A Transgenic Mouse Model for Studying the Lineage Relationships and Differentiation Program of Type II Pneumocytes at Various Stages of Lung Development*

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A pedigree of transgenic mice has been characterized that contains a H2-KK/LacZ fusion gene that exhibits integration site-dependent expression from the earliest stages of lung development through adulthood. Histological and immunocytochemical studies indicate that the LacZ reporter appears throughout the pulmonary endoderm by embryonic day 11 (E11). A proximal-to-distal wave of extinction of transgene expression occurs during E13–14 that parallels the wave of cytodifferentiation of the pulmonary endoderm. By E16, the LacZ reporter is restricted to the distal portion of epithelial tubules and by birth to scattered cells located in alveoli. Crude epithelial cell suspensions were prepared from lungs harvested from E16 and 14 day postnatal transgenic mice, labeled with the fluorescent LacZ substrate fluorescein di-(β-galactopyranoside), and the LacZ expressing population isolated by fluorescence-activated cell sorting. Electron microscopic, immunocytochemical and histochemical studies of this purified cell population establish that type II pneumocytes are the only cell lineage that support H2-KK/LacZ expression in the mature postnatal lung. Fluorescence-activated cell sorting of E16 lung suspensions yielded a homogeneous population of cells that produced surfactant protein A, that could be maintained in cell culture, and that are likely precursors of adult type II pneumocytes. Together these studies indicate that (i) expression of the transgene in this pedigree of mice provides a marker for describing early differentiation of the pulmonary epithelium; (ii) the transgene may be useful as an enhancer trap to isolate cis-acting sequences that regulate gene transcription within this lineage; (iii) the LacZ reporter expression can be used to purify specific embryonic pulmonary epithelial cell populations; and (iv) primary cultures of these embryonic populations represent a potentially useful model system for analyzing the cellular components and signaling pathways necessary to support and complete passage through the type II pneumocyte differentiation program.

The pulmonary epithelium of the adult mouse undergoes slow but continuous renewal of its eight principal terminally differentiated cell types: ciliated cells, nonciliated basal cells, mucous and serous secretory cells, neuroendocrine cells, Clara cells, and type I and type II pneumocytes. Despite their continuous renewal, each cell type is able to establish and maintain a characteristic regional distribution along the tracheal-alveolar axis of the lung (1). However, details about how this epithelium is normally renewed are lacking. Basal, secretory, Clara, and type II epithelial cells are able to re-enter the cell cycle after the completion of their differentiation program, but it is unclear whether the pulmonary epithelium contains unipotent or multipotent stem cells that sustain a process of perpetual proliferation and spatially well organized differentiation as they do in the mouse intestinal epithelium (reviewed in Refs. 2 and 3).

One approach to understanding the lineage relationships and renewal pathways for pulmonary epithelial cell populations is to examine their evolution during development. The mouse lung arises as an outgrowth of the embryonic foregut. Lung buds are first evident by embryonic day (E)10, branching morphogenesis is initiated by E12, and the precursor conducting airways are evident by E16 (1). Cytodifferentiation of the pulmonary epithelium occurs in a proximal-to-distal wave which is initiated at E13-E14 in the trachea (4). Morphologic studies using transmission electron microscopy suggest that the first phase of this cytodifferentiation results in the allocation of embryonic epithelial cells into two populations: one destined to populate the alveoli (prealveolar cells) and the other destined to form the differentiated epithelium of more proximal components of the airway (the precursors broncholar epithelium) (5–7). However, further analysis of the molecular events that regulate initial cytodifferentiation of the pulmonary epithelium has been hampered by (i) the limited number of genes that have been isolated which can serve as lineage markers and model systems for analyzing how this process is regulated; (ii) an inability to recover and characterize a relatively homogeneous population of prealveolar epithelial cells at early points in lung development; and (iii) the lack of cell culture systems (either primary or permanent) that mimic the biological properties of embryonic epithelial cell populations and/or their subsequent differentiation pathways.

Transgenes have provided powerful tools for examining...
lineage relationships and differentiation programs in other perpetually renewing epithelia, e.g. the gut and skin (8–16). A recent lineage ablation study, conducted in transgenic mice containing fusion genes composed of transcriptional regulatory elements from the surfactant protein C (SP-C) gene linked to Diphtheria toxin A gene, suggested that type I and II cells share a common progenitor which is represented in the late gestation developing lung (17). In the present study, we describe a pedigree of transgenic mice containing a chimeric construct composed of the 5'-nontranscribed domain of the mouse major histocompatibility class I gene, H-2 Kb, linked to the coding region of the Escherichia coli β-galactosidase gene (LacZ). These mice first express the LacZ reporter in the E11 mouse lung bud. This transgene has been used as (i) a marker of early lung development, (ii) to describe the potential pathways by which type II pneumocytes arise, and (iii) to recover and culture populations of LacZ-positive (pre) type II cells at several stages of lung development by fluorescence-activated cell sorting (FACS).

These mice first show lacZ expression in the endoderm at the earliest stages in lung bud formation (by E11), which during subsequent lung development follows an expression pattern consistent with the presumed differentiation to prealveolar cell types, and finally is restricted to pulmonary epithelial type II cells of the postnatal animal. Furthermore, we demonstrate the use of fluorescence-activated cell sorting to isolate homogenous embryonic epithelial and type II epithelial cell populations based on the lacZ expression of the transgene. This study shows the utility of this pedigree as a novel lineage marker for pulmonary epithelial cell differentiation in fetal and postnatal life and also that the use of FACS can provide a powerful method for the isolation of subpopulations of pulmonary pretype I epithelial cells at any point in lung development based on transgenic lacZ expression.

**EXPERIMENTAL PROCEDURES**

**Generation and Characterization of a Pedigree of Transgenic Mice Containing H2-Kβ15 to +12/LacZ That Exhibits Reporter Expression in the Lung.—**Fig. 1 illustrates the features of the Kβ15 to +12/LacZ DNA used to generate transgenic mice. Nucleotides −2015 to +21 of the murine H2-Kβ class I gene (18) were isolated as a NruI/EcoRI fragment, subcloned into Bluescript at the EcoRI and EcoRV sites, and then resoldated at the HindIII site of a Bluescript which contained a 3.6-kb BamHI fragment obtained from pMMuLV-SV-LacZ (19) and a 3' splicing and polyadenylation signal from a BamHI/BamHI digest of pSV2-CAT (20). Both of which had been previously ligated into the polylinker (as HindIII and EcoRI/BamHI fragments, respectively, utilizing subcloning of the original fragments into Bluescript vectors to produce the desired flanking restriction sites). The 3.6-kb fragment from pMMuLV-SV-LacZ contains the entire coding region of the E. coli β-galactosidase gene, and the 516-kb fragment originally derived from pSV2-CAT (nucleotides 3369-4285) contains the splicing/polyadenylation signal from nucleotides 2533-2774 and 4100-4773 of the simian virus 40 (SV40) genome (20). Transgenic mice were produced by microinjection of the 6.7-kb fusion gene (recovered as a SalI/NotI fragment) into the pronuclei of fertilized oocytes using standardized techniques (21). Nine pedigrees of H2-Kβ15 to +12/LacZ mice were established. All nine pedigrees exhibited remarkably different cellular patterns of LacZ expression as determined by histochemical surveys (see below). The tissue and cellular distribution of LacZ expression was constant within multiple members of a given pedigree (22). However, no pedigree manifested a pattern of reporter expression which mimicked that of the intact endogenous mouse MHC class I H-2R gene (18), suggesting that the patterns of transgene expression were influenced by transcriptional regulatory elements located in genomic sequences that flanked the site of insertion of the transgene. One of these lines, derived from founder G12)5 (22), demonstrated high levels of LacZ expression in the lung and was chosen for further analysis. This line was maintained by mating animals who were hemizygous for the transgene to C57Bl/6 mice. Transgenic mice were identified using (i) tail DNA, (ii) two oligonucleotide primers, one derived from nucleotides 5162-5171 of the 6706-bp H2-Kβ15 to +12/LacZ fusion gene (this primer anneals to the 3' region of the LacZ coding sequence) and the other from nucleotides 5801-5820 (5' region of the SV40 splicing and polyadenylation region), (iii) Tag polymerase (United States Biochemical, Cleveland, OH), and the following cycling conditions for the Taq DNAase chain reaction: (i) denaturation, 1 min at 90 °C (annealing), 2 min at 72 °C (extension). Thirty cycles yielded a unique 668-bp DNA fragment. Alternatively, Southern blots of EcoRI-digested tail DNA were probed with 32P-labeled DNA fragments derived from the LacZ or SV40 components of the H2-Kβ15 to +12/LacZ fusion gene using hybridization and washing conditions detailed elsewhere (23). Southern blot analyses were also used to determine that the transgene in this line had inserted at a unique site in the mouse genome and that the transgene copy number was one haploid genome.

Developmental studies were initiated by mating a male mouse, hemizygous for the H2-Kβ15 to +12/LacZ transgene, to a normal C57Bl/6 female. The day a vaginal plug was first noted was scored as day 0 of gestation. Females were sacrificed at various stages of pregnancy by cervical dislocation, their embryos/fetuses were recovered by Caesarean section, and subsequently killed by decapitation. Developmental stages were confirmed using parameters described by Thaller (24). The presence or absence of the transgene in embryos/fetuses was ascertained by polymerase chain reaction or Southern blot analysis of carcass DNA.

**Isolation of Reporter-positive Pulmonary Epithelial Cell Populations from Fetal and Postnatal Transgenic Mice by Fluorescence-activated Cell Sorting.—**Pulmonary epithelial and type II epithelial cells were recovered from 2- to 4-week-old transgenic mice and their normal littermates using a modification of the procedure of Massey et al. (25). Animals were sacrificed by cervical dislocation, the chest cavity was opened, the trachea was cannulated with a 20–22-gauge plastic catheter, and the lungs lavaged with 2 ml of phosphate-buffered saline (PBS), pH 7.4. The right ventricle was then cannulated with a 22-gauge plastic catheter, a small incision was made in the left atrial appendage, and the pulmonary vasculature perfused with a solution of PBS containing 10 units/ml of heparin. The lungs were then removed en block by transecting the tracheas and the pulmonary artery/veins. To determine whether the lung was derived from a transgenic mouse, a wedge biopsy of the right or left lower lobe was taken, placed in PBS at pH 7.2 with 20 mM potassium ferricyanide, 20 mM potassium ferrocyanide, 2 mM MgCl₂, and 1% dimethyl sulfoxide, 5-bromo-4-chloro-3-indoyl β-d-galactoside (X-Gal, 0.8 mg/ml), and the solution incubated at room temperature. Immediately after the wedge biopsy was taken, the remaining lung was placed in a glass Petri dish, inflated by infusing 2 ml of PBS containing 0.5% (w/v) of a bovine pancreatic protease preparation (Type I, Sigma; specific activity = 10 units/mg, units defined according to Sigma) and 0.4 mM EDTA through the tracheal catheter. The catheter was clamped and the lung tissue placed in a holding solution of PBS containing EDTA and X-Gal. Following a 5-min incubation, the lung was allowed to deflate by removing the clamp from the catheter; fresh PBS/bovine protease/EDTA solution was instilled, and a 5-min incubation at 37 °C repeated. After a total of three cycles of infusion, incubation, deflation, and reinfusion were completed, the lung from a single animal was minced into 1- to 2-mm pieces with scissors and the pieces added to an equal volume of PBS containing CaCl₂ (2.5 mM), MgSO₄ (1.5 mM), and bovine pancreatic deoxyribonuclease (Type II, Sigma; specific activity 2, 500 Kunitz units/mg, final concentration = 200 units/ml). The suspension was incubated for 10 min at 37 °C, fetal bovine serum (Gibco) was added to a final concentration of 5%, and the mixture was passed through a 70-μm nylon filter (Falcon, Lincoln Park, N2). The flow-through fraction was subjected to centrifugation at 1600 × g for 10 min at 4 °C. The pellet was washed once with PBS, 5% fetal bovine serum and then resuspended in PBS, 5% fetal bovine serum, 10 mM HEPES, pH 7.2, with frequent flow cytometry or suspended in tissue culture media (see below).

The procedure described in the preceding paragraph was modified when dealing with E16 lungs. The left and right lungs were isolated from fetuses using the dissecting microscope. Small segments of lung tissue from each embryo were added to a solution of PBS/potassium ferricyanide/potassium ferrocyanide/dimethyl sulfoxide/X-Gal to determine whether they were derived from animals which expressed the H2-Kβ15 to +12/LacZ transgene. LacZ-positive and LacZ-negative fetal lung cells from multiple animals were allocated to control and experimental groups, minced in a volume of PBS (maintained at 4 °C), and the suspension allowed to settle by gravity. Following two to three cycles of resuspension in fresh PBS and settling, the final
washed cell pellet was taken up in 3 volumes of PBS, 0.5% bovine pancreatic protease, 0.4 mM EDTA, and the suspension was incubated with agitation for 20 min at 37 °C. The material was then processed using a protocol identical to that employed for 2-week-old lungs.

The crude epithelial cell suspensions were labeled for flow cytometry and FACS sorting using the LacZ substrate fluorescein di-(β-galactopyranoside) (FDG, Molecular Probes, Inc., Eugene, OR). The suspension was added to an equal volume of 2 mM FDG (prepared in deionized water containing 1% dimethyl sulfoxide), incubated at 37 °C for 2 min, and then diluted 10-fold with a solution of PBS, 5% fetal bovine serum and 10 mM HEPES, pH 7.2. Cells were assayed for the presence of a reporter transgene using 50 mM 3-0-methyl-α-D-galactose to block specific MPA binding (26).

Flow cytometry and FACS sorting were carried out using an EPICS 753 flow cytometer (Coulter, Hialeah, FL) or a FACSCAN system (Becton Body, CA.). The suspension was incubated under subdued light for 1 h at 4 °C. Cells were washed twice as before and resuspended in PBS/ fetal bovine serum/HEPES for flow cytometry and FACS. Control studies were carried out using a Phillips PW 6006 transmission microscope (Phillips Electronics Inc., Mahwah, NJ).

**Immunochemical and Histochemical Studies—** Tissue samples were fixed in 1% paraformaldehyde (prepared in PBS) for 1-2 h at room temperature. They were subsequently rinsed with PBS and with 10% sucrose, PBS during an overnight incubation conducted at 4 °C. Samples were embedded in O.C.T. (Miles Inc., Elkhart, IN), quickly frozen in liquid nitrogen, and stored at −70 °C until 5-10-μm sections were prepared. Cell suspensions were fixed in 1% paraformaldehyde, PBS as above and then attached to lysine-coated slides by centrifugation (for cytokeratin staining, unfixed cells were attached to the slides). Slides were rinsed in PBS, incubated in blocking buffer (PBS supplemented with 2% bovine serum albumin, 0.2% fetal calf serum, 0.1% normal goat serum (Dako, Carpinteria, CA), and 0.3% Triton X-100) for 30 min at room temperature. Primary antisera were diluted in blocking buffer and applied to the sections. Slides were incubated for 12-16 h at 4 °C, then washed with PBS, and overlaid with gold-labeled goat anti-rabbit serum (Amerham Corp.). After PBS and water washes, slides were developed using a silver enhancement method described by Amerham Corp. The sources and final dilutions of primary antisera were: rabbit anti-E. coli β-galactosidase (1:1000, a gift from Joshua Stern, Washington University), rabbit anti-cytokeratin (1:1000, Dako), and rabbit anti-rat surfactant protein A (1:1000, SP-A, kindly supplied by Jeffrey A. Whitsett, University of Cincinnati).

**RESULTS**

**Integration Site-Specific Expression of an H2-K^b^ to ^c^ LacZ Transgene in the Epithelium of the Developing Lung—** Histochemical and immunochemical surveys of the patterns of expression of transgenes, composed of the β'-non-transcribed domain of the mouse major histocompatibility gene H2-K^b^ linked to the E. coli β-galactosidase gene (Fig. 1) in 2-4-week-old mice that were members of nine different pedigrees of transgenic mice, revealed remarkable differences in the cellular patterns of reporter accumulation between but not within lines. None of the patterns of expression mimicked that of the intact mouse H2-K^b^ gene locus. Nonetheless, reporter gene expression appeared to transcriptional regulatory elements that flanked the site of insertion of the transgene (e.g. see Weis et al., 1991). Histochemical surveys of tissues harvested from young adult (2-6-week old) members of one such pedigree revealed β-galactosidase activity primarily, if not exclusively in the lungs. The tissues surveyed included lung, heart, brain, kidney, muscle, skin, liver, stomach, intestine, and colon. In embryos of various developmental stages, LacZ expression was also detected in the brain and skin; however, this expression diminished and disappeared in latter stages of development and in postnatal life.

Developmental studies of five litters belonging to this line indicated that the reporter was present at the earliest points in embryonic lung development (E11/E12) and limited to endodermal rather than mesenchymal compartments (Fig. 2A). The pattern of X-Gal staining was uniform and of equal intensity throughout all endodermal cells of the lung bud from E11 through E13 and was not inhibited by 300 μM chloroquine, an inhibitor of lysosomal galactosidase activity. No X-Gal staining was detected in any cell populations present in the lungs of comparably aged normal (nontransgenic) littersmates. By E13/E14 there was a dramatic shift in the distribution of LacZ-positive cells: transgene expression had been extinguished in the proximal epithelium (with little to no staining in the trachea and main stem bronchi) but was sustained in the distal epithelial cell populations, particularly in the region of active branching morphogenesis (Fig. 2B). The temporal and regional patterns of transgene expression very closely mirror the process of initial cytodifferentiation of the lung epithelium into precursor broncholar and prealveolar components. At E14, a gradient of X-Gal expression was evident within epithelial tubules: the intensity of the blue-staining reporter-positive cells was considerably higher in distal compared to proximal portions of the tubules (Fig. 2C). The steady state level of LacZ activity and immunoreactive protein was identical between epithelial cells distributed around the tubule when surveyed at a particular position along the proximal to distal axis of the tubule (Fig. 2C and data not shown). Further restrictions in transgene expression were evident between E19 and the end of the suckling period (postnatal day 14): lacZ-positive cells were limited to the avelli and not represented in the bronchioles (Fig. 2, D-H) or more proximal regions of the tracheal-bronchial tree (data not shown).

**Characterization of Reporter-positive Cells Recovered at Various Stages of Development by Flow Cytometry and Fluores-
cience-activated Cell Sorting—Flow cytometry and FACS were employed to characterize the population of alveolar cells that supported synthesis of the LacZ reporter and to determine whether the capacity to support transgene expression could be used as a tool to isolate specific pulmonary epithelial cell lineages during early phases of their cytodifferentiation in fetal life.

Lungs were recovered from E16- and 14-day postnatal transgenic mice or their normal littersmates, using a protocol described under Experimental Procedures, cellular suspensions incubated with the β-galactosidase substrate, FDG, and then subjected to FACS. Analysis of samples from E16 transgenic mice or from 2-week-old H2-Kb^2015 to +12/LacZ animals (n = 5 experiments for each time point) revealed a peak of cells with fluorescence intensities 50-1000-fold greater than that seen with a second population of cells (designated as reporter-negative) and from any population of cells contained in preparations of nontransgenic littermate lung. Representative results are shown in Fig. 3A and B. The FDG-loaded cells that exhibited intense fluorescence appeared to represent a distinct and relatively homogeneous population as independently judged by two parameter contour plots of their forward and side light scattering features (see Fig. 3C for an analysis of cells obtained from E16 transgenic mice). The forward and side light scattering features of lacZ-positive cells recovered from 14-day-postnatal transgenic mice were very similar to those previously obtained from laser flow cytometric analyses of normal (i.e., nontransgenic) type II pneumocytes (28, 29).

Additional studies supported the notion that the reporter-positive cells present in the alveolar epithelium of 14 day old H2-Kb^2015 to +12/LacZ mice were type II pneumocytes. The modified Papanicolaou stain was used to demonstrate that 92 ± 2% of the highly fluorescent cell fraction obtained by FACS contained lamellar bodies (i.e., they contained three or more dark staining cytoplasmic granules). In contrast, only 7 ± 4% of the low fluorescence (“LacZ minus”) fraction contained these positively staining granules (n = 4 experiments, 100-200 cells scored/experiment). Transmission electron microscopy of osmium tetroxide and lead citrate stained “LacZ- positive” fraction (defined by FACS) confirmed that the great majority of cells had cytoplasmic lamellar granules and other morphologic features characteristic of type II cells including lamellar bodies and multivesicular granules (Fig. 4A). Surveys of several hundred cells in this fraction indicated that the small subpopulation which lacked lamellar bodies also had swollen vacuoles and mitochondria plus evidence for disruption of the integrity of nuclear and cytoplasmic membranes (data not shown). The representation of these unidentified, damaged cells was similar to the percentage of (i) lamellar body negative cells scored by light microscopic surveys of Papanicolaou-stained LacZ-positive cells and (ii) the number of nonviable cells scored by propidium staining (30, data not shown). At least some of this apparent cell damage could have been induced by the hypotonic conditions used for FDG loading. In contrast, transmission electron microscopy of the LacZ-negative fraction indicated that >90% of these cells lacked cytoplasmic lamellar granules or other features of type II pneumocytes. Together the data establish that (i) those alveolar cells which support transgene expression in 2-week-old members of this H2-Kb^2015 to +12/LacZ pedigree are type II pneumocytes and (ii) the LacZ reporter can be exploited to recover a very homogenous population of an epithelial cell lineage from crude lung cell suspensions. Moreover, the state of differentiation of these cells can be operationally defined in part by their ability to support transgene expression.

The intensely fluorescent population of cells recovered from E16 transgenic mice also appeared to be homogenous based on a number of criteria. First, the LacZ-positive fraction of cells recovered by FACS was stained with MPA lectin, which has specificity for α-D-galactose residues, and then resorted (see “Experimental Procedures”). These studies revealed a single peak of highly fluorescent cells, indicating that the great majority (97%) of FDG-positive cells were MPA-positive (Fig. 5 which shows representative results from one of three independent experiments, each involving a minimum of 10,000 sorted cells). 1-O-methyl-α-D-galactose (50 mM) blocked staining with this lectin which reacts with epitopes known to be expressed in a variety of late gestation prealveolar/alveolar epithelial cell populations (including most prominently type II pneumocytes, (31, 32)). Second, 99% of the LacZ-positive E16 cells contained detectable levels of immunoreactive cytokeratin which is known to be synthesized by embryonic pulmonary epithelial cells (33, 34) (n = 2 experiments, ≥250 cells scored/experiment). Third, immunocytochemical studies indicated that 97% of the sorted LacZ-positive cells also produced surfactant protein A (SP-A, n = 2 experiments, ≥250 cells counted/experiment; see Fig. 6).

Previous work suggested that SP-A staining in the embryonic lung may be specific for prealveolar epithelial cells (91-33). Finally, primary cultures of the LacZ-positive/MPA-positive E16 cells were established to independently assess their degree of homogeneity. Intact, monolayers rapidly form in Eagle's minimal essential medium, 5% fetal bovine serum (e.g., within 2-3 days after plating 50,000-100,000 cells in 24.5-mm plastic dishes). These monolayers were composed of cells with very similar morphologic features as judged by light and transmission electron microscopy (Fig. 7). Demosomal tight junctions were evident (Fig. 7, D and E) consistent with an epithelial origin. Histochemical studies confirmed that these confluent monolayers retained the ability to express LacZ for a period of at least several days. However, electron microscopic surveys conducted as early as 36 h after plating indicated these LacZ-
FIG. 2. Histochemical and immunocytochemical studies of the cellular patterns of H2-Kb-21 to -21/LacZ expression in E11-E19 and 2-week postnatal transgenic mice. Whole tissues and cryostat sections were stained with X-Gal as described under "Experimental Procedures" to identify LacZ-producing cells. Panel A, lung buds from a E12 embryo show intense blue staining of...
Fig. 3. Flow cytometry of E16- and 14-day postnatal lung suspensions obtained from H2-Kb<sup>wt</sup> to +<sup>12</sup>/LacZ transgenic mice or their normal littermates. Cell suspensions were prepared as described under "Experimental Procedures" and loaded with FDG using a procedure modified from Ref. 61. Cells were analyzed for cellular fluorescence by flow cytometry. Shown are representative results from five independent experiments performed on E16- and 14-day postnatal lungs. Panel A, FACS analysis of cells harvested from 2-week-old lungs. Note the population of highly fluorescent cells (peak at 10<sup>5</sup> log fluorescent units) that is present in the sample prepared from the transgenic animal but not a sample prepared from its normal littermate's lung. Panel B, flow cytometry of FDG-treated cells harvested from E16 lung. A distinct peak of highly fluorescent cells (LacZ-positive) is present in the cell suspension obtained from transgenic embryos but not in the cell suspension prepared from normal littermate embryos. Panel C, two parameter contour plot of the light scattering characteristics of the crude epithelial suspension obtained from E16 transgenic lung. The dense concentration of cells indicated by the arrow contains essentially all of the FDG-loaded highly fluorescent cells (the LacZ-positive fraction).

Fig. 4. Transmission electron microscopy of LacZ-positive cell fractions recovered by FACS analysis. A typical type II pneumocyte isolated by fluorescence-activated cell sorting of FDG-loaded lung suspensions prepared from a 14-day-postnatal transgenic mouse. (Original magnification = ×24,000.)

positive cultured cells did not have cytoplasmic lamellar bodies (data not shown). By contrast, the morphologic appearance of cells cultured from the sorted LacZ-negative fraction was quite distinct from the features of cultured LacZ-positive cells (compare panels A and B of Fig. 7). Our data suggest that the LacZ reporter can be used to recover a homogenous population of pulmonary epithelial cells during a critical period of cytodifferentiation of the fetal lung epithelium. The LacZ-positive and the LacZ-negative cell populations are able to enter the cell cycle (i.e. proliferate) and can be maintained for periods up to 7 days in culture using standard conditions. Although it appeared that the cultured descendants of the LacZ-positive FACS fraction rapidly dedifferentiated and did not maintain features of type II pneumocytes over an 2-day period, the system for recovery and culture described in this report offers an opportunity for exploring conditions that may allow further expression of their differentiation programs <i>ex vivo</i>.

**DISCUSSION**

We have analyzed a pedigree of transgenic mice whose integration site-dependent expression of a H2-Kb<sup>wt</sup>/LacZ fusion gene is initiated at the very earliest phases of lung development. Expression of the LacZ reporter is perpetuated in an epithelial cell lineage that gives rise to type II pneumocytes. The LacZ reporter can be used to rapidly recover homogeneous populations of epithelial cells from crude lung suspensions (prepared at various stages of development) by fluorescence-activated cell sorting. These LacZ-positive or LacZ-negative populations can be maintained in primary culture, which should allow for further study of this highly purified epithelial cell population.

Expression of the H2-Kb<sup>wt</sup> to +<sup>12</sup>/LacZ transgene in this pedigree occurs at a critical period in the cytodifferentiation of the embryonic lung epithelium. The reporter is first detectable at E11 in the lung bud endoderm. Restriction of transgene expression occurs subsequently taking the form of a proximal-to-distal wave of extinction of cellular LacZ accumulation between E13-E14. This wave of silencing of transgene expression parallels a wave of morphologic transformation that involves allocation of the endoderm to precursor bronchiolar and prealveolar epithelial components. By E16, the reporter is limited to distal epithelial tubules and by birth to type II pneumocytes in the alveoli. Remarkably, the transgenic endodermal compartment but not of the surrounding mesoderm. (Original magnification = ×50.) Panel B, lung from an E13 embryo shows prominent staining in the distal pulmonary epithelium with faint staining in the embryonic tracheal and main stem bronchial epithelium. (Original magnification = ×25.) Panel C, E18 lung with diffuse parenchymal staining. (Original magnification = ×6.) Panel D, cryostat section prepared from an E16 transgenic mouse lung stained with X-Gal and counterstained with eosin. Blue, LacZ-positive cells are located in distal epithelial tubes (indicated by the closed arrows) but not in their proximal portions (open arrows). (Original magnification = ×100.) Panels E and F, low and high power views (<i>x</i>100 and <i>x</i>400, respectively) of a cryostat section prepared from a 14-day-postnatal transgenic mouse. Note the distinct, blue-staining cells confined to the alveolar region (closed arrows). This LacZ-positive cell population is not evident in the bronchiolar airway (<i>B</i>) or in blood vessels (<i>V</i>). Note that control experiments showed no detectable staining in samples prepared from the comparably aged, non-transgenic littersmates of H2-Kb<sup>wt</sup>/LacZ mice shown in panels A–D. Panels G and H, cryostat sections were prepared from a 2-week-old transgenic mouse (<i>G</i>) and its normal littermate (<i>H</i>) and stained with a rabbit anti-LacZ antisera. Antibody complexes were subsequently detected using gold-labeled goat anti-rabbit IgG and silver enhancement. Sections were viewed using reflected light polarization microscopy. The scattered LacZ immunoreactive cells are confined to the alveolar epithelium. (Original magnification = ×400.)
gene is not expressed in other lineages in the postnatal lung, including type I pneumocytes, Clara cells, and the bronchial epithelium. This constellation of features makes the H2-K''/LacZ transgene and the genomic sequences which flank its site of insertion a unique genetic marker for developmental studies of the pulmonary epithelium.

The data presented also support the notion that H2-K''/LacZ is a very early marker of the type II cell lineage in the embryonic lung epithelium. Specifically, the initial uniform cellular pattern of transgene expression noted at E11-E13, the subsequent restriction of expression to the distal pulmonary epithelium after E14, and the persistent expression in type II in late fetal life are consistent with the results of histologic studies of the origins and differentiation of type II cells in the developing lung (4-7). The gene encoding mouse surfactant apoprotein C (SP-C) is the only reported marker whose expression is limited to members of the type II lineage. In contrast to the expression of endogenous SP-C protein, the LacZ expression in this transgenic pedigree is detectable much earlier in embryonic development (35). Additionally, several pedigrees of transgenic mice, containing up to 3.7 kb of the 5' upstream regulatory elements from SP-C gene linked to a principal cell types of the lung are derived from common progenitors (stem cells).

The restricted expression of the H2-K''^5 to +12/LacZ transgene appears to reflect the more dominant effects of cis-acting elements that flank its site of insertion in the mouse genome compared to elements located within the transgene. This conclusion is supported by prior observations that multiple pedigrees containing this transgene each have markedly different tissue-specific patterns of expression (22). Thus, recovery of regions flanking the unique site of insertion of H2-K''/LacZ in the pedigree described in this report may provide a potential opportunity for identifying new regulatory elements or endogenous mouse genes that are expressed in the developing pulmonary epithelium. The use of transgenes with weak promoters as "enhancer traps" has been proposed by others (36, 37).

Cultured eukaryotic cells that contain the LacZ gene have been labeled with a fluorescent substrate of E. coli β-galactosidase (fluorescein di-(β-D-galactopyranoside) and subjected to FACS to recover subpopulations that produce this bacterial enzyme. We adapted this powerful approach to recover (i) a highly purified (near homogeneous) preparation of type II pneumocytes from a very heterogeneous population of cells present in crude protease digests of minced 14-day postnatal lung and (ii) what appears to be a antecedent to this lineage cytodifferentiation of this endoderm. Moreover, crossing these animals to other transgenics containing fusion genes composed of transcriptional regulatory elements expressed in other pulmonary epithelial cell lineages linked to different reporters should allow us to determine which if any of the principal cell types of the lung are derived from common progenitors (stem cells).

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present in the E16 lung. Other methods have been used to obtain cell populations enriched for type II pneumocytes (e.g., 28, 29, 33, 38–40). Acridine orange and phosphine 3R staining of lamellar bodies followed by FACS can yield a >90% pure preparation of type II cells from adult rat lung (28, 29). However, this approach should not be useful when the goal is to isolate fetal type II pneumocytes and their precursors which lack or contain low numbers of cytoplasmic lamellar bodies (7, 41). In addition, it is not clear what metabolic effects these dyes have on the isolated type II cells in contrast to the nontoxic FDG LacZ substrate. In addition, this FACS-based technique using LacZ transgene expression appears to produce significantly higher purity of isolated epithelial cells than differential adherence techniques previously used for the isolation of fetal alveolar epithelial cells (33, 42). The combination of transgenes that direct expression of a bacterial gene product (LacZ) in specific lineages and FACS should be a generally applicable scheme for recovering specific cell populations from transgenic mouse tissues. cDNA libraries produced from these sorted, reporter-positive cells should yield markers of endogenous gene transcripts that are characteristic for that cell type at a particular stage of its differentiation program. In this scheme, differentiation is initially defined operationally as the ability to support transgene expression. However, transgenes have proven to be remarkably sensitive tools for defining states of cellular differentiation. For example, subpopulations of cells within a given lineage have been identified by virtue of the fact that accumulation of the products of transgenes (their reporters) can be affected by subtle variations in differentiation programs. These differences are not often readily appreciated from an analysis of expression of endogenous mouse genes (e.g. see Roth et al. (8)) and mean that a "homogeneous" population of reporter-positive cells is more uniform with respect to its state of differentiation than a population of cells recovered based on the presence of an endogenous gene product.

Finally, the ability to recover a preparation of reporter-positive cells from the developing lung provides an opportunity to examine the factors which contribute to its subsequent differentiation programs and/or its allocation to various cell lineages ex vivo. The LacZ-positive cell preparation obtained by FACS from E16 lung had features of early type II pneumocytes including the capacity to produce surfactant protein A. Once established in primary culture, they proliferated forming monolayers of cells with uniform morphologic features. These morphologic features were distinct from the features of cells generated after plating the LacZ-negative fraction obtained after FACS. However, although virtually all members of the reporter-positive FACS fraction which were represented in primary cultures retained their ability to produce β-galactosidase for up to 2 days, none of these epithelial cells appeared to yield fully differentiated type II pneumocytes. Previous studies have indicated that adult type II pneumocytes rapidly dedifferentiate in culture (43–47). Moreover, the mesodermal components are thought to be essential for induction of endodermal differentiation during the early phases of lung development (48–50) just as it appears to be necessary for the differentiation of gut epithelial cell lineages (51–60). The molecular basis for this proposed "cross-talk" between mesoderm and endoderm is not known. Primary cultures of LacZ-positive cells obtained from E16 H2-Kb/LacZ transgenic mouse lung suspension by FACS may represent a useful assay system for investigating the role of specific growth factors in the differentiation of type II pneumocytes, for assessment of the differentiation potential of isolated cells at various points in development, and/or for identifying cellular populations present in embryonic lung that are required for supporting evolution of this cell lineage.

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