* gene results in a constitutively activated protein product that, in turn, activates the MAP kinase pathway. Constitutive activation and increased mRNA expression for many of the MAP kinases, including *RAF1*, *MEK*, and *TAK*, also confer tumorigenic potential, but, as with *MAP3K8*, mutations are rarely found in these genes in lung cancer (Miwa et al., 1994; Bansal et al., 1997; Kondo et al., 1998). Although these data support a role for *MAP3K8* activity in lung tumorigenesis, the mutation identified in this report probably occurred through a rare genetic rearrangement during the progression to a cancerous cell. Moreover, it is interesting that the mutations in the thyroid and lung tumors both occurred in exon 8, creating speculation about whether this DNA sequence may be susceptible to recombination.

Although additional mutations of *MAP3K8* were not found, the PCR amplifications of the lung cancer cell line cDNA indicated that altered levels of expression might exist in lung tumors (data not shown). Such alterations of *MAP3K8* mRNA transcription levels have been significantly associated with human breast cancer, and when overexpressed, the wild-type gene displays mild transforming potential (Chan et al., 1993; Sourvinos et al., 1999). Thus, it is possible that, although mutational activation is a rare event, aberrant transcriptional regulation or gene amplification of *MAP3K8* may be a common alteration in lung cells and may contribute to tumorigenic progression.

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