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Frequent Aberrant Methylation of *p16*^{INK4a} in Primary Rat Lung Tumors

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The *p16*^{INK4a} (*p16*) tumor suppressor gene is frequently inactivated by homozygous deletion or methylation of the 5′ CpG island in cell lines derived from human non-small-cell lung cancers. However, the frequency of dysfunction in primary tumors appears to be significantly lower than that in cell lines. This discordance could result from the occurrence or selection of *p16* dysfunction during cell culture. Alternatively, techniques commonly used to examine tumors for genetic and epigenetic alterations may not be sensitive enough to detect all dysfunctions within the heterogeneous cell population present in primary tumors. If *p16* inactivation plays a central role in development of non-small-cell lung cancer, then the frequency of gene inactivation in primary tumors should parallel that observed in cell lines. The present investigation addressed this issue in primary rat lung tumors and corresponding derived cell lines. A further goal was to determine whether the aberrant *p16* gene methylation seen in human tumors is a conserved event in this animal model. The rat *p16* gene was cloned and sequenced, and the predicted amino acid sequence of its product found to be 62% homologous to the amino acid sequence of the human analog. Homozygous deletion accounted for loss of *p16* expression in 8 of 20 cell lines, while methylation of the CpG island extending throughout exon 1 was observed in 9 of 20 cell lines. 2-Deoxy-5-azacytidine treatment of cell lines with aberrant methylation restored gene expression. The methylated phenotype seen in cell lines showed an absolute correlation with detection of methylation in primary tumors. Aberrant methylation was also detected in four of eight primary tumors in which the derived cell line contained a deletion in *p16*. These results substantiate the primary tumor as the origin for dysfunction of the *p16* gene and implicate CpG island methylation as the major mechanism for inactivating this gene in the rat lung tumors examined. Furthermore, rat lung cancer appears to be an excellent model in which to investigate the mechanisms of de novo gene methylation and the role of *p16* dysfunction in the progression of neoplasia.

Inactivation of the *p16* tumor suppressor gene on chromosome 9p21 has been detected in >70% of cell lines derived from human non-small-cell lung cancers (NSCLCs) (19, 37, 39). The importance of *p16* inactivation in disrupting control of the normal cell cycle lies within its regulatory role in the retinoblastoma (Rb) pathway. *p16* is an inhibitor of the cyclin-dependent kinases 4 and 6, which bind *cyclin D1* and phosphorylate the *Rb* gene (25, 46). Thus, *p16* contributes to the maintenance of *Rb* in the unphosphorylated state, which inhibits cell cycle progression. This regulation is lost if either *p16* or *Rb* is inactivated. The reciprocal relationship between *Rb* alterations in small-cell lung cancer (13) and *p16* alterations in NSCLC suggests that dysfunction within the Rb pathway could be a major target in the genesis of this disease (37).

The predominant mechanisms for inactivation of the *p16* gene in human tumors include homozygous deletion (19) and aberrant methylation of a CpG island which extends from the promoter region into exon 1 (33). Both events occur at frequencies of 40 to 50% (7, 38), while mutations are observed in <10% of cell lines (35, 42). Despite the strong evidence for the tumor-suppressing function and frequent inactivation of *p16* in cell lines derived from NSCLCs, a dominant role for the *p16* gene in the genesis of primary NSCLCs has not been clearly substantiated due to a significantly lower frequency of dysfunction in primary tumors (7, 33, 36). The discordance in frequency of inactivation between cell lines and primary tumors could result from the occurrence or selection of *p16* dysfunction in cell culture. Alternatively, this difference in frequency may stem from a lack of sensitivity by the assays used to detect homozygous deletion (microsatellite markers) and methylation (Southern analysis) within tumors that may have heterogeneous cell populations for *p16* dysfunction and may also be contaminated by normal tissue. Support for the latter hypothesis comes from immunohistochemical studies, which indicate a lack of *p16* expression in 50% of primary NSCLCs examined (21, 43). In addition, fluorescence in situ hybridization studies indicate that the percentage of primary tumor cells exhibiting loss of both alleles may often be <40% (47), a frequency that may go undetected by microsatellite assays.

The question of whether *p16* inactivation arises within primary or cultured tumors is particularly important in light of the involvement of CpG island methylation in the development and progression of many human cancers (18, 22, 28). CpG island hypermethylation has been identified within specific chromosome loci prior to allelic loss (29, 31), and the expression of several tumor suppressor genes (14, 30, 48), including *p16* (12, 27, 33), is silenced in tumors in association with aberrant methylation within the CpG island of the gene. However, aberrant CpG island methylation of tumor suppressor genes, including *p16*, is especially common in cultures of transformed cells (14, 30, 33, 48). Therefore, it is critical to define the dynamics underlying the evolution of *p16* gene hypermeth-

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ylation during tumorigenesis and its role in establishing cultures from primary cancers.

The present investigation addressed these issues by using a rat model for lung cancer in which the frequency and mechanism for inactivation of *p16* can be determined in matched primary lung tumors and derived cell lines. In conjunction with cloning and sequencing of the rat *p16* cDNA, these studies were facilitated by a new PCR method (15), methylation-specific PCR (MSP), which readily detects methylation of *p16* alleles in heterogeneous cell populations. The potential involvement of the *p16* gene in rodent cancers has been suggested by the loss of heterozygosity on mouse chromosome 4 (8, 16) and by the monosomy of rat chromosome 5 (10), which have been observed in rodent tumors; these regions contain the alpha interferon gene cluster that is syntenic to human chromosome 9p21-22. In addition, deletion of *p16* has been detected in cell lines derived from murine lung tumors (1) and rat kidney tumors (20), and low expression has been observed in some primary murine lung tumors (1).

Recently, a second protein, $p19^{ARF}$ (p19), has been identified (40) that arises from the *p16* gene sequence but is encoded by an alternate first exon (designated 1β). Together with exon 1β, the second and third exons of *p16* are used to encode a protein, p19, that is unrelated to the amino acid sequence of p16 due to a frameshift but can also inhibit cell cycle progression (40). Because deletion of the *p16* gene or alterations within exon 1β would affect the function of p19, this transcript was also cloned and examined for inactivation.

MATERIALS AND METHODS

Cloning and sequencing. A 304-bp fragment containing portions of the first two exons of *p16* was generated from cDNA prepared from normal lung tissue by reverse transcriptase PCR (RT-PCR) using primers based on the mouse sequence (39); this fragment was ligated into the TA cloning vector (Invitrogen, San Diego, Calif.) and sequenced with the Sequenase kit (United States Biochemicals, Cleveland, Ohio). With this sequence information, the remaining portions of the *p16* and *p19* messages were obtained by rapid amplification of cDNA ends (RACE) (5′ and 3′ RACE systems; Life Technologies, Gaithersburg, Md.) by using two rounds of nested PCR. The *p19* cDNA fragments were generated from 5′ RACE experiments in which the gene-specific antisense primers were located in the second exon. Although these primers were designed to amplify both *p16* and *p19*, they produced no sequences matching mouse *p16*. The 5′ end of the *p16* cDNA was generated by locating the antisense primers in the exon 1 sequence, which is unique to *p16*. The 3′ sequence was generated by using *p16* exon 1 sense primers. RACE products were cloned into the TA vector. Three or more 5′ RACE clones each for *p16* and *p19* were sequenced with the Sequenase kit, and two 3′ RACE clones were cycle sequenced with an ABI automated sequencing system (Perkin-Elmer, Foster City, Calif.).

Tissue origins and exposures. The experiments described here were carried out in facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care. Eighteen primary rat lung tumors (T1 through T18) and corresponding cell lines (CL1 through CL18) were analyzed. Lung tumors were induced in F344/N rats exposured to either X rays (2), beryllium metal (34), cigarette smoke (9), plutonium-239 oxide (9), or cigarette smoke and plutonium-239 oxide (9). Excluding X rays, all exposures were via inhalation. Separate portions of each lung tumor were fixed in 4% buffered paraformaldehyde for histological classification, frozen by immersion in liquid nitrogen, and used to establish a cell line. Cell lines were derived from tumor samples that were first xenografted in athymic nude mice and then established as in vitro epithelial cell lines. All derived cell lines produced tumors when reinjected into nude mice; the resulting histotypes were either adenocarcinoma (AC), squamous cell carcinoma (SCC), adenosquamous carcinoma (ASC), or pulmonary osteosarcoma (OS). The histotypes of these explants generally recapitulated that of the primary tumor. The exceptions were tumors of mixed histotype, i.e., ASC, in which the squamous phenotype was usually selected through initial propagation in the nude mouse. Spontaneously transformed cell lines were derived from type II lung epithelial cells isolated from an F344/N rat (CL 19 [24]) and a Sprague-Dawley rat (CL 20 [23]). Both of these cell lines produced ACs when injected into nude mice.

Tissue culture and nucleic acid isolation. Cell lines were grown in ITRI-1 medium (4) to 80% confluence, harvested by trypsinization, pelleted, and snap frozen in liquid nitrogen prior to isolation of RNA and DNA. Selected cell lines were grown to 20% confluence in ITRI-1 medium prior to demethylation of genomic DNA. Culture medium was replaced with ITRI-1 containing 1 μM 2-deoxy-5-azacytidine (DAC) for 3 to 4 days to allow several cell doublings. Cells were then trypsinized and snap frozen. Total RNA was prepared with the Trizol reagent (Life Technologies), and DNA was isolated by standard phenol-chloroform extraction. Because tumor architecture was disrupted by freezing, the contaminating normal tissue was not removed by microdissection prior to isolation of DNA from primary tumors. However, the percent neoplastic cells estimated from the paraformaldehyde-fixed sections was ≥50% in all tumors analyzed.

***p16* and *p19* expression.** First-strand cDNA was synthesized from 3 μg of total RNA primed with random hexamers by using the Superscript Preamplification System (Life Technologies). To eliminate the possibility of false positives caused by residual genomic DNA, samples were treated with DNase (41), and primers were designed to cross exon splice junctions. Amplification products comprising a portion of exons 1 and 2 for *p16*, exons 1β and 2 for *p19*, and exons 5 through 8 of the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) were generated from the cDNA template in parallel PCRs. Following cDNA synthesis, 10% of the product from each sample was used for each PCR. Three parallel PCRs were performed: *p16* and *p19* were amplified to assess expression, and *GAPDH* was amplified as a control for RNA integrity. Amplifications were carried out in 50-μl reaction volumes for 35 cycles, each consisting of denaturation at 94°C for 1 min, annealing for 45 s, and extension at 72°C for 2 min (1 min for *GAPDH*). Primer pairs and annealing temperatures were as follows: for *p16*, 5′-CTTCACCAACGCCCCGAACAC-3′ (sense) and 5′-CGGGAGAGGGTG GTGGGGTC-3′ (antisense), 68°C; for *p19*, 5′-GCAGAGCATGGGTCGCAG GTTC-3′ (sense) and the same antisense primer as that used for *p16*, 66°C; and for *GAPDH*, 5′-GGTGCTGAGTATGTCGTGGA-3′ (sense) and 5′-GCCATG CCAGTGAGCTTCCC-3′ (antisense), 59°C. The amounts of amplified products analyzed by electrophoresis were 10 μl for *p16* and *p19* and 5 μl for *GAPDH*. Each RT-PCR assay was repeated at least once for confirmation. PCR products generated from cDNA template were authenticated as *p16* or *p19* by direct sequencing.

Amplification of genomic DNA. *p16* exon 1 and *p19* exon 1β were amplified by PCR from tumor cell lines and primary tumors. After an initial 5-min denaturation at 94°C, amplifications were carried out for 35 cycles, each consisting of denaturation at 94°C for 45 s, annealing for 30 s, and extension at 72°C for 45 s. For *p16*, primers 5′-ATGGAGTCCTCTGCAGATAGA-3′ (sense) and 5′-ATC GGGGGTACGACCGAAAGTGTT-3′ (antisense) were used, with annealing at 58°C. For *p19*, primers 5′-GCAGAGCATGGGTCGCAGGTTC-3′ (sense) and 5′-TGGTCCAGGATGTGGCTGCGGC-3′ (antisense) were used, with annealing at 71°C. PCR products generated from genomic DNA were authenticated as *p16* or *p19* by direct sequencing. Exon 6/7 of the rat *p53* gene was also amplified from each DNA sample as a control for DNA integrity, using conditions previously described (44). Water blanks, in which no template DNA was added, were included in each experiment, and no PCR product was detected in these controls. Products were resolved by electrophoresis on 2.0% agarose gels.

Simple sequence repeat markers were amplified by PCR using 22 primer pairs dispersed (distance between markers ranged from 7 to 22 centimorgans) across chromosome 5 (Research Genetics, Huntsville, Ala.) under the following conditions: 16.5 pmol of each primer and 400 ng of genomic DNA, denaturation at 95°C for 5 min followed by 35 cycles, each consisting of denaturation at 95°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 75 s. Products were analyzed on 6% nondenaturing polyacrylamide gels poststained in ethidium bromide.

Northern and Southern analysis. Twenty micrograms of total RNA from each sample was electrophoresed on 1% agarose formaldehyde gels, transferred to nylon membranes by capillary blotting, and cross-linked to the membrane by irradiation from a UV Stratalinker (Stratagene, La Jolla, Calif.). The *p16* probe was limited to the first exon for specificity. The *GAPDH* probe was an RT-PCR product from a normal lung sample. Probes were gel purified and ^{32}P (Amersham, St. Louis, Mo.) labelled by random priming according to the manufacturer's instructions. After overnight hybridization at 65°C in 0.5 M Na_2HPO_4–7% sodium dodecyl sulfate, membranes were washed twice at 65°C in 40 mM Na_2HPO_4–1% sodium dodecyl sulfate, dried, and exposed to X-ray film with an intensifying screen. For Southern analysis, 5 μg of DNA from each sample was digested with *Sac*I or double-digested with *Sac*I and *Hpa*II and then electrophoresed on a 1% agarose gel. Membrane transfer and probe hybridizations were as described above.

Methylation-specific PCR. A new method, MSP, in which site-specific methylation can be determined even for CpG sites not located in methylation-sensitive restriction enzyme sites has been developed (15). In MSP, genomic DNA is modified by treatment with sodium bisulfite, which converts all unmethylated cytosines to uracil and then to thymidine during the subsequent PCR step. Two sets of primers are used to amplify each region of interest: one pair recognizes a sequence in which CpG sites are unmethylated (modified by bisulfite to UpG), and the other recognizes a sequence in which CpG sites are methylated (unmodified by bisulfite). Primers are localized to regions containing frequent cytosines (to distinguish unmodified from modified DNA), and CpG pairs near the 3′ end of the primers provide maximal discrimination in the PCR between methylated and unmethylated DNA. Annealing at the CpG sites located at the growing end strongly affects primer extension, and thus the methylation status of these sites is discerned.

Sodium bisulfite modification of DNA and PCR were performed by the tech-

TABLE 1. Amplification primers for MSP analysis of rat *p16*

Direction	Designation[a]	Sequence[b]
Sense	BS-1	5′-GTGAATTTGAGGAGAGTGATTTG-3′
Antisense	BS-2	5′-CAAAACATTTAATAAAACCCCAA-3′
Antisense	BS-3	5′-CAACCAAAAATATTCAAAACATT-3′
Antisense	BS-4	5′-CACCTATATCAAAATACAACCAA-3′
Sense	BSM-1	5′-AATTCGAGGAGAGCGATTCG-3′
Antisense	BSM-2	5′-AACGTTTAATAAAACCCCGA-3′
Antisense	BSM-3	5′-GACCGAAAATATTCGAAACG-3′
Antisense	BSM-4	5′-ACCTATATCGAAATACGACCGA-3′

[a] Primers designated BS anneal to unmethylated sequences, while primers designated BSM anneal to methylated sequences. Primer locations within exon 1 are indicated in Fig. 5, with antisense primers 2, 3, and 4 located sequentially toward the 3′ end of exon 1.

[b] Primer sequences reflect those required to amplify the sense strand of bisulfite-modified DNA.

nique described by Herman et al. (15). Briefly, 2-μg aliquots of DNA were denatured with sodium hydroxide, treated with 3 M sodium bisulfite and 10 mM hydroquinone, purified with the Wizard DNA purification system (Promega, Madison, Wis.), and desulfonated with 0.3 M sodium hydroxide. Treated DNA was precipitated with ethanol and resuspended in 50 μl of Tris-EDTA buffer. PCR amplification was performed with approximately 150 ng of treated DNA as the template. Two sets of primers were designed at the same position, one set specific for DNA methylated at CpG sites and one specific for fully unmethylated DNA. After bisulfite treatment, double-stranded DNA is no longer complementary, so primer pairs were designed to amplify only the modified sense strand. Both pairs were used for each sample in separate reactions as follows: 94°C for 5 min; 35 cycles each of 94°C for 30 s, 51°C for 30 s, and 72°C for 30 s; and finally 72°C for 5 min. Negative control reactions were performed by using both sets of modified primers with untreated DNA, to confirm that unmodified DNA could not be amplified in the event of incomplete bisulfite reactions. One common 5′ primer was paired with three different 3′ primers to examine methylation status throughout exon 1 of the *p16* gene. Primers are listed in Table 1. Products were visualized on 2.0% agarose gels.

Nucleotide sequence accession numbers. Accession numbers through GenBank for the *p16* and *p19* sequences are L81167 and L81168, respectively.

RESULTS

Sequences of rat *p16* and *p19* cDNAs. The sequence of the rat *p16* cDNA is depicted in Fig. 1A. Its identity as *p16* was supported by (i) its conservation within the ankyrin repeat regions of the gene, (ii) its 75% homology to the mouse *p16* gene within the coding region and 88% homology within exon 1, and (iii) only 65% exon 1 homology and 63% overall homology to the mouse homolog of the closely related $p15^{INK4b}$ gene. The first exon of the rat *p16* cDNA was defined as a CpG island based on established criteria (11). The deduced amino acid sequence of the rat *p16* product (Fig. 1B) was 75 and 62% homologous to mouse and human sequences, respectively.

The nucleotide sequence of the *p19* transcript, formed by using the alternate first exon (exon 1β), is shown in Fig. 1C. This exon was spliced onto the common exon 2 and produced a second open reading frame, with a size similar to that reported for mouse *p19* (40). The nucleotide sequence within the protein-coding region was 63% homologous to the mouse *p19* gene. Exon 1β was also defined as a CpG island.

Expression of *p16* and *p19* in normal tissues and tumor-derived cell lines. The *p16* and *p19* transcripts were not expressed at levels sufficient for detection by Northern blotting in any normal rat tissues (data not shown). Similar to expression in mouse and human tissues (39, 43), *p16* expression by RT-PCR was clearly present in rat spleen, liver, and lung and absent in kidney, colon, stomach, and brain (data not shown). Expression of *p19* was detected in all tissues, corresponding to its ubiquitous expression in the mouse (40). However, only trace levels of transcript were detected in kidney and colon (data not shown).

p16 mRNA was not detected in 11 of 20 tumor-derived cell lines, and the level of expression varied significantly in the 9 lines for which a transcript was observed (Fig. 2). Only three cell lines (CL10, CL15, and CL19) had expression levels similar to that of normal lung (Fig. 2; Table 2), while only a trace amount of *p16* transcript was evident in three other cell lines (CL2, CL6, and CL9). Expression of *p16* in the remaining three cell lines (CL4, CL7, and CL14) appeared to be higher than in normal lung. The relative intensities of product bands shown in Fig. 3 were reproduced in three separate experiments. Moreover, Northern analysis confirmed that *p16* transcript from the three cell lines that appeared to be expressed most abundantly by RT-PCR analysis was also detected by hybridization with an exon 1-specific probe, while transcript from other cell lines was not detected (data not shown).

p19 mRNA was detected in 12 of 20 cell lines (Fig. 3). Only a trace of *p19* transcript was evident in two cell lines (CL8 and CL12). Expression in two cell lines (CL9 and CL10) approximated that observed in normal lung, while expression in the remaining cell lines appear to be increased.

Deletions of *p16* exon 1 and *p19* exon 1β. The lack of *p16* expression seen in 11 of 20 cell lines and only trace expression in 3 others could stem from gene deletion in some cells or from transcriptional silencing associated with methylation of the CpG island located within exon 1. If the structure of the rat *p16* gene is similar to the structures of its mouse and human homologs (32, 39), exon 1β could be located several kilobases upstream of exon 1, so that the *p19* transcript could be lost as a result of the deletion of exon 1β and/or deletion of exon 2, which is shared by *p16* and *p19*. Therefore, tumor cell lines were first analyzed by PCR for deletion of exons 1 and 1β. Homozygous deletion of *p16* exon 1 was detected in four cell lines (Fig. 3; Table 2) which showed loss of *p16* expression (CL8, CL11, CL16, and CL18). Four additional cell lines which had little or no *p16* expression (CL1, CL9, CL10, and CL12) showed markedly reduced amplification products, suggestive of gene deletion (Fig. 2 and 3). These results were produced in a replicate experiment. In addition, Southern analysis of CL9 and CL10, which showed reduced amplification product, failed to detect hybridization signal in these samples, confirming results from the PCR-based assay (data not shown). The weak *p16* amplification products in these cell lines were not due to DNA degradation, because the *p53* control fragments were present at similar levels (Fig. 3) among all cell lines. Because these cell lines are not clonal and were examined at an early passage (<10 passages), the weak *p16* amplification products may reflect heterogeneity of the tumor cell populations in these cultures.

To determine whether the deletions could be physically mapped, the four cell lines with homogeneous *p16* exon 1 deletions were analyzed for homozygous deletions of simple sequence repeat markers dispersed across rat chromosome 5. None of the 22 markers were deleted in any cell lines (data not shown), validating the interstitial nature of the deletion but not allowing the boundaries of the deletions to be defined.

Evidence for homozygous deletion of *p19* exon 1β was detected in six cell lines (CL1, CL8, CL12, CL16, CL18, and CL20 [Fig. 3]). Two cell lines had complete loss of the exon (CL8 and CL16), while the remaining lines had reduced amplification products. Five of these cell lines also contained deletion of *p16* exon 1, while this exon was present in CL20. All cell lines with evidence for homozygous deletion showed reduced or no expression of the *p19* transcript compared to the other expressing cell lines (Fig. 2). The exon 1β gene was

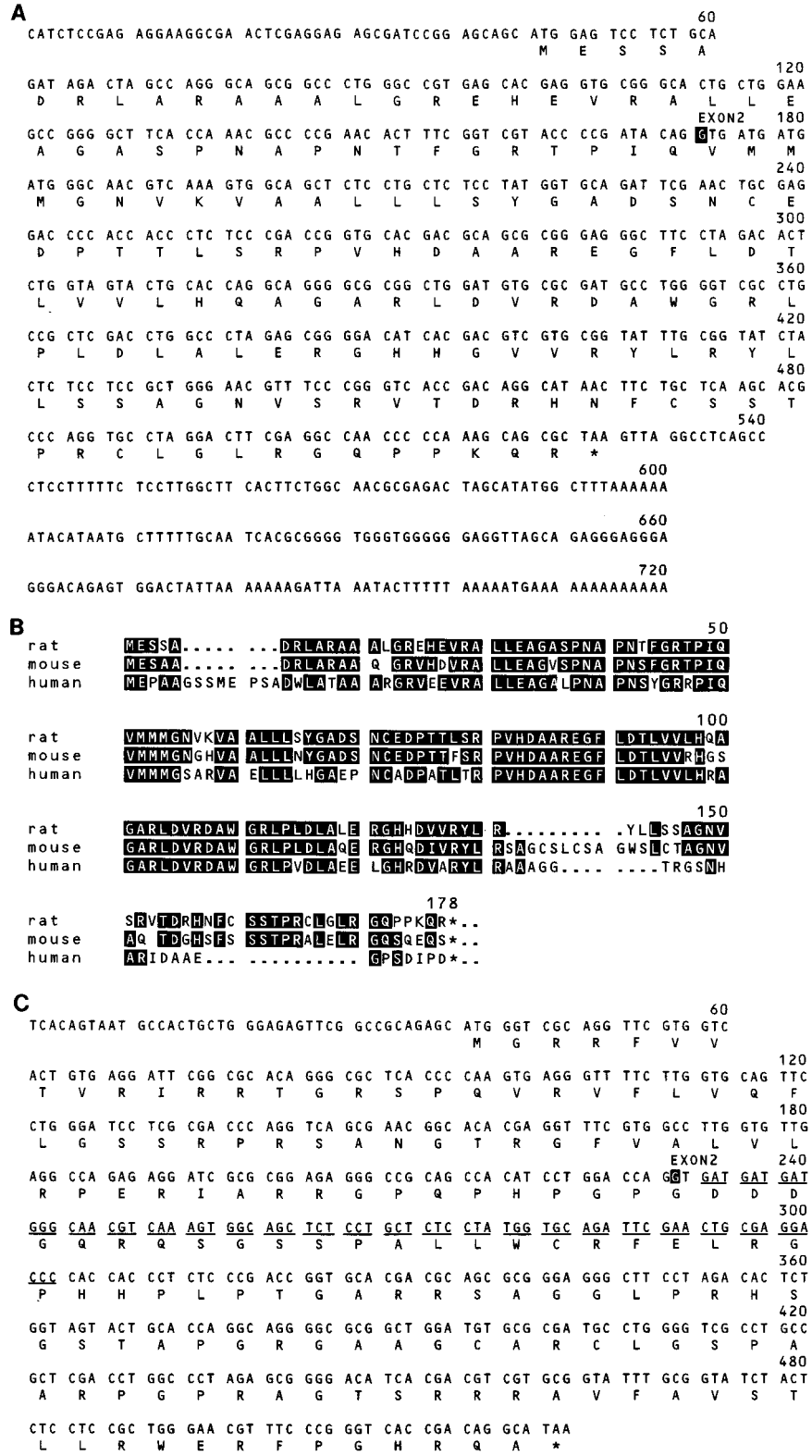

FIG. 1. Nucleotide sequences of *p16* and *p19* and deduced amino acid sequences of their products. (A) Sequences for the rat *p16* cDNA. The exon 2 boundary is indicated by the highlighted guanine nucleotide. (B) Comparison of the rat *p16* amino acid sequence to the mouse and human homologs. Conserved residues are highlighted. (C) Sequences for *p19*. Sequence data from cloned *p19* fragments defined the exon 1 region and confirmed that the nucleotide sequence from position 230 to 303 (indicated by underlining) is identical to the sequence of *p16*; the remainder of the nucleotide sequence shown was inferred from the sequence of the *p16* 3′ RACE clone. The exon 2 boundary is indicated by the highlighted guanine nucleotide.

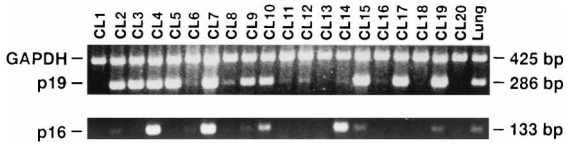

FIG. 2. RT-PCR analysis of *p16* and *p19* gene expression in rat lung tumor cell lines. Expression of *GAPDH* was determined as a control for RNA integrity.

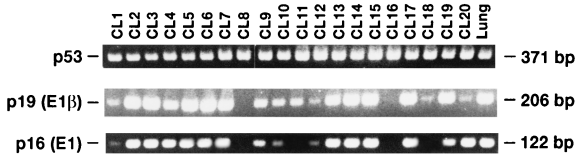

FIG. 3. Detection of deletion of *p16* exon 1 and *p19* exon 1β in rat lung tumor cell lines by PCR. Amplification of *p53* served as a control for DNA integrity.

present in DNA from all other cell lines that expressed *p19* and in four cell lines (CL6, CL11, CL13, and CL14) that showed no *p19* transcript (Fig. 2 and 3).

Induction of *p16* expression by DAC. To determine whether the reduced or absent *p16* expression in cultures retaining a *p16* gene might be due to gene methylation, eight cell lines were grown in the presence of DAC to achieve widespread demethylation of genomic DNA. Treatment of human cancer cell lines with DAC has been shown to result in demethylation of the *p16* gene and reexpression of the *p16* transcript (38). RT-PCR analysis of treated cultures (Fig. 4) showed that *p16* expression relative to that of *GAPDH* was induced, in contrast to untreated samples examined at the same passage. Two of the four cell lines that failed to express *p19* but retained exon 1β were in the DAC-treated group (CL6 and CL13); neither line reexpressed *p19* after treatment (data not shown), suggesting that methylation may not be a mechanism for silencing of this transcript. The deletion of exon 1β in CL20 did not prohibit expression of *p16* after treatment with DAC, indicating that the deletion within this cell line was confined to exon 1β and did not include the *p16* promoter.

***p16* gene methylation in cell lines.** To initially establish the concordance of the new MSP analysis with standard Southern analysis for determining DNA methylation status, two *Hpa*II methylation-sensitive restriction sites were examined in the *p16* CpG island (Fig. 5) by both techniques. Three of the cell lines selected (CL13, CL17, and CL20), which showed no expression of *p16* but reexpressed the gene after DAC treatment, were densely methylated, as assessed by both techniques (Fig. 6). CL7, which expressed the gene, had an unmethylated *p16* gene, as did normal lung, as determined by Southern analysis. However, MSP indicated the presence of both methylated and unmethylated DNA in this cell line. The methylated PCR product in this sample was much less abundant than the product observed in cell lines in which methylation was evident by both assays (Fig. 6B). These results are consistent with the greater sensitivity of MSP over Southern analysis (15). Examination of CL15 by Southern analysis revealed the presense of 3.0- and 2.1-kb bands, indicating that some alleles were methylated at both *Hpa*II sites while others were methylated at only one site. MSP analysis detected only methylated product in this cell line, as predicted, because the methylation-specific primer pair will amplify only template which has both *Hpa*II sites methylated. Together, these results confirmed that the MSP assay accurately indicated the methylation state of the rat *p16* gene at specific CpG sites.

MSP, using the primers located across the *Hpa*II sites (Fig.

TABLE 2. *p16* expression and mechanism of inactivation in primary lung tumors and derived cell lines

Cell line		Exposure[a]	Histology[b]	*p16* transcript in cell line[c]	*p16* gene dysfunction	
No.	Designation				Cell line	Primary tumor[d]
1	C006	Pu	ASC	−	Deleted[e]	Methylated
2	X404	X ray	AC	Tr	Methylated	Methylated
3	U635	X ray	AC	−	Methylated	Methylated
4	R623	Pu+Sm	SCC[f]	+++	Unmethylated	Methylated
5	L173	Pu+Sm	SCC	−	Methylated	Methylated
6	J429	Pu+Sm	SCC[f]	Tr	Methylated	Methylated
7	D434	Be	SCC	+++	Methylated[g]	Methylated
8	D391	Sm	SCC[f]	−	Deleted	Unmethylated
9	U640	X ray	AC	Tr	Deleted[e]	Methylated
10	W173	X ray	AC	+	Deleted[e]	Unmethylated
11	J546	Pu+Sm	SCC	−	Deleted	Methylated
12	N204	Pu	AC	−	Deleted[e]	Unmethylated
13	H177	Pu	AC	−	Methylated	Methylated
14	B444	Pu	OS	+++	Unmethylated	Unmethylated
15	U649	X ray	SCC[f]	+	Methylated	Methylated
16	V304	X ray	SCC	−	Deleted	Methylated
17	I149	X ray	SCC	−	Methylated	Methylated
18	V309	X ray	SCC	−	Deleted	Unmethylated
19	LEC	Spont.	AC	+	Methylated	NA[h]
20	FRLE	Spont.	AC	−	Methylated	NA

[a] Abbreviations: Pu, plutonium; Sm, smoke; Be, beryllium; Spont., spontaneous.
[b] Histology observed in the nude mouse tumor produced from each cell line. The resulting tumor generally recapitulated the histology of primary tumors.
[c] −, no transcript; +, expression roughly equal to that seen in normal lung; +++, expressed at levels detectable by Northern analysis.
[d] Normal lung tissue was unmethylated.
[e] This cell line had reduced *p16* exon 1 amplification product compared to the *p53* control amplimer.
[f] SCC which showed an adenosquamous phenotype in the primary tumor.
[g] Both methylated and unmethylated alleles were present in this cell line.
[h] NA, not applicable.

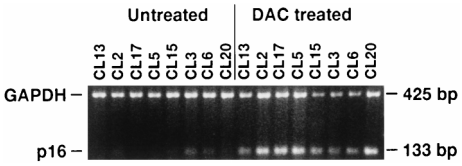

FIG. 4. Expression of *p16* transcript in cell lines following treatment with DAC. Untreated cell lines served as controls for change in expression. Expression of *GAPDH* was determined as a control for RNA integrity.

5), was then used to analyze the methylation status of the 12 cell lines that had no evidence for *p16* gene deletion (Fig. 7; Table 2). Only three lines (CL4, CL7, and CL14) produced strong amplification by using the primers specific for unmethylated DNA; these cell lines had abundant expression of the *p16* transcript. The remaining 9 cell lines with little or no *p16* expression produced amplification product only with the methylation-specific primers, suggesting that the majority of the *p16* exon 1 alleles in these samples were methylated. The density of methylation within the *p16* gene was examined further by using two additional antisense primers with 3′ ends located over CpG sites 2 and 3 or sites 4 and 5 (Fig. 5). The distribution of methylated and unmethylated products among cell lines was identical to that observed with the first set of MSP primers (data not shown).

Analysis of primary tumors for *p16* deletion and methylation. The 18 primary rat lung tumors from which the tumor cell lines were established were analyzed by PCR for presence of *p16* exon 1 and *p19* exon 1β. Both fragments were amplified from all primary tumor samples (data not shown). Primary lung tumors were then analyzed for methylation of *p16* exon 1 by the MSP assay. Seventeen of 18 tumors produced amplimers from the unmethylated primer pairs, consistent with the presence of nonneoplastic tissue and/or neoplastic cells with unmethylated alleles. However, eight tumors also clearly produced amplimers by using the methylation-specific primers that examine the *Hpa*II sites and CpG site 1, 2, and 3 (Fig. 8; Table 2), indicating substantial levels of methylated CpG sites within *p16* exon 1. The methylated primer pair that used the antisense primer with the 3′ end located over CpG sites 4 and 5 (Fig. 5) detected methylation not only within these eight tumors but also within an additional five tumors (Fig. 8). Methylation of *p16* was not detected by any of the MSP primer pairs (data not shown) in normal rat tissues that had been analyzed for gene expression, indicating that contaminating normal tissue could not account for the methylation detected in the primary tumors. All cell lines in which the *p16* gene was extensively methylated were derived from tumors that also had methylation of this gene (Table 2). Interestingly, of the eight

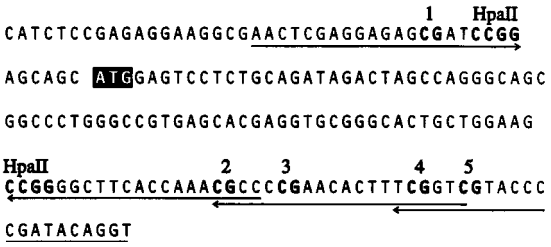

FIG. 5. Exon 1 of the rat *p16* gene. The two *Hpa*II restriction sites and five additional CpG sites (numbered 1 through 5) examined by MSP primers are shown in bold type. The location and orientation of the MSP primers are indicated by the arrows under the sequence. The start codon is highlighted.

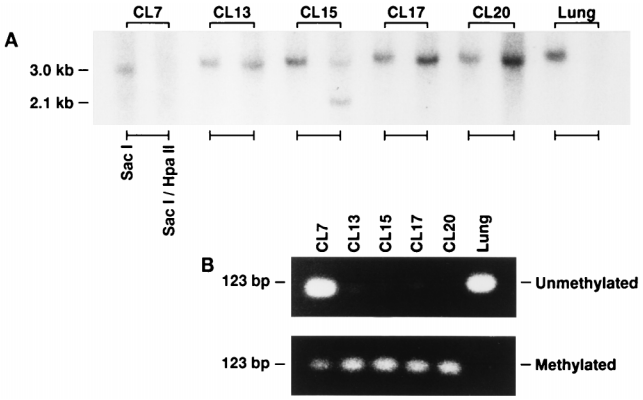

FIG. 6. Detection of *p16* methylation by Southern (A) and MSP (B) analyses. DNAs from normal lung and five lung tumor cell lines were digested with *Sac*I alone or in combination with the methylation-sensitive *Hpa*II restriction enzyme. Hybridization to the *p16* exon 1 probe detected a 3.0-kb band in DNA digested with *Sac*I. This band is retained following double digestion with *Sac*I and *Hpa*II if both *Hpa*II sites are methylated, while no bands are visible if both sites are fully unmethylated (small fragments generated by restriction digestion are not retained on the gel). Partial methylation is indicated by the presence of a 2.1-kb band in DNA digested with *Sac*I and *Hpa*II. The same samples were also analyzed by MSP, using primers that encompass the *Hpa*II sites and a CpG site 5′ of the first *Hpa*II site (primers BS-1/BSM-1 and BS-2/BSM-2) (Fig. 5). An amplification product of the primers specific for unmethylated sequence indicates the presence of unmethylated DNA at the *Hpa*II sites, while the converse applies to the primers specific to methylated sequence.

cell lines with homozygous deletion of the *p16* gene, four were derived from primary tumors that contained methylated *p16* alleles (Table 2). Methylation was detected in one tumor (T4) but not in its derived cell line. The phenotype of this tumor was adenosquamous and the derived cell line was an SCC, suggesting that the methylation detected in the primary tumor may have been localized to the adenomatous portion, which was not propagated in culture.

DISCUSSION

Results of this investigation clearly demonstrate that aberrant *p16* CpG island methylation leading to the loss of *p16* expression originates in primary tumors. Moreover, de novo methylation of the *p16* exon 1 CpG island occurs frequently in primary lung tumors induced in rats. The mechanisms for *p16* dysfunction in the rat lung tumor cell lines examined in this study parallel those seen in human cell lines (33, 37), validating the rat as a model for studying the role of *p16* in lung cancer.

The presence of aberrant *p16* methylation in cell lines was strongly correlated with absent or low expression of the gene. Only CL7 which expressed *p16* at high levels showed evidence for both methylated and unmethylated alleles, indicating heterogeneity within this cell line. The fact that trace or low levels of expression were detected in some cell lines with apparent

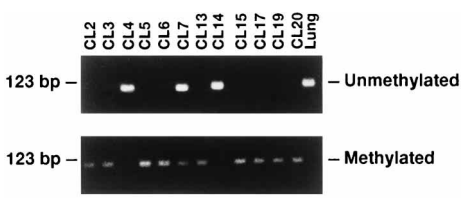

FIG. 7. MSP analysis for *p16* methylation in lung tumor cell lines. Bisulfite-modified DNA was amplified with primer pairs specific for unmethylated and methylated template as described for Fig. 6.

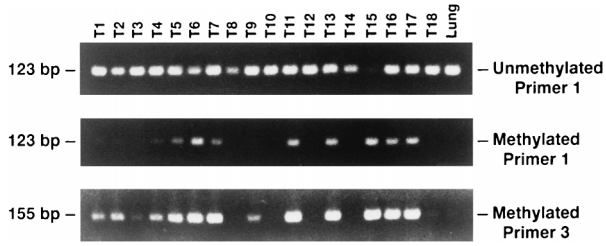

FIG. 8. MSP analysis for *p16* methylation in primary lung tumors. Bisulfite-modified DNA was amplified with the primer pairs (specific for unmethylated and methylated template) that encompassed CpG site 1 and both *Hpa*II sites (top two panels) as described for Fig. 6 and with the methylation-specific primer (bottom panel) that encompassed CpG sites 4 and 5 (primer BSM-4) (Fig. 5).

complete methylation of exon 1 may indicate a lack of methylation at sites within the promoter region, leading to minimal levels of transcription. Although the methylated phenotype showed an absolute correlation between cell cultures and parent tumors, both methylated and unmethylated alleles were detected by the MSP procedure in most primary tumors. Estimating the extent to which this heterogeneity of methylation reflects contamination of the tumor by normal tissue or heterogeneity within the tumor cell population is difficult. In tumor 15, no unmethylated alleles were observed, and contamination of this tumor with normal cells was estimated to be only 2%. The fact that methylation was detected in tumors contaminated by up to 50% with normal tissue indicates that methylation must be extensive within many of these tumors.

The studies described here did not attempt to show that methylation detected in primary tumors resulted in loss of *p16* expression. Such experiments are difficult without specific antibodies that are reactive to rat p16 because tumor heterogeneity and contamination by normal tissues would render RT-PCR-based assays ineffective. However, several lines of evidence support the hypothesis that tumor cells harboring methylated *p16* alleles would not express the transcript. First, Kratzke et al. (21) showed by immunohistochemistry that 47 of 100 human NSCLCs completely lacked p16 protein. Second, expression stemming from inactivation of only one *p16* allele is unlikely given the fact that allelic loss is frequently detected in rodent and human tumors at the *p16* locus (10, 16, 19, 20, 33). In the rare situation in which cell lines had retained both alleles, both were methylated (33). This indicates a strong selection pressure for inactivating this gene. Finally, methylation of the *p16* gene shown in cell lines from this study clearly resulted in a lack of expression.

It is clear that *p16* inactivation via homozygous deletion is also frequent in rat cell lines, and this finding parallels that reported by Otterson et al. (38) in cell lines from human NSCLCs. Cell line deletions were too small to include loss of nearby microsatellite markers, and in one case the deletion was small enough to encompass *p16* exon 1 but not *p19* exon 1β. This is also consistent with homozygous deletions of human *p16*, which in some tumors involved a region of only 170 kb (7). Homozygous deletions of *p16* were not detected in the matched primary tumors; however, this probably reflects cell heterogeneity stemming from contamination of tumor by normal tissue. In addition, fluorescence in situ hybridization studies indicate that when total deletion of *p16* is detected, it often occurs in <40% of tumor cells from NSCLCs (47), a frequency which would have been undetected in our study. Interestingly, CpG island methylation was detected in four of the eight primary tumors corresponding to the eight cell lines with evidence of gene deletion. Hypermethylation within CpG islands has been hypothesized to lead to changes in chromatin structure which may predispose the islands to genetic instability. For example, a previous study (31) with renal tumors demonstrated that regional hypermethylation on chromosome 17p, in an area subsequently shown to harbor a candidate tumor suppressor gene (30), preceded allelic loss. Our results may indicate such a relationship between methylation and homozygous deletion of the *p16* locus.

This study is the first to examine dysfunction of *p19* in cell lines derived from lung tumors. The absence of methylation within *p19* exon 1β, an observation consistent with studies of human tumors (15a), and the lack of coordinate tissue-specific expression are further evidence that this gene is controlled by its own promoter (40) and that its regulation is independent and distinct from that of *p16*. A lack of *p19* expression was observed in four cell lines in which exons 1β, 2, and 3 were present. This effect could stem from mutations within exon 1β or the *p19* promoter. Studies to define the mechanism for loss of expression and its effect on cellular phenotype may better define the role of this alternate transcript in normal cellular functions.

The frequency of *p16* methylation observed in primary rat lung tumors (72%) surpasses the frequency of 26% obtained by Southern analysis in human lung tumors (33). A similar frequency has also been observed in primary rat lung tumors induced by plutonium from which cell lines were not established (5a), indicating that the high frequency of aberrant methylation of *p16* was not a function of selecting tumors for analysis from which cell lines were established in culture. This difference may be due in part to the greater capability of MSP analysis to detect methylation, but it could also reflect the fact that the majority of rat lung tumors examined were induced by radiation exposure. A recent study by Issa et al. (17) revealed a carcinogen-specific effect on methylation of the estrogen receptor gene in rat and human lung tumors. Specifically, a higher frequency of methylation was observed in lung tumors from individuals who had never smoked than in tumors from smokers and in rodent tumors induced by radiation than in rodent tumors induced by the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Alternatively, factors involved in initiating the spread of methylation through the *p16* CpG island may differ between humans and rodents. Vertino et al. (45) demonstrated that CpG island loci differ in their inherent tendency to undergo de novo methylation.

The potential contributions of species and exposure differences to the frequency of CpG island methylation will remain elusive until events that lead to de novo methylation and the targeting of specific genes are clearly defined. Our rat model may be an excellent setting for such studies. Recent studies in our laboratory (3) have shown that carcinogen exposure can induce the activity of DNA methyltransferase, the enzyme responsible for methylation of CpG sites (6). Moreover, this activity is induced selectively in the alveolar type II pneumocyte, the cell of origin for the resulting murine lung tumors (5), and is an early event in lung cancer evolution which increases during tumor progression. DNA methyltransferase activity is also elevated in rat and human lung tumors (5a). Overexpression of DNA methyltransferase in human fibroblasts has been associated with hypermethylation of promoter region CpG islands of some cancer-related genes (e.g., the estrogen receptor gene), while the *p16* gene remains unmethylated in this setting (45). However, these fibroblasts already have a functionally inactivated *Rb* gene due to simian virus 40 T antigen, and thus, there is no selective advantage for loss of *p16*. Effects on locus-specific factors such as Sp1 (26) may protect CpG islands from de novo methylation and could be required for initiating

aberrant methylation within the *p16* gene. Studies of *p16* throughout tumor evolution in rodent carcinogenesis models should help identify factors important in aberrant methylation of this gene and define its role in the progression of neoplasia.

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