Cyclin-dependent Kinase Inhibitor Expression in Pulmonary Clara Cells Transformed with SV40 Large T Antigen in Transgenic Mice

Susan M. Magdaleno, Guiying Wang, Veronica L. Mireles, Manas K. Ray, Milton J. Finegold, and Francesco J. DeMayo

Departments of Cell Biology [S. M. M., G. W., V. L. M., M. K. R., F. J. D.] and Pathology [M. J. F.], Baylor College of Medicine, Houston, Texas 77030

Abstract
Expression of cell cycle regulatory genes in mouse lung was investigated in transgenic models for Clara cell transformation. Clara cells were transformed by generating transgenic mice in which the SV40 large T antigen was expressed under the control of the mouse Clara cell M, 10,000 protein promoter. The resulting lung tumors express the large T antigen in normal Clara cells and in tumors, and these tumors express reduced levels of CC10 mRNA. The expression of cell cycle regulatory protein, p53, and the cyclin-dependent kinase inhibitors was analyzed by Northern blot analysis and in situ hybridization throughout the progression of Clara cell transformation in the lung. Increases in specific cyclin-dependent kinase inhibitor steady-state mRNA levels were detected in p15, p18, p27, and p57 during tumor progression. The expression of p15, p57, and p21 mRNAs were verified by in situ hybridization. Using this approach, regulatory genes have been identified that may be involved in the regulation of Clara cell differentiation.

Introduction
The lung functions as the site for gas exchange with the external environment and the body tissues. To facilitate this function, the lung develops into an organ that contains conducting airways that warm, moisten, and filter the inspired air before its delivery to the alveoloi where gas exchange occurs. The lung develops from a ventral thickening of the epithelium of the foregut in the 9.5-day-old mouse embryo to form a tube-like structure lined by cuboidal epithelium. This organ rudiment is subsequently subdivided into lobes with continually branching bronchiole airways and distal alveolar gas exchange regions. The mature proximal pulmonary airways are lined by a specialized epithelium, characterized as pseudostratified ciliated epithelium. In the most distal respiratory bronchiole, this epithelium abruptly changes into the flattened squamous cells that line the pulmonary alveoli, providing the surface for gas exchange (reviewed in Ref. 1). The pulmonary epithelium is composed of many different specialized cells, each of which contribute to normal lung physiology. Controlled cellular proliferation, differentiation, and cell turnover are critical events that maintain the pulmonary epithelium in homeostatic balance. This strict control of cell differentiation in a complex mucosa makes the lung a good model to study the mechanisms that lead to the differentiation of specific cell types.

One of the largest cells of the proximal pulmonary epithelium is a specialized cell called the Clara cell. The Clara cells are the nonciliated secretory cells of the pulmonary epithelium characterized by a large apical dome shape and abundant endoplasmic reticulum. Clara cells secrete a number of proteins, including SP-A, SP-B, and SP-D (2-4), a leukocyte protease inhibitor (5), and a trypsin-like protease (6). The major secretory product of the Clara cells is a specific protein called the Clara cell M, 10,000 protein, also termed CC10 or CCSP (7). This protein has been implicated in an anti-inflammatory role in pulmonary physiology (8, 9). The genetic elements regulating expression of the mouse CC10 gene have been identified and can be used to target transgenes specifically to the Clara cells of the lung (10, 11). In the present study, 2.1 kb of the mouse promoter, mCC10, was used to target the oncogene SV40 TAg to the Clara cells of the lung to challenge the normal processes of cell proliferation and differentiation. TAg has been shown to disrupt normal cell cycle control by binding and inhibiting the cell cycle checkpoint proteins Rb and p53 (12). Removal of these proteins, concomitant with secondary genetic events, leads to the loss of cell cycle control, increased cellular proliferation, and eventual tumor development. The purpose of this report was to specifically disrupt the cell cycle of the Clara cells and investigate the molecular changes that result in these cells. Expression of cell cycle regulatory genes, p53, and CDKIs was analyzed for mRNA steady-state levels throughout lung tumor development.

Many molecules that regulate the cell cycle have been identified and characterized, and recently, the CDKII families, p21, and INK4 families were described. CDKIs have been shown to regulate the cell cycle by binding and inhibiting the cyclin dependent kinases CDK2, CDK4, and CDK6 from

Received 5/3/96; revised 9/4/96; accepted 12/17/96.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This research is supported by American Cancer Society Grant CN-135, NIH Grant HL 47620, and a National Science Foundation Minority Graduate Fellowship.

2 To whom requests for reprints should be addressed, at Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030. Phone: (713) 798-6241; Fax: (713) 790-1275.

3 The abbreviations used are: CC10, Clara cell M, 10,000 protein; mouse promoter, mCC10; TAg, large T antigen; CDKI, cyclin-dependent kinase inhibitor; SP, surfactant protein.
Pulmonary CDK1 mRNA Expression in CC10Tag Transgenic Mice

forming the functional holoenzymes necessary for cell cycle progression (13, 14). The p21Cip1 inhibitor has been implicated as an important regulator of terminal cell differentiation. Reports from Halevy et al. (15) and Parker et al. (16) demonstrated the induction of p21 mRNA and protein expression after treatment of myogenic cells with MyoD, which triggers terminal differentiation. They further showed that p21 induction was a p53-independent event in the MyoD-treated cells. These studies indicate that the p21 protein may be a factor that regulates exit from the cell cycle and terminal differentiation in muscle. Therefore, these CDKIs may be important regulatory proteins for determining cell differentiation in other tissues, such as the lung.

To study the molecular controls for Clara cell proliferation and differentiation, a transgenic mouse was generated to specifically challenge this cell type with the SV40 Tag oncogene. The transgenic mice were subsequently characterized for expression of the CDKIs in whole lung throughout the progression of lung tumor development. Understanding the mechanisms invoked by challenge with an oncogene in the Clara cell will identify those molecules that are important for maintaining the control of proliferation and Clara cell phenotype.

**Results**

**CC10Tag Transgenic Mice Develop Multifocal Pulmonary Adenocarcinomas.** Microinjection of the mCC10Tag construction (Fig. 1A) into one-cell FvB mouse embryos resulted in the generation of two transgenic founder mice, 7593

---

**Fig. 1.** Generation of transgenic mice and analysis of transgene expression. A, structure of the CC10Tag transgene. Double-headed arrow indicates the DNA fragment used for cDNA probes in Southern and Northern blot analysis. B, total RNA from nontransgenic mouse lung and liver and from lung tissue of the CC10Tag 7736 and 7593 mouse lines was isolated and analyzed by Northern blot analysis for expression of the SV40 Tag. Tag mRNA levels are higher in the 7736 mouse line compared with Tag levels in the 7593 mouse line. Lu, lung; Li, liver. Loading of total RNA was evaluated by ethidium bromide staining of RNA. C, ten μg of total RNA from a series of mouse tissues of the CC10Tag 7736 mouse line were analyzed for Tag expression by Northern blot analysis. The Tag 2.4-kb message was localized specifically in the lung. Loading of sample RNA was evaluated by ethidium bromide staining of the 28S and 18S rRNA. Br, brain; He, heart; Ki, kidney; St, stomach; Ut, uterus. D, immunohistochemistry was performed on lung sections from an F1 7736 offspring to localize Tag protein. Tag protein was detected in the nuclei of Clara cells of the bronchiolar epithelium (arrows) and in the nuclei of transformed tumor cells of the adenocarcinoma (not shown), but was not detected in alveoli.
Fig. 2. Morphological analysis of lung tumor progression in the 7736 mouse line. Mouse lungs were collected at 2, 3, and 4 months of age, sectioned, and stained with hematoxylin. A, CC10Tag lung at 2 months of age showing normal alveolar spaces and a small bronchiole cut in cross-section (b). B, high-power magnification of previous section of 2-month-old lung. Arrowheads indicate normal bronchiolar epithelium, and arrows point to regions of hyperplastic growth. C, CC10Tag lung from 3-month-old mouse showing a small tumor focus (d). D, CC10Tag lung at 4 months of age. Large adenocarcinomas are found to compose the majority of the lung section. A and C, ×100; B and D, ×200.

and 7736. Transgenic mice were identified by Southern blot analysis when probed with a HindIII fragment of the SV40 Tag cDNA (data not shown). Southern blot analysis demonstrated that more than 10 copies of the transgene had integrated in the 7736 mouse line, with a lower copy number (between 1 and 5 copies) in the 7593 mouse line. The level of pulmonary Tag expression was analyzed by Northern blot analysis, shown in Fig. 1B. The level of expression varied, with mouse line 7736 showing the highest level of Tag expression. The level of expression corresponded with the timing of the onset of the Tag phenotype. Transgenic offspring from the 7736 line developed multifocal pulmonary adenocarcinoma of the lung and succumbed consistently at 16-20 weeks of age. Transgenic offspring from the 7593 mouse line developed lung cancer at a later onset and succumbed at 11 months of age with pathology similar to the 7736 line.

Tissue and Cell Specificity of SV40 Tag Expression. The pathology and expression of Tag was localized to the lungs. Northern Blot analysis was used to determine the tissue specificity of Tag expression. As shown in Fig. 1C, the expression of Tag mRNA in the 7736 line was restricted to the lungs. The second line, 7593, displayed a similar Tag tissue distribution. This specificity of Tag expression was also verified by reverse transcription-PCR (data not shown). Antibodies to Tag were used to determine Tag protein localization in normal and transformed lung in the 7736 CC10Tag mouse. Immunohistochemistry showed that Tag protein (Fig. 1D) is restricted to expression in the Clara cells of the lung in both the 7736 and 7593 transgenic lines. Due to the long latency of disease onset of the 7593 line, the remainder of the investigations were limited to the rapid onset, 7736 line.

Tumor Development. Histological analysis was conducted to investigate the progression of the Tag phenotype. CC10Tag lungs were examined at 2, 3, and 4 months of age to characterize the morphological progression of lung cancer. At 2 months of age, the lung appears morphologically normal upon gross examination compared with nontransgenic lung (Fig. 2A). However, at higher magnification, areas of hyperplasia of the bronchiolar epithelium are evident (Fig. 2B, arrow), and normal bronchiolar epithelium is abundant (arrowhead). At 3 months of age, a number of tumor foci could be observed (Fig. 2C), and 4 weeks later, the majority of the lung is composed of transformed cells (Fig. 2D). Having established time points of distinct morphological
changes of tumor growth, these time points were used to correlate the molecular changes involved in tumor progression.

Expression of Lung Molecular Markers. The expression of lung-specific markers was analyzed in situ using immunohistochemistry. The CC10Tag mouse lungs showed normal levels of CC10 expression in the Clara cells in the bronchiolar epithelium; however, at 3 months, the tumor displayed reduced and sporadic expression of the endogenous CC10 marker protein (Fig. 3, arrows). Therefore, expression of the endogenous CC10 gene coincides with the expression of TAg (compare Fig. 1D with Fig. 3) in the bronchiolar epithelium and shows a reduced level of expression with cellular transformation. This suggests that TAg expression alone does not affect the expression of the endogenous marker protein, and additional molecular events lead to changes in gene expression in the tumor itself. Clonal Clara cell lines were generated from the lungs of the CC10Tag mice for further analysis on the expression of the Clara cell marker protein and the SPs. These cells maintain an epithelial morphology and have been cultured for more than 30 passages (data not shown). Using a panel of DNA probes specific for SP-A, SP-B, SP-C, and SP-D, and a cDNA probe for CC10, the expression of these mRNAs was analyzed by Northern blot analysis. Within the bronchiolar epithelium, Clara cells express SP-A, SP-B, and SP-D; however, the transformed Clara cells in culture express SP-B mRNA at normal levels detectable by Northern blot analysis and showed a reduction in the level of CC10 mRNA. SP-A and SP-D mRNA was not detected in these cells (data not shown). Therefore, either upon transformation or in the present culture conditions, the expression of SP-B is not affected. However SP-A, SP-D, and CC10 expression is reduced by transformation of the Clara cells.

Cell Cycle Regulatory Protein Expression Analysis. An analysis was undertaken to identify the progressive changes in cell cycle regulatory proteins during the progression of lung cancer in these mice. This was initiated by Northern blot analysis of total lung RNA to identify the changes in the expression of the mRNA for p53, as well as for the cyclin-dependent kinase inhibitors. This strategy was chosen because it allowed for a rapid triage for changes in the steady-state mRNA levels of cell cycle regulatory genes. However, this approach was limited due to the heterogeneous cell
population in whole lung. Therefore, subtle changes or decreases in CDKI expression may not be detected due to masking from normal cells in the lung. Changes detected by Northern blot analysis were verified by in situ hybridization to determine the cell type expressing the specific mRNA.

This investigation began with analyzing the expression of p53 during the development of tumors in these mice. By immunohistochemistry, the expression of the p53 protein was evaluated in lungs of a 3-month-old CC10Tag mouse of the 7736 transgenic line. At low power, a section containing bronchioles and two large tumors was stained with antibodies to p53. Dense staining was observed in the Clara cells of the hyperplastic proximal airways (Fig. 4A, marked with b) and in the lung tumors (marked with f). Higher magnification shows that the p53 protein is strictly nuclear in the Clara cells in the proximal airways (Fig. 4B) and in lung tumors (Fig. 4C). The increases in p53 protein observed in the Clara cells and in the tumor may be due to a stabilization of the p53 protein by Tag (17). Northern blot analysis on the lungs from transgenic mice at 2, 3, and 4 months of age showed that p53 mRNA levels were also increased in transgenic mouse lung over that of control lung mRNA. The increase in the steady-state levels of p53 mRNA was detected in all positive CC10Tag lung (indicated by + symbol) as early as 2 months of age compared with nontransgenic lung (indicated by – symbol; Fig. 4D). Expression of Tag in the Clara cell results in increased p53 protein, as well as p53 mRNA levels in transgenic mouse lung tumors. The integrity and quantitation of total RNA were determined by 28S and 18S rRNA with ethidium bromide staining (Fig. 4D).

CDKI Expression Analysis. The CDKI p21Cip1 is transcriptionally regulated by p53 (18). This led to the initial analysis of the members of the p21 CDKI family to determine the steady-state mRNA levels by Northern blot analysis. In these analyses, total RNA was harvested from nontransgenic mouse lung at 2, 3, and 4 months of age, and from the lungs of two CC10Tag transgenic mice at 2, 3, and 4 months of age. The total RNA was subjected to denaturing electrophoreses and analyzed by Northern blot with cDNA probes for p27, p57, and p21. Expression of p27 and p57 mRNA in nontransgenic lung at 2, 3, and 4 months (indicated by –) was low (Fig. 5). A similar pattern was observed in mice positive for the CC10Tag transgene. Expression of p27 and
p57 in 2- and 3-month-old transgenic lung was low and did not significantly differ from control lung mRNA levels, but significant increases in the steady-state levels of both p27 and p57 were detected at 4 months of age (Fig. 5, B and C) in CC1OTAg lungs. However, Northern blot analysis of p21 mRNA showed that expression levels of the 2.1-kb message varied between normal and CC1OTAg lung in an irregular pattern (Fig. 5A). Longer exposure detected fainter bands of lower molecular weight, and these bands displayed a similar pattern of expression as the 2.1-kb message. From these analyses, it was observed that the expression of p21 mRNA levels did not change, whereas, the levels of p27 and p57 were elevated at the latest stage of tumor development in the CC1OTAg lung. The integrity and loading of total RNA in the agarose gel were determined by 28S and 18S staining with ethidium bromide. These analyses were performed on a single blot of Hybond N membrane, which was stripped completely of the previous probe and verified by overnight exposure to film.

In situ hybridization analysis was performed to analyze the localization of the p57 mRNA expressed in the CC1OTAg lung tumors. Fig. 6A shows a light micrographic section of 3-month-old CC1OTAg lung probed with an antisense riboprobe to p57 cDNA. Fig. 6B shows the same section as above using dark-field microscopy to localize the p57 autoradiographic silver grains. The level of p57 message was high in the bronchiole (b) and in the lung tumors (t), and at lower levels in the alveolar spaces (a). The arrows in the figure depict the boundaries of hyperplastic epithelium and lung tumor. This is in contrast to the in situ localization of p21 in both normal and CC1OTAg lung. Lower levels of p21 mRNA were shown in the tumor (t) and bronchiolar epithelium (b) of the lung compared with alveolar distal lung (a; Fig. 7, A and B). The low levels of expression of p21 in the tumors could be due either to the inactivation of p53 by TAg, leading to lower levels of p21, or to the fact that p21 may not be significantly expressed in the Clara cells of the proximal airways. To address this question, in situ hybridization analysis of normal lung was performed. These analyses showed that higher levels of p21 mRNA is expressed in the cells of the alveoli type I and type II cells (a), but is expressed at much lower levels in the proximal airways, such as the bronchioles (b; Fig. 7, C and D). Therefore, it appears that p21 is not significantly expressed in the proximal airways, and any changes in p21 mRNA levels during Clara cell transformation could not be detected in situ.

Northern blots on the INK4 family of CDKis were performed on lungs from nontransgenic and CC1OTAg mice at 2, 3, and 4 months of age. The CDKis are highly homologous; therefore, cross-hybridization between family members in this study was observed. The appropriate molecular sizes were used to determine the correct hybridized band in these analyses. To evaluate more closely the expression of the different members, DNA probes specific for p15 and p16 were generated by PCR and used for Northern blot analysis. Although these probes proved to be more specific, they were less sensitive than the full-length cDNAs (data not shown). Therefore, for interpretation of the data, the Northern blots probed with the cDNA probe are shown, despite the additional bands. The expression of these inhibitors was examined throughout a time course of 2-, 3-, and 4-month-old CC1OTAg mouse lung, and compared with wild-type mouse lung at the same ages. Two transgenic mice positive for the CC1OTAg transgene (+) and one wild-type mouse (−) were analyzed for each time point.

Using a cDNA probe for p16 and p19, changes in mRNA levels of the 1.9-kb p16 band and the 1.4-kb p19 band were not detected when total RNA from CC1OTAg lung was compared with that of normal lung at any time point (Fig. 8, B and D). In the Northern blot for p16, an additional higher molecular weight band is evident in the +CC1OTAg mice at 3 and 4 months of age (Fig. 8B). This additional band corresponds to cross-hybridization with p15 mRNA, because subsequent analysis showed a single band of 1.9 kb when probed with the p15 PCR-derived probe. Analysis of the other members of the INK4 family was performed, and additional changes were detected in the CC1OTAg lung. Expression of p18 mRNA was elevated at 4 months of age and was slightly elevated in one of the lungs from a 3-month-old CC1OTAg mouse. In this blot, an additional band of 1.4 kb was seen in all the lung samples analyzed, including control nontrans-
genic and CC10Tag lung. This band corresponds to the correct molecular weight of p19 and is due to cross-hybridization between these family members. This result coincides with the Northern blot analysis using a probe for p19, and this band does not change in intensity throughout the course of the disease.

A distinct pattern of expression was observed for the CDKI p15. A major band at 2.2 kb was detected for p15 in all of the samples evaluated; however, an increase in the intensity of this band was observed at the earliest time point evaluated (2 months), and this increase persists throughout the progression of the disease (Fig. 8A, arrow). It is interesting that an additional band was observed in the 3- and 4-month-old mouse lung at a lower molecular weight than the major p15 band at 2.2 kb. Whether this band is due to cross-hybridization with a yet unidentified CDKI family member or due to changes in the p15 gene is yet to be determined. From these analyses, the expression of both p15 and p18 increases through the progression of the tumor growth in the CC10Tag mouse lung, with p15 levels increasing early, and the other CDKI levels increasing later in tumorigenesis.

In situ hybridization was performed with an antisense riboprobe for p15 cDNA in 3-month-old CC10Tag lung to confirm the Northern blot analysis (Fig. 9, A and B). In situ hybridization with a riboprobe for p15 demonstrated that although the levels of p15 in distal alveoli (a) was high, increases in p15 could be specifically detected in lung tumors (t) and in the hyperplastic bronchiolar epithelium (b).

**Discussion**

In this report, cell-specific expression of SV40 Tag was used to create an animal model for Clara cell transformation and to characterize the molecular events that occur upon transformation throughout the progression of lung cancer in these mice. This animal model is advantageous in that both lung and Clara cell-specific transformation is achieved, which is in contrast to other lung cancer models. The rabbit uteroglobin promoter, the rabbit CC10 homologue, was used to drive the expression of Tag to the lungs. In the latter mouse model, tumors in lung, as well as in secretory epithelium such as gastrointestinal tract and prostate, developed (19, 20). The oncogene SV40 Tag is a potent stimulator of proliferation. Tag has been shown to bind and inhibit the cell cycle regulatory proteins p53 and Rb (12), which confer a significant
growth advantage over non-TAg-expressing cells. Because TAg binds to cell cycle regulatory proteins to deregulate the cell cycle, it serves as a useful tool to investigate transforming effects on other cell cycle regulatory molecules, such as the CDKIs. Using this animal model, changes in the expression of regulatory proteins could be investigated during the progression of the disease in situ. This allows the development of models describing the molecular events that occur in cells in response to specific oncogenic insults.

This animal model was analyzed for the expression of the Clara cell marker protein CC10 in the CC10TAg lung. Expression of CC10 was normal in nontransformed Clara cells in the pulmonary epithelium (Fig. 3) and is reduced upon transformation. Upon transformation, multiple events are suffered, and the development of tumors is induced. From this study, several molecular changes were identified throughout the progression of the lung cancer in the CC10TAg mouse model. The evaluation of lung marker proteins, such as the SPs, demonstrates the changes in gene expression that transformed Clara cells undergo. Normal nontransformed Clara cells produce SP-A, SP-B, and SP-D, but upon transformation, these cells lose the ability to express SP-A and SP-D; however, high levels of SP-B are maintained. Since changes in marker proteins were detected, this analysis was conducted to determine changes in the expression of cell cycle regulatory proteins.

This model was used to identify the Clara cells’ response to oncogenic insult by TAg. One of the first changes that was detected in this model was the increase in p53 protein and mRNA in the lung tumors. As early as 2 months of age, before the development of lung tumors in the CC10TAg mouse, an increase in the steady-state levels of p53 is observed (Fig. 4D) in the CC10TAg lung. Because p53 is capable of detecting DNA damage, it can be inferred that expression of TAg is inducing multiple events at the DNA level. The role of p53 in the maintenance of the Clara cell differentiation is yet to be determined.

Using Northern blot analysis, it was determined that the levels of the CDKIs p15, p18, p27, and p57 mRNA levels increased, whereas changes in the levels of mRNA expression of other family members, such as p21, p16, and p19, were not detected. The CDKIs that did not increase may indicate that they are not involved in Clara cell differentiation.
or that the sensitivity of this assay was not sufficient to detect these changes. Further study on protein expression in the CC10Tag lung may help this evaluation. It is interesting that one CDKI, p15, was shown to increase early, before tumors are detected by gross morphological analysis. The early increase may indicate that a mechanism involving p15 is induced in the Clara cell to control cell proliferation upon initial disruption of the cell cycle. Additionally, the appearance of a lower molecular weight band in the p15 analysis may indicate a rearrangement of the p15 gene, leading to a smaller mRNA species. However, further analysis is required to fully understand the changes in p15 expression that occur throughout Clara cell transformation.

The later increases in other members may suggest a hierarchy of cell cycle control, in which early transformation involves p15 and later transforming events recruit other members of the CDKI families for cell cycle control. In addition, elevated levels of p15 mRNA may be used as a potential marker for transformation of cells of the proximal epithelium at early stages of oncogenesis. The events identified in this study may reflect the individual activities of the CDKIs themselves.

The p21 family has been shown to have a global inhibition of CDKs, demonstrating an ability not only to inhibit CDK4 and CDK6, but also CDK2 (reviewed in Refs. 13 and 14). Expression of p21Gaz has been shown to be induced not only by p53 (18), but also by p53-independent mechanisms (21). In situ hybridization of sections of the CC10Tag tumors showed that p21 levels were lower compared with cells in the distal pulmonary airways (Fig. 7, A and B). Evaluations of proximal airways of normal lung show that p21 mRNA is also low (Fig. 7, C and D). These findings may indicate that p21 may not be the predominant regulator of differentiation of the cells in the proximal epithelium; however, the high levels of p21 mRNA in the distal airways may indicate that p21 is important in the maintenance of the type I and II alveolar cells, which are the predominant cell type in this region. On the other hand, p57 is increased in the Clara cell-derived tumors compared with alveolar epithelium (Fig. 6, A and B). These data concur with the localization of p57 in lung, where p57 mRNA is localized to the bronchiolar epithelium at higher levels than the distal alveolar epithelium, indicating its importance in the differentiation pathways of these cell types (22).

This study highlights the importance of transgenic animals in the study of pulmonary oncogenesis. (a) Lung tumors can be generated that originate from a specific cell type, in this case allowing the mechanisms of differentiation of the Clara cell type to be investigated. (b) By conducting an in vivo analysis of cell cycle gene expression, cell cycle regulator gene expression was initially shown to increase. This is apparently contradictory to the findings of other researchers who have characterized the mutations and losses of p16 and other CDKIs in the development of non-small cell lung cancer in numerous lung cancer cell lines (23, 24). The difference may be resolved by the fact that the later studies were performed on specimens taken from human lung tumors and in vitro, on cell cultures of lung tumors. Thus, these samples represent late events in tumorigenesis and therefore molecular changes before tumor development would not be detected. The current analysis, using an in vivo model, has identified events early in cell transformation and during tumor progression. In this in vivo animal study, the elevated levels of several cell cycle control molecules may indicate that the Clara cells maintain homeostatic regulation of the control of cellular differentiation in response to oncogene insult by inducing expression of the CDKIs along with p53 as early events in tumorigenesis.

An important caveat to the present study is that it was designed to identify increases in mRNA expression. Subtle changes or decreases in cell cycle gene expression were not identified. The question of functionality of the proteins generated from the elevated levels of the CDKI mRNA must be determined. This may prove crucial to understanding the role these molecules play, since it was recently shown that a major regulatory mechanism of CDKI action is mediated through Ubiquitin (25). When this potential candidate of regulation of Clara cell differentiation in this transformation model has been identified, further analysis will be required to determine how these CDKIs influence Clara cell development in normal lungs.

This study involves the generation of an animal model for Clara cell transformation and characterization of specific molecules involved in cell cycle control of transformed Clara cells. Identification of molecules that maintain Clara cell differentiation may lead to gene targets for therapy of lung cancer arising from the Clara cell. Once a gene therapy strategy is designed, the CC10Tag mouse model will serve as a useful tool to test these methods in vivo in an animal predisposed to the development of lung cancer.

Materials and Methods
Generation of mCC10Tag Transgenic Mice. The mCC10Tag transgene was made by fusing the coding sequences of the SV40 TAg gene with 2.1 kb of upstream 5' flanking sequences of the mCC10 promoter (Ref. 10; see Fig. 1A). Briefly, a BglI-BamHI (Promega, Madison, WI) fragment of the SV40 TAg was cloned into the EcoRI site in the multicloning region of pBluescript (pSK-); Stratagene, La Jolla, CA) producing the starter plasmid pSKTag. A 2.1-kb EcoRI fragment of the mCC10 5' flanking region was cloned into the EcoRI site of pSKTag upstream of TAg. The transgene was liberated from vector by a digestion with NotI and KpnI. This generated a transgene fragment of 5.0 kb. The transgene fragment was gel purified and isolated using the Qiaex (Qiagen, Inc., Chatsworth, CA) protocol for isolation of DNA from agarose. The transgene was microinjected into one-cell-fertilized FvB mouse embryos at a concentration of 2 ng/ml (26).

Transgenic founders positive for the transgene were identified by screening genomic Southern blots from DNA isolated from tail biopsies (27). Briefly, genomic DNA from each potential F1 was digested overnight with HindIII (which liberates a 1.1-kb fragment of the transgene) and electrophoresed through a 1% agarose gel. The digested DNA was then transferred to Zetaprobe (Bio-Rad) membrane and hybridized with a HindIII DNA probe specific for the SV40 TAg coding sequences (see below for preparation of probe) by the method described by Church et al. (28). The blots were then washed and subjected to autoradiography to identify the transgenic F1 founder mice.

Analysis of mRNA Expression. Expression of the mCC10Tag transgene and CDKIs were performed by Northern analysis. Total RNA was isolated from mouse lung and other tissues from both CC10Tag and nontransgenic mice at 2, 3, and 4 months of age using a phenol-chloroform extraction of a guanidinium preparation from RNASol (BioTec, Houston, TX; Ref. 29). Ten µg of total RNA were subjected to denaturing electrophoresis in a 2.2 M formaldehyde agarose gel and transferred to
Hybond N (Amersham, Arlington Heights, IL) nylon membrane. Membranes were prehybridized in prehybridization solution [45% formamide, 4× SSC, 0.1× sodium phosphate (pH 6.5), 5× Denhardt’s, 0.1% sodium pyrophosphate, 0.1% SDS, and 250 mg/ml salmon sperm DNA] for 1-5 h at 42°C. Membranes were then hybridized with 2 × 10^6 total counts of an [α-32P]dCTP-labeled DNA probe. Hybridizations were performed for 18 h at 42°C in 10 ml of hybridization buffer [45% formamide, 4× SSC, 0.1× sodium phosphate (pH 6.5), 1× Denhardt’s, 0.1% sodium pyrophosphate, 0.1% SDS, 100 mg/ml salmon sperm DNA, and 10% dextran sulfate]. After hybridization, the blots were washed at 60°C in 1× SSC and 0.1% SDS for a total of three 15-min washes. The blots were then subjected to autoradiography for 1-4 days to analyze the expression of mRNA. Membranes were stripped of radiolabeled probe by boiling in 0.1% SDS for 20 min. To ensure complete stripping, membranes were subjected to autoradiography for 24 h after subsequent hybridization. Northern blot analysis for each probe was repeated at least twice to ensure reproducibility.

Full-length cDNA probes for CDKIs p15, p16, p18, and p19 were obtained from Dr. Charles J. Sherr (St. Jude’s Hospital, Nashville, TN). The cDNA probes for p21, p27, and p57 were obtained from Dr. Wade Harper and Dr. Steven Elledge (Department of Biochemistry, Baylor College of Medicine). A cDNA probe for p53 was obtained from Dr. Gorgo Lopez (M. D. Anderson Cancer Center, Houston, TX). The 1.1-kb cDNA probe for SV40 TAg was isolated by a restriction digest with HindIII. The cDNA probe for p53 was subjected to restriction digest with KpnI to isolate a 0.65-kb fragment. cDNA probes for p21 and p57 were digested with XhoI, and probe for p27 was digested with SacI and EcoRI, to yield fragments of 0.75, 0.5, and 0.5 kb, respectively. A cDNA probe fragment for p15 was digested with XhoI, p16 was digested with EcoRI, and p18 and p19 were restriction digested with BamHI and EcoRI, yielding DNA fragments of 1.3, 1.0, 0.5, and 0.5 kb, respectively. The fragments labeled for the CDKI probes contained the coding regions for these respective probes. The cDNA fragments were subject to gel electrophoresis through a 1% low melting point agarose gel (Sea Plaque, FMC Bioproducts, Rockland, ME) to separate them from vector. The cDNA fragment was liberated from agarose using the protocol of Qiagen (Chgain). Approximately 50 ng of DNA were labeled with [α-32P]dCTP to a specific activity of 0.8-2 × 10^6 cpm using random hexamers as primers (Pharmacia, Milwaukee, WI).

Immunohistochemistry. Tissues were fixed in 10% buffered formalin and in 96% alcohol and 4% glacial acetic acid, and stained using either anti-CC10 (30), anti-TAg or anti-p53 (Novocastra Laboratories, Ltd., Newcastle Upon Tyne, UK) antibodies and citrate enhancement with microwave heating for antigen retrieval. Immunohistochemical staining for CC10, TAg, or p53 was accomplished by using immunoperoxidase staining as described by Sepulveda et al. (31). Briefly, purified rabbit anti-CC10, anti-TAg, or anti-p53 antibodies were used to detect TAg or p53 protein, respectively. Biotin-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) were used as secondary antibody. The sections were penetrated with 0.3% pepsin (Sigma Chemical Co., St. Louis, MO) for 12 min at 37°C. The endogenous peroxidase activity was blocked with 3% H2O2 in methanol for 10 min at room temperature. The slides were blocked with normal goat serum, diluted primary antibody (in 0.2% BSA, 0.1% sodium azide in PBS) was added, and the slides were incubated for 3 h at 42°C. The slides were rinsed with PBS, and diluted biotinylated secondary antibody was added. Incubation with the 2nd antibody was performed for 30 min at room temperature. The slides were rinsed, and the peroxidase activity was detected using the ABC kit (Vector Laboratories). The peroxidase activity was enhanced with NiCl2-enhanced diaminobenzidine. The sections were then counterstained with Nuclear fast red and examined. Nontransgenic mouse lung sections were analyzed concurrently as normal lung control, and to control for specific protein staining, immunohistochemistry was performed in the absence of the anti-CC10, anti-TAg, or anti-p53 primary antibody, respectively.

In Vitro Hybridization. In situ hybridization was performed as described by Cox et al. (32) with slight modifications. Lung was excised under sterile conditions and fixed in 4% paraformaldehyde in PBS overnight at 4°C. The tissue was then dehydrated and embedded in paraffin. Sections were cut and mounted on silane-coated (3-aminopropyltriethoxysilane) slides. According to the method of the Riboprobe in Vitro Transcription System (Promega), [35S]UTP-labeled RNA probes were prepared from the specific CDKI probes. The antisense and control sense probes were generated by linearizing the plasmids with the specific restriction endonucleases. Slides were deparaffinized, hydrated, and de-gested with 20 mg/ml proteinase K in 50 mM Tris (pH 8.0) and 5 mM EDTA for 10 min. Afterward, sections were washed with PBS, and slides were post-fixed with 4% paraformaldehyde, treated with 0.25% acetic anhydride, washed in PBS, and then dehydrated. Slides were hybridized with riboprobe at 55°C overnight, then washed in 2× SSC containing 50% formamide and 50 mM m-mercaptoethanol at 65°C for 30 min. Sections were then treated with RNase A in 0.5 μg/ml NaCl, 10 mM Tris (pH 7.5), and 5 mM EDTA at 37°C for 30 min. Slides were then washed with 0.1× SSC and dehydrated. For signal detection, the slides were exposed to Amersham Hyperfilm (Amersham) and then dipped in Kodak NTB-2 liquid emulsion (Eastman Kodak, Rochester, NY) for autoradiography for 4–10 days.

Acknowledgments
We thank Louise A. Hadsell, John D. Stockton, and Joyce L. Pike for generating and screening the transgenic mice, and Angela Majors for immunohistochemical analysis. We also thank John Ellsworth for preparation of this manuscript.

References


