Simian Virus 40 Large T Antigen Directed by Transcriptional Elements of the Human Surfactant Protein C Gene Produces Pulmonary Adenocarcinomas in Transgenic Mice

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ABSTRACT

A model of pulmonary adenocarcinomas was produced in transgenic mice harboring a chimeric gene comprising the SV40 large T antigen under the control of a transcriptional region derived from the human surfactant protein C (SP-C) gene. Transgenic mice succumbed with pulmonary tumors within 4-5 months of age. By histology, the tumors were adenocarcinomas with lepidic, papillary, and solid growth patterns that were indistinguishable from adenocarcinomas occurring in humans. Immunocytochemistry demonstrated the lack of staining for neuroendocrine markers, consistent with the identification of the tumors as non-small cell rather than small cell carcinomas. The presence of SV40 large T mRNA in the lung and tumors was detected by in situ hybridization and Northern blot analysis. Exogenous SV40 large T mRNA and endogenous CC10 (a nonciliated respiratory epithelial cell marker) and SP-C (a Type II alveolar cell marker) mRNAs were expressed at variable levels in the lung tumors. SV40 large T mRNA and CC10 mRNA were detected in the majority of tumors, while SP-C mRNA was detected less frequently. The heterogeneity of bronchiolar and alveolar cell markers in the tumors from the transgenic mice supports the concept that tumorigenesis was initiated in distinct subsets of epithelial cells that produce characteristic adenocarcinomas of the lung.

INTRODUCTION

Lung cancer is the most frequent cause of cancer deaths in men and women in the United States and is associated with a 90% death rate within the first year of diagnosis (1, 2). Lung cancer now accounts for 34% and 21% of cancer deaths in men and women, respectively (2). Pulmonary tumors are divided into small cell and non-small cell lung cancers for biological and clinical reasons. Non-small cell lung cancers comprise approximately 75% of all lung tumors and include large cell, squamous cell, and adenocarcinoma cell types (1, 3, 4). Pulmonary adenocarcinomas are classified into four subtypes: acinar; papillary; bronchioloalveolar; and solid (4). Adenocarcinoma is the most frequent lung cancer in nonsmokers and women (3, 5), and the incidence of adenocarcinomas is increasing in the United States (6). Diagnosis of pulmonary adenocarcinoma is complicated by the lack of clinical signs and symptoms until the disease is well advanced. Pulmonary adenocarcinomas are also generally refractory to conventional antitumor therapies. Although a definitive cell of origin has not been identified, the four subtypes of adenocarcinomas are thought to be derived from epithelial cells lining the peripheral regions of the respiratory tract, including mucus-producing cells, nonciliated bronchiolar (Clara) cells, and Type II alveolar cells (5, 7).

SV40\(^3\) large T antigen has been used to produce tumors in transgenic mice by regulating its expression with a variety of transcriptional elements (8). SV40 large T antigen binds the tumor suppressor gene products p53 and Rb, resulting in uncontrolled cellular proliferation (9). Mutations in Rb or p53 occur in many small cell lung carcinomas (10-12), and a high incidence of p53 mutations is found in lung cancers of the non-small cell type (12, 13). Moreover, the mutations in Rb and p53 preferentially occur in regions known to be important in forming complexes with SV40 large T antigen (9, 14, 15). The overexpression of mutant p53 results in a high incidence of lung tumors in transgenic mice, further supporting a role for p53 in lung carcinogenesis (16).

Previously, this laboratory isolated and characterized genes encoding SP-C, an abundant hydrophobic protein present in pulmonary surfactant. SP-C is closely associated with surfactant phospholipids and aids in the reduction of surface tension in the lung alveoli upon expiration (for a review, see Ref. 17). In the mouse, the SP-C gene is expressed in Type II epithelial cells lining the alveoli (18). In the human, proSP-C is present in bronchiolar and alveolar epithelial cells in the neonatal and adult lung.\(^{4}\) A 3.7-kilobase fragment derived from the 5'-flanking region of the human SP-C gene was previously used to direct expression of chimeric genes in transgenic animals in a lung epithelial cell-specific manner (18, 19). Expression of the CAT reporter gene under the control of the SP-C promoter was detected early in fetal lung development\(^5\) and was confined to peripheral epithelial cells in the adult, consistent with the localization of Type II and nonciliated bronchiolar epithelial (Clara) cells (18). The human SP-C transcriptional element was therefore chosen to express SV40 large T antigen in the lungs of transgenic mice to produce an animal model of lung adenocarcinomas derived from distal respiratory epithelial cells. Such an animal model will provide a tool for studying the development and function of the distal respiratory epithelium as well as its role in lung tumorigenesis. The transgenic mice generated consistently developed pulmonary tumors morphologically indistinguishable from that seen in human adenocarcinomas, including the solid, papillary, and bronchioloalveolar subtypes.

MATERIALS AND METHODS

Construction of the SP-C-SV40 Large T Antigen Chimeric Gene.

SP-C-TAg consisted of the S'-flanking region from the human SP-C gene fused to SV40 early region sequences. The SP-C promoter region, comprising 3701 base pairs, nucleotides (—) 3683 to (+) 18, was under the control of a transcriptional region derived from the human SP-C gene was previously used to direct expression of chimeric genes in transgenic animals in a lung epithelial cell-specific manner (18, 19). Expression of the CAT reporter gene under the control of the SP-C promoter was detected early in fetal lung development\(^5\) and was confined to peripheral epithelial cells in the adult, consistent with the localization of Type II and nonciliated bronchiolar epithelial (Clara) cells (18). The human SP-C transcriptional element was therefore chosen to express SV40 large T antigen in the lungs of transgenic mice to produce an animal model of lung adenocarcinomas derived from distal respiratory epithelial cells. Such an animal model will provide a tool for studying the development and function of the distal respiratory epithelium as well as its role in lung tumorigenesis. The transgenic mice generated consistently developed pulmonary tumors morphologically indistinguishable from that seen in human adenocarcinomas, including the solid, papillary, and bronchioloalveolar subtypes.

\(^5\) Wert, S. E., Classer, S. W., Korfhagen, T. R., and Whitsett, J. A. Transcription of the SP-C gene in the lung and tumors was detected by in situ hybridization and Northern blot analysis. Exogenous SV40 large T mRNA and endogenous CC10 (a nonciliated respiratory epithelial cell marker) and SP-C (a Type II alveolar cell marker) mRNAs were expressed at variable levels in the lung tumors. SV40 large T mRNA and CC10 mRNA were detected in the majority of tumors, while SP-C mRNA was detected less frequently. The heterogeneity of bronchiolar and alveolar cell markers in the tumors from the transgenic mice supports the concept that tumorigenesis was initiated in distinct subsets of epithelial cells that produce characteristic adenocarcinomas of the lung.

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3 The abbreviations used are: SV40, simian virus 40; Rb, retinoblastoma; SP-C, surfactant protein C; SP-C-TAg, SP-C-SV40 large T antigen construct; CAT, chloramphenicol acetyltransferase; cDNA, complementary DNA.

4 M. Stahlman and J. Whitsett, unpublished observations.

5 Wert, S. E., Glasser, S. W., Korfhagen, T. R., and Whitsett, J. A. Transcriptional elements from the human SP-C gene direct expression in the primordial respiratory epithelium of transgenic mice, submitted for publication.
obtained from Bethesda Research Laboratories (Gaithersburg, MD) and extends from the StuI to the BamHI site (nucleotides 5190 to 2533). The DNA fragment contains 29 base pairs of sequence upstream from the ATG, the initiation codon for large T antigen, and the polyadenylation signal. The SP-C-TAg plasmid was constructed by introducing a 2657-base pair StuI/BamHI SV40 DNA fragment into the BamHI site of pUC18 containing the 3.7-kilobase PstI/HindIII human SP-C promoter cloned into the HindIII site. The StuI/BamHI fragment was modified by the addition of BglII linkers (New England Biolabs, Inc., Beverly, MA) prior to ligation.

Generation and Identification of Transgenic Mice. The SP-C-SV40 large T DNA was excised from the parental plasmid by digestion with SacI and purified by agarose gel electrophoresis and CsCl gradient centrifugation in the absence of ethidium bromide. Purified DNA was dialyzed extensively in 5 mM Tris- HCl (pH 7.5)/0.1 mM EDTA prior to pronuclear injection. Donor eggs were prepared from FVB/N females. Transgene-positive mice were identified by Southern blot analysis of tail DNA using the 2.7-kilobase SV40 large T antigen DNA fragment from the original construct as the probe. The integrity of the construct was analyzed by BamHI/XbaI restriction enzyme digestion of tail DNA followed by Southern blot analysis. The abundance of the DNA was assayed by staining the agarose gel prior to transfer to nylon membranes.

Northern Blot Analysis. Northern blot analysis was performed as previously described (20). Total tissue RNA (15 μg) was isolated and separated by electrophoresis, transferred to Nytran (Schleicher and Schuell, Keene, NH), and stained with 0.02% methylene blue/0.3 N sodium acetate (pH 5.0) to ensure the presence and integrity of the RNA. Northern blots were hybridized with a nick-translated [32P]-dCTP-labeled probe consisting of the SV40 large T DNA derived from the original construct. Following hybridization, membranes were washed in 0.2× saline-sodium-phosphate-EDTA (1× saline-sodium phosphate-EDTA is 0.15 M NaCl, 0.02 M NaPO4, pH 7.4, and 0.001 M EDTA) with 0.1% sodium dodecyl sulfate at 55°C for 30 min and exposed to Kodak XAR-2 film in the presence of an intensifying screen at −80°C.

Processing of Tissues for Histopathology. Lung tissue was fixed in 4% paraformaldehyde in phosphate-buffered saline for approximately 20 h. Tissue was then washed in phosphate-buffered saline, dehydrated through a series of alcohols, and embedded in paraffin. Tissue sections (5 μm) were melted onto 3-aminopropyltriethoxysilane-coated slides and subsequently stained with hematoxylin and eosin.

In Situ Hybridization. A 2.7-kilobase SV40 large T DNA fragment was isolated from the original construct by BamHI/XbaI digestion and ligated into a pBluescript II SK vector (Stratagene, La Jolla, CA) for riboprobe synthesis. A 452-base pair rat CC10 cDNA clone in pGEM4Z (21) and a 758-base pair murine SP-C cDNA clone in riboprobe synthesis. A 452-base pair rat CC10 cDNA clone in pGEM4Z (21) and a 758-base pair murine SP-C cDNA clone in pGEM4Z (21) and a 758-base pair murine SP-C cDNA clone in System II transcription kit (Promega Biotech, Madison, WI). Riboprobe synthesis and in situ hybridization were performed as previously described (21). Riboprobes were applied to lung sections and hybridized for approximately 15 h at 55°C. Nonspecific hybridization was minimized by subjecting the tissue sections to a series of washes under the conditions previously described (21). The sections were subsequently exposed to Ilford K5 nuclear track emulsion for 1 to 11 days at 4°C prior to development with Kodak D19 developer. Tissue sections were stained with hematoxylin or hematoxylin and eosin and analyzed by bright- and dark-field illumination microscopy.

RESULTS

Generation of Transgenic Mice. A 6.4-kilobase SacI SP-C-TAg fragment was microinjected into fertilized eggs, and the eggs were transferred to surrogate dams (Fig. 1). Twenty pups survived to weaning. Three founder animals bearing the 3.7-kilobase SP-C-TAg chimeric gene were identified by Southern blot analysis of tail DNA. The integrity of the transgene was confirmed by the generation of diagnostic fragments upon restriction enzyme digestion of tail DNA and subsequent Southern blot analysis (Fig. 2). The founder mice died at 4 to 5 months of age with signs of respiratory distress. Multiple lung nodules were noted at autopsy (Fig. 3). Founder 5.1 produced offspring harboring the transgene, and the 5.1 line has been propagated for six generations in the hemizygous state to date. Animals from all subsequent generations have consistently died or showed signs of respiratory distress and were sacrificed between 4 and 5.5 months of age (over 35 animals to date). All analyses were performed on mice sacrificed at this age. Multiple lung tumors indistinguishable from the tumors seen in the founder animals were observed upon autopsy. No tumors were apparent in any other tissues upon autopsy including heart, brain, adrenal glands, liver, submandibular gland, spleen, pancreas, gastrointestinal tract, or reproductive organs (over 15 animals observed to date, including Founder 2 and animals of the 5.1 line). Founder 2 was a female and gave birth to two litters; however, all the pups died within the first day after birth. Founder 5.2 was a male and produced 38 offspring, all of which were negative for the transgene. The failure of transmission of the transgene to the offspring from this founder is most likely due to mosaicism of the founder animal, with the germline cells being free of the transgene.

Histopathology. Adenocarcinomas were detected in the lungs upon histopathological analysis of Founder 2 and four mice representing the 5.1 line. Three distinct patterns of tumor growth were noted in each mouse: (a) small foci of tall columnar cells using the existing alveolar walls for support of growth (lepidic growth) (Fig. 4A); (b) nodules of cuboidal cells with lepidic and papillary growth (Fig. 4B); and (c) solid areas consisting of pleomorphic tumor cells and necrotic debris (Fig. 4C). Neoplastic cells involving bronchioles contained large, pleomorphic nuclei when compared to normal bronchial epithelium (Fig. 4F). Reactive alveolar cell hyperplasia with marked cellular atypia was also noted in areas adjacent to the tumors (Fig. 4G). No clearly invasive properties such as vessel invasion or pleural extension were noted. Macrophages, but not lymphocytes or polymorphonuclear leukocytes, were commonly observed in the regions of tumor formation. To test whether the tumors demonstrated neuroendocrine features characteristic of human small cell lung carcinomas, sections from the lungs of transgenic animals were immunostained using the peroxidase-antiperoxidase method for the general
Tissues of two male mice representing the 5.1 line were sectioned and examined histologically in order to detect microscopic lesions. Several small lesions were noted in the brain of one mouse; however, it was impossible to determine whether the lesions represented primary foci or metastatic lesions from the lung. There were also nodules in both animals, which possibly represented metastases to mediastinal lymph nodes, but there was no normal architecture left to determine whether the tissue was lymph node or represented a lung area completely grown over with tumor. There were no significant lesions in any other tissue sections examined including heart, liver, kidney, bladder, intestines, spleen, adrenal glands, pancreas, and reproductive organs.

Expression of SV40 Large T mRNA. Tissues derived from Founder 2 and representatives of the 5.1 line were analyzed by Northern blot analysis for expression of SV40 large T mRNA. A single species migrating at 2.7 kilobases was detected at high levels in the lung and at lower levels in the brain and ovaries of Founder 2 (Fig. 5B and data not shown). High levels of SV40 large T mRNA were also detected in the lungs derived from four transgenic mice representing the 5.1 line (Fig. 5 and data not shown). No detectable message was observed in the ovaries screened from two transgenic mice representing the 5.1 line even after prolonged exposure times (Fig. 5A and data not shown). Low levels of SV40 large T mRNA were noted in all three brains analyzed from transgenic mice of the 5.1 line (Fig. 5A and data not shown). SV40 large T mRNA was also detected at low levels in one of two testes samples analyzed which were derived from two distinct transgenic mice of the 5.1 line (Fig. 5B and data not shown). All corresponding tissues derived from nontransgenic animals were negative for SV40 large T mRNA expression (Fig. 5 and data not shown).

The cellular localization of SV40 large T mRNA in the lung was determined by in situ hybridization studies utilizing a riboprobe generated from the SV40 large T DNA fragment of the original construct (Fig. 1). SV40 large T mRNA was detected in the majority of the tumors (Fig. 6, A and B). The level of SV40 large T mRNA varied among tumors and among individual cells within each tumor (Fig. 6). Despite the variability of SV40 large T mRNA expression, the mRNA was detected in tumors of all morphological types, and hybridization patterns were indistinguishable in all animals analyzed, including representatives from the 5.1 line and Founder 2.

SV40 large T mRNA was also detected in areas of the lung lacking apparent tumor formation. Discrete sites of SV40 large T mRNA were detected in bronchial and bronchiolar epithelial cells (Fig. 6, G and H). Occasionally, isolated foci of hybridization were observed in the alveolar parenchyma not involved in tumor formation; however, such foci were rare, and it was not possible to determine whether the underlying cells represented alveolar Type II cells or peripheral aspects of bronchioles or tumor (Fig. 6, E and F). More typically, the morphologically normal areas of lung parenchyma consisted of alveolar cells devoid of SV40 large T mRNA.

Expression of Cell Markers. Lung sections from the transgenic animals were analyzed by in situ hybridization using known lung epithelial cell markers in order to further characterize the cells comprising the tumor. CC10 was utilized as a distinct marker of nonciliated bronchiolar epithelial cells (Clara cells). CC10 protein has previously been localized to Clara cells in several species including human, dog, and rat (22, 23). A riboprobe generated from a rat CC10 cDNA clone was utilized as a probe for in situ hybridization studies (21). CC10 mRNA

neuroendocrine marker neuron-specific enolase as well as neurotropetides including bombesin, calcitonin and calcitonin gene-related peptide, and serotonin. No immunoreactivity was detected with antibodies to these markers in any of the tumors (data not shown), despite positive immunoreactivity to human tissue for bombesin; human, sheep, rat, and hamster tissue for calcitonin-gene related peptide and serotonin; and human, sheep, rat, and normal mouse tissue for neuron-specific enolase and calcitonin.6 These observations are consistent with the classification of the tumors as pulmonary adenocarcinomas rather than small cell carcinomas.

6 M. Stahlman and Dako Corporation (Carpinteria, CA), personal communications.
was detected in tracheal, bronchial, and bronchiolar but not alveolar epithelial cells in nontransgenic mice and in similar nonneoplastic regions in the transgenic mice (Fig. 7; Fig. 8, G and H). CC10 mRNA was detected in the majority of tumor nodules, as well as in tumors with the lepidic growth pattern (Fig. 7, D, E, G, and H). Similar to the distribution of SV40 large T mRNA, the levels of CC10 mRNA varied among tumors and individual cells within the tumors (Figs. 7 and 8). Discrete nodules lacking detectable CC10 mRNA were also noted, although rarely (Fig. 7, G and H; Fig. 8, G and H).

SP-C mRNA was utilized as a marker for Type II epithelial cells. Endogenous SP-C mRNA was detected with a riboprobe generated from a murine SP-C cDNA (18). SP-C mRNA was detected exclusively in the alveolar region of nontransgenic mice as previously reported (Fig. 7, B and C) (18). In morphologically normal tissue of lung sections derived from transgenic animals, SP-C mRNA was detected in discrete cells in the lung parenchyma in a pattern consistent with the localization of Type II cells (Figs. 7 and 8). This pattern of expression of SP-C mRNA in the transgenic mice was indistinguishable from that seen in nontransgenic animals. High levels of SP-C mRNA were noted in several tumors (Fig. 7, H and I), although the majority of tumors were devoid of detectable SP-C mRNA (Figs. 7 and 8). Heterogeneity in levels of SP-C mRNA was noted among individual cells comprising the tumor nodules (Fig. 8, E and F).

The expression patterns of SP-C and CC10 in the pulmonary tumors were compared by probing serial lung sections with the respective riboprobes. Single tumor nodules with detectable levels of both CC10 and SP-C mRNA expression were observed (Fig. 8, D–F). In addition, SP-C mRNA was detected in tumors devoid of CC10 mRNA (Fig. 7, G–I), and CC10 mRNA was detected in tumors lacking detectable SP-C mRNA (Fig. 7, G–I; Fig. 8, A–C). Rare tumors lacking hybridization for both CC10 and SP-C mRNA were also observed (Fig. 8, G–I). The different expression patterns of CC10 and SP-C mRNA were not correlated with the histological growth patterns of the tumors, and similar patterns of expression were noted in tumors derived from Founder 2 and two animals of the 5.1 line.

Immunostaining of nontransgenic animals with rabbit anti-human proSP-C demonstrated reactivity in alveolar lining cells in a pattern consistent with the localization of Type II cells and in agreement with the SP-C mRNA observed by in situ hybridization (data not shown). Anti-proSP-C staining was not detected in the lung tumors of two transgenic mice representing the 5.1 line, despite reactivity in the normal-appearing parenchymal regions (data not shown). Likewise, immunoreactivity to SP-A or SP-B (surfactant proteins normally detected in bronchiolar and Type II cells) was not detected in any of the lung tumors (data not shown).

DISCUSSION

Expression of SV40 large T antigen under the transcriptional control of 3.7 kilobases of 5' sequence from the human SP-C gene produced pulmonary adenocarcinomas in transgenic mice. Histopathological analysis of the lungs revealed papillary, lepidic, and solid tumor growth patterns indistinguishable from that seen in human adenocarcinomas. The cellular morphology and lack of immunoreactivity to neuroendocrine small cell carcinoma markers further support the classification of the tumors as non-small cell lung adenocarcinomas.

Expression of SV40 Large T mRNA. SV40 large T mRNA was detected in the lung, brain, and reproductive organs by Northern blot analysis. Localization of mRNA to tissues other than lung was unexpected, since strict lung specificity was observed in transgenic mice bearing the 3.7-kilobase SP-C-CAT chimeric gene (18). Transgene expression was detected in the
Fig. 4. Histopathological analysis of lungs from transgenic mice. Lung tissue was fixed in paraformaldehyde and subsequently embedded in paraffin. Sections were analyzed after staining with hematoxylin and eosin. Three types of morphological growth patterns were noted in the lungs derived from the transgenic mice. A, tumor growth in small foci of tall columnar cells with a lepidic (scale-like) growth pattern using the existing alveolar walls for support. Bar, 100 µm. B, tumor nodules with lepidic and papillary growth patterns composed of cuboidal cells. Bar, 100 µm. C, solid areas of tumor growth with pleomorphic cells and necrotic debris. Bar, 50 µm. D, normal peripheral lung from a nontransgenic mouse depicting two bronchioles and normal alveolar parenchyma. Bar, 100 µm. E, normal peripheral lung from a nontransgenic mouse depicting a high-power view of normal alveolar parenchyma. Note the thinness of the alveolar septae. Bar, 50 µm. F, neoplastic cells involving a bronchiole in a transgenic mouse. Note the large, pleomorphic nuclei of the neoplastic cells on the right as compared to the nuclei of the more normal (arrow) appearing bronchial epithelial cells. Bar, 50 µm. G, reactive alveolar cell hyperplasia and tumor cells in a transgenic mouse. Note the contrast of the reactive and neoplastic alveolar septae with the thin, morphologically normal appearing alveolar septae in the transgenic mice (arrows) and nontransgenic mice (E). Bar, 50 µm. The figure is representative of the lungs from Founder 2 and four animals of the 5.1 line.
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Fig. 5. Tissue distribution of SV40 large T mRNA in transgenic mice by Northern blot analysis. Total tissue RNA was subjected to Northern blot analysis and probed with the XbaI/BamHI SV40 large T DNA fragment of the original construct. A, total tissue RNA from a transgenic mouse representing line 5.1 (Tg+) and total lung RNA from a nontransgenic littermate (Tg-). Tissues are as indicated. Sm./int., small intestine; Sub./Man., submandibular gland. SV40 large T mRNA migrating at 2.7 kilobases was detected in lung, brain, and resected tumor from transgenic mice. No detectable message was observed in lung from the nontransgenic mice. Radiographie film was exposed for 5 h. A similar analysis was done on tissue derived from Founder 2, and SV40 large T mRNA was detected in lung, tumor, brain, and ovaries. B, total tissue RNA derived from Founder 2 (F.2) and a representative from line 5.1 (5.1). SV40 large T mRNA was detected in the ovary of Founder 2 and testes and lungs of mice from line 5.1. Radiographie film was exposed for 20 h. A similar analysis was done on the brain tissue of three mice, the ovary tissue of two mice, and the testes tissue of an additional mouse of the 5.1 line. SV40 large T mRNA was detected in all of the brain samples, but no detectable message was observed in the two ovary samples or additional testes sample analyzed.

The levels of SV40 large T mRNA varied among the distinct pulmonary adenocarcinomas generated in the transgenic mice. While SV40 large T antigen mRNA was detected in most tumors, the mRNA was also detected in nonneoplastic epithelial cells. Such heterogeneity of expression of SV40 large T antigen was noted in tumors of other transgenic mouse model systems (25–28). Nonneoplastic lung epithelial cells expressing the SV40 large T mRNA may represent distinct cell populations resistant to the effects of SV40 large T antigen. Alternatively, these cells may require additional events for the initiation of tumorigenesis and thus represent cells with premalignant changes which are not detected morphologically. This model system may, therefore, be helpful in studying the progression of tumorigenic events leading to the development of pulmonary adenocarcinomas.

Expression of Epithelial Cell Markers. Pulmonary adenocarcinomas of all subtypes are thought to originate from the cells of the peripheral airway epithelium, namely Clara, mucinous, and Type II epithelial cells. The assignment of cellular origin to human pulmonary adenocarcinomas has been based upon morphological, ultrastructural, and immunological analysis of resected tumors and biopsy specimens. These types of analyses are complicated by the variability in cell phenotype in the distal regions of the respiratory tract. Type II cells are characterized by the presence of lamellar bodies; however, Clara cells also contain lamellar bodies indistinguishable from those of Type II cells after exposure to carcinogens (29). In addition,
Fig. 6. Cellular localization of SV40 large T mRNA in transgenic mice using in situ hybridization. Lung sections derived from transgenic mice were analyzed by in situ hybridization using a riboprobe generated from the Xbal/BamHI SV40 large T DNA fragment of the original construct. Dark-field illumination photomicrographs are shown to depict localization of signal (left), and bright-field illumination photomicrographs are shown to depict morphology (right). The figure is representative of the lungs derived from Founder 2 and two transgenic mice of the 5.1 line. In A and B, SV40 large T mRNA was detected in the majority of the tumors and was not correlated with the growth pattern of the tumor. Heterogeneity in the levels of expression was observed among the individual tumors. Regions of normal appearing tissue devoid of expression were also noted. Bar, 3 mm. In C and D, low levels of SV40 large T mRNA were detected in some of the tumors. Expression at the cellular level was heterogeneous, with some cells lacking a detectable signal and a small number of cells with high levels of detectable signal. Bar, 300 μm. In E and F, SV40 large T mRNA was detected in alveolar parenchymal cells outside the immediate regions of tumor growth (arrowhead). Such foci were rare, and it was impossible to determine whether they represented normal alveolar cells or distal aspects of tumors. SV40 large T mRNA was also detected at variable levels in cells comprising tumors with a lepidic growth pattern (arrow). Bar, 300 μm. In G and H, distinct foci of SV40 large T mRNA were localized to epithelial cells lining bronchi uninvolved in tumor growth. No signal over background levels were observed in accompanying vessels (top). A tumor nodule with a papillary growth pattern expressing high levels of SV40 large T mRNA is also shown (left). Bar, 300 μm.
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Fig. 7. Cellular localization of CC10 and SP-C mRNAs in transgenic and nontransgenic mice. Lung sections derived from transgenic and nontransgenic mice were subjected to in situ hybridization analysis. Serial sections were probed with a riboprobe generated from a rat CC10 cDNA (left) or a murine SP-C cDNA (right). Dark-field illumination photomicrographs are shown to depict localization of signal (left and right), and bright-field illumination photomicrographs are shown to depict morphology (center). The figure is representative of the lungs derived from Founder 2 and two transgenic mice of the 5.1 line. A, CC10 mRNA localized to the bronchial and bronchiolar epithelium in nontransgenic mice. No signal above background was detected in the alveolar regions, even after overexposing the sections. B, bright-field illumination photomicrograph corresponding to A and C. Arrow, a bronchus in the hilar region of the lung; arrowhead, a branching terminal bronchiole. Bar, 2 mm. C, SP-C mRNA localized to the alveolar epithelium in nontransgenic mice. No signal above background was detected in the bronchial or bronchiolar epithelium even after overexposure. D, CC10 mRNA was detected in the majority of the tumors in the transgenic mice. The levels of CC10 mRNA were heterogeneous among distinct tumors and did not correlate with the morphological growth pattern. E, bright-field illumination photomicrograph corresponding to D and F. Bar, 3 mm. F, SP-C mRNA was not detected in the majority of the tumors in the transgenic mice. The expression pattern of SP-C mRNA in the morphologically normal alveolar regions of the lung was indistinguishable from that observed in nontransgenic mice. G, CC10 mRNA expression was heterogeneous among distinct tumors and among cells comprising individual tumor nodules. High levels of CC10 mRNA were detected in some tumors (tumor on left) adjacent to tumors with low or no signal above background levels (tumor on right). CC10 mRNA was detected in morphologically normal bronchiolar regions of the lung in a pattern indistinguishable from that observed in nontransgenic mice. Note the contrast in expression patterns with SP-C mRNA in both the tumor and nontumor cells (compare with I). H, bright-field illumination photomicrographs corresponding to G and I. *, A bronchiole. Bar, 1 mm. I, SP-C mRNA was detected in a minority of the tumors of the transgenic animals (tumor on right). Adjacent tumors (tumor on left) and bronchiolar structures were devoid of signal above background levels. SP-C mRNA was detected in morphologically normal alveolar regions surrounding tumors. Note the contrast in expression patterns with CC10 mRNA in both the tumor and nontumor cells (compare with G).

Lamellar bodies have been observed in tumors of the Clara cell type (30). Immunological identification is also complicated by the fact that Clara cells and Type II cells share many antigenic markers. The surfactant proteins previously believed to be expressed only in the alveolar epithelium were localized to the bronchiolar epithelium in several species (17, 31, 32). The CC10 and SP-C cell markers utilized in the present study were restricted to bronchiolar and alveolar cells, respectively, in the mouse (18, 21). The restricted localization of CC10 and SP-C was also maintained after oxygen-induced lung injury (21). Therefore, the presence of CC10 or SP-C mRNA distinguished bronchiolar versus alveolar epithelial cell type in the normal mouse lung and distinguished subsets of the pulmonary tumors produced in the SP-C-SV40 large T antigen transgenic mice.

In the present study, CC10 mRNA was detected in the majority of pulmonary tumors. SP-C mRNA but not proprotein was detected in some of the tumors, although much less frequently than CC10 mRNA. The discrepancy between the detection of SP-C mRNA and the lack of proSP-C immunoreactivity in the tumors may relate to the higher sensitivity of in situ hybridization analysis as compared to immunocytochemistry or to posttranscriptional events required for SP-C synthesis, processing, or storage. Nonetheless, the presence of SP-C and CC10 mRNAs in the tumors supports the hypothesis that both Type II and Clara cells or precursor cells committed to these

7 K. Wikenheiser and J. Whitsett, unpublished observations.
Fig. 8. CC10 and SP-C mRNA expression patterns in lung tumors of transgenic mice. Lung sections derived from transgenic mice were subjected to in situ hybridization analysis. Serial sections were probed with a riboprobe generated from a rat CC10 cDNA (left) or a murine SP-C cDNA (right). Dark-field illumination photomicrographs are shown to depict the localization of signal (right and left), and bright-field illumination photomicrographs are shown to depict morphology (center). The figure is representative of lungs derived from Founder 2 and two transgenic mice of the 5.1 line. A-C, CC10 mRNA (A) was detected in tumors devoid of detectable SP-C mRNA above background levels (C). SP-C mRNA was detected in morphologically normal alveolar cells surrounding the tumor nodules (C). D-F, CC10 (D) and SP-C mRNA (F) were colocalized in some tumors. The levels of CC10 and SP-C mRNAs were heterogeneous among cells comprising an individual tumor. Bar, 500 μm (E), magnification in all panels. G-I, CC10 (G) and SP-C (I) mRNAs were both absent in rare tumors. The lack of signal in the tumor was evident despite expression of CC10 mRNA in morphologically normal bronchiolar epithelium (G and H, arrow) and SP-C mRNA in morphologically normal alveolar epithelium (I and H, arrowhead).

Comparison of Mouse Model to Human Pulmonary Tumors. The heterogeneity in tumor growth patterns and expression of cell markers observed in the present study are similar to the findings in human pulmonary adenocarcinomas. The CC10 protein was found in 10% of human non-small cell lung tumors, and nearly one-half of peripheral adenocarcinomas with ultrastructural features of Type II and/or Clara cells were immunoreactive for CC10 protein (23). In situ hybridization analysis of human non-small cell lung tumors demonstrated CC10 mRNA only rarely; however, the tumors analyzed were not selected with regard to specific tumor type or ultrastructural characteristics (33). Surfactant proteins are expressed in human pulmonary adenocarcinomas with variable incidence and levels (30, 33–35). SP-A mRNA or protein and SP-B mRNA were detected in a small fraction of cell lines established from human adenocarcinomas with ultrastructural features of Clara cells or Type II epithelial cells (35). SP-C mRNA was detected in a single cell line (H-820), but only after exposure to dexamethasone (35). In contrast, SP-A mRNA was detected more frequently in pulmonary adenocarcinomas by in situ hybridization analysis of lung tissue sections (33). The lack of SP-A or SP-B protein in lung tumors seen in the present study contrasts with the previous findings in human pulmonary adenocarcinomas wherein SP-A expression was readily detected (34), raising the possibility that SV40 large T antigen and subsequent cell transformation may alter SP-A and SP-B protein expression. The variable incidence and levels of peripheral airway cell markers in human lung adenocarcinomas support previous morphological observations demonstrating the heterogeneity of the cells comprising these tumors (5, 7). The mixed cell populations observed in human adenocarcinomas are in accordance with the hypothesis that lung tumorigenesis results from the transformation of a pluripotent progenitor cell (36). This hypothesis was based upon the “unitarian theory” of the origin of lung epithelial cells, wherein stem cells differentiate into an intermediate, differentiation-committed cell capable of differentiating along multiple pathways before terminal differentiation. Transformation of a subpopulation of cells in the intermediate stage of differentiation could result in clonal tumors expressing cell markers of several distinct but closely related
cell types. The current tumor model may, therefore, represent transformation of cells at distinct times in the differentiation process. The 3.7-kilobase SP-C promoter enhancer region is active in Clara cells and Type II cells, both known to serve as progenitor cells of the distal respiratory epithelium in the adult lung (18, 37). The tumors produced in the 3.7-kilobase SP-C-SV40 large T antigen transgenic mice may, therefore, result from the transformation of precursor cells prior to terminal differentiation or transformation of differentiated cells in which SV40 large T antigen is continually expressed.

SV40 Large T Antigen Transformation. Unlike most other oncocenes, SV40 large T antigen is sufficient to establish and transform primary cells in culture (38). In the present work, the observation that only a subpopulation of cells expressing SV40 large T antigen undergoes tumor formation may be explained by the differential expression of the cellular proteins required for SV40 large T antigen-mediated transformation. Binding of SV40 large T antigen to the cellular protein p53 is believed to be a critical factor in cell transformation (9, 39). It is, therefore, possible that expression patterns of p53 could affect the transformation potential of SV40 large T antigen in the lung. Schmid et al. (40) recently reported that p53 mRNA expression is temporally and spatially regulated in the embryonic mouse lung, appearing in the bronchiolar epithelium on gestational days 14.5 to 16.5. This pattern of expression correlated with the temporal and spatial expression of both CC10 and the 3.7-kilobase SP-C-directed CAT mRNA in fetal mouse lung. The present transgenic mouse model may, therefore, be useful to determine the function of p53 and the relationship between SV40 large T antigen and p53 in the process of cellular transformation in the lung.

Other Models of Lung Tumorigenesis. Pulmonary epithelial tumors were observed in transgenic mice using a variety of oncogenes and promoter/enhancer sequences. Mice expressing an activated form of ras under the control of the mouse mammary tumor virus (41), the human albumin promoter (42), and a human immunoglobulin enhancer/SV40 early region promoter sequences (43) developed pulmonary tumors. Lung tumors were also recently produced in transgenic mice harboring the SV40 large T antigen under control of the rabbit uteroglobin promoter (44). Histopathology of the lung tumors generated in the albumin/ras and the uteroglobin/SV40 large T antigen transgenic mice appear to be similar to that observed in the lung adenocarcinomas described in the present study. Ultrastructural features of the tumors produced in the albumin/ras transgenic mice were described as a heterogeneous population of cell types composed primarily of Clara and Type II epithelial cells (42). Phenotypic cell markers were not reported in these previous studies.

In summary, a mouse model for pulmonary adenocarcinomas was produced in transgenic mice harboring a chimeric SP-C-SV40 large T antigen gene. The histopathology of the tumors was indistinguishable from that seen in human adenocarcinomas, including the papillary, solid, and bronchioloalveolar subtypes. The expression of the epithelial cell markers CC10 and SP-C mRNAs was consistent with a close relationship of these tumor cells to bronchiolar Clara cells and alveolar Type II cells, further substantiating the roles of these two cell types (or their precursors) in the formation of adenocarcinomas. The SP-C-SV40 large T antigen transgenic mice provide a useful model system for the study of lung carcinogenesis, lung development, and the process of epithelial cell transformation mediated by SV40 large T antigen. The present study demonstrates the utility of the 3.7-kilobase SP-C promoter region in directing an oncogene to cells capable of forming pulmonary adenocarcinomas. Similar constructs may be useful in testing the possible roles of other oncogenic proteins in the pathogenesis of human pulmonary tumors.


Simian Virus 40 Large T Antigen Directed by Transcriptional Elements of the Human Surfactant Protein C Gene Produces Pulmonary Adenocarcinomas in Transgenic Mice

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