

# Intraamniotic Administration of an Adenoviral Vector for Gene Transfer to Fetal Sheep and Mouse Tissues

ANDREAS HOLZINGER, BRUCE C. TRAPNELL, TIMOTHY E. WEAVER, JEFFREY A. WHITSETT,  
AND HARRIET S. IWAMOTO

*Divisions of Pulmonary Biology [A.H., T.E.W., J.A.W.] and Neonatology [H.S.I.], Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio 45229, and Genetic Therapy, Inc. [B.C.T.], Gaithersburg, Maryland 20878*

## ABSTRACT

Replication-deficient adenoviruses have been used to transfer various genes of interest to mammalian tissues *in vivo*. Effective gene therapy for inborn genetic defects presenting with significant morbidity and mortality at birth will require correction of the defect prenatally. To test the hypothesis that intraamniotically administered adenovirus transfers gene expression to fetal tissues, replication-deficient human type 5 adenovirus carrying the *lacZ* gene which encodes nuclear-targeted bacterial  $\beta$ -galactosidase (Av1LucZ4) was instilled into the amniotic cavity of fetal sheep ( $10^{10}$  to  $1.5 \times 10^{11}$  pfu) and fetal mice ( $10^9$  pfu) at 0.8 term gestation. Amniotic membranes and gastrointestinal and respiratory tract tissues were harvested after 3 d, bacterial  $\beta$ -galactosidase activity was determined by 5-bromo-4-chloro-3-indoyl- $\beta$ -D-galactopyranoside (X-gal) enzyme-histochemistry, and tissue integrity was assessed in sections stained with hematoxylin and eosin. Bacterial  $\beta$ -galactosidase activity was abundant in amniotic membranes and present in lower levels in

esophagus, stomach, and small intestine as well as in conducting airways and pulmonary alveoli. To determine whether gene transfer by intraamniotic injection of adenovirus was dose-dependent, Av1Luc1, an adenoviral vector carrying the gene for luciferase ( $10^5$ – $10^9$  pfu), was injected intraamniotically into fetal mice at 0.8 term gestation. Luciferase activity measured after 3 d in tissue homogenates of Av1Luc1-treated fetal mice revealed a linear dose response in amniotic membranes and gastrointestinal and respiratory tract organs. Intraamniotic administration of an adenoviral gene vector leads to expression of the transferred gene in amniotic membranes as well as in fetal gastrointestinal and respiratory tract tissues in a dose-dependent manner. (*Pediatr Res* 38: 844–850, 1995)

### Abbreviations

pfu, plaque-forming unit

X-gal, 5-bromo-4-chloro-3-indoyl- $\beta$ -D-galactopyranoside

A variety of techniques for gene transfer to mammalian tissues *in vivo* are currently under investigation. Recombinant adenoviral vectors rendered replication-deficient by virtue of deletions of the E1 and E3 transcriptional units have been used to transfer a variety of genes to mammalian epithelial cells *in vivo* (1–7). DNA transferred into mammalian cells by an adenoviral vector is thought to remain episomal and is not integrated into the host cell genome. This technique holds promise for somatic gene therapy for inheritable diseases such as cystic fibrosis or  $\alpha_1$ -antitrypsin deficiency. Other genetic diseases—such as surfactant protein B deficiency (8–10)—present with severe morbidity and mortality immediately after birth, and successful gene therapy will likely require the cor-

rection of the defect prenatally, before irreversible structural changes develop. Prenatal diagnosis—as a prerequisite for such an approach—has been demonstrated to be feasible for this defect (11).

Fetal tissues *in utero* are accessible for gene transfer with adenovirus. Previous studies have demonstrated successful gene transfer by adenovirus to fetal sheep lung *in utero* using chronically implanted intratracheal catheters (12, 13). The amniotic cavity is more easily accessible, and intraamniotically administered substances have been demonstrated to distribute freely even to the most distal parts of the respiratory tract (14–16). This is made possible by simple diffusion and fetal breathing activity, although there is a net outflow of lung fluid into the amniotic cavity. Adenoviruses administered into the amniotic fluid can also gain access to the gastrointestinal tract by fetal swallowing.

In this study, transfer of the reporter genes *lacZ* and *luc* to fetal tissues by intraamniotic injection of an adenoviral vector is investigated. *lacZ* is an *Escherichia coli*-derived gene en-

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Correspondence: Harriet S. Iwamoto, Ph.D., Department of Pediatrics, Neonatology/TCHRF Division, Children's Hospital Medical Center, 3333 Burnet Ave., Cincinnati, OH 45229-2899.

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coding  $\beta$ -galactosidase (EC 3.2.1.23). This enzyme, which contains a nuclear localization sequence, can be detected histologically by enzyme-histochemical methods (17). Luciferase (EC 1.13.12.7), the corresponding product of the *luc* gene derived from the common firefly (*Photinus pyralis*), is used for quantitation in tissue homogenates in the dose response study because it does not occur naturally in mammalian tissues, and highly sensitive luminometric quantitation protocols are available (18).

## METHODS

Replication-deficient human type 5 adenoviruses Av1LacZ4 and Av1Luc1 were provided by Genetic Therapy Inc., Gaithersburg, MD, in titers from 1.0 to  $2.3 \times 10^{11}$  pfu/mL. Portions of the E1 and E3 regions of the adenoviral genome were deleted to render the adenoviruses replication-deficient and to create space for the insertion of the gene to be transferred. The virus contained a transgene expression cassette consisting of the Rous sarcoma virus promoter, the transgene [*E. coli*-derived nuclear-targeted  $\beta$ -galactosidase (Av1LacZ4) or firefly luciferase (Av1Luc1)] followed by the Simian virus 40 poly(A) signal inserted into the site of the deleted E1 region (19, 20). Viruses were constructed, propagated, purified and titered as previously described (3, 19).

### Fetal Sheep

**Surgical procedures.** Five ewes with singleton or twin pregnancies were obtained from a commercial supplier at least 1 wk before surgery. They had free access to standard laboratory diet and water until 24 h before surgery, when they had access to water only.

At 109–112 d of gestation the ewes were sedated by i.v. injection of pentobarbital (30 mg/kg), intubated, and ventilated with oxygen. Low spinal anesthesia was achieved by injection of 4 mL of 1% tetracaine hydrochloride, and the ewes were secured in a supine position. Pentobarbital (150–200 mg) was injected i.v. every 10–15 min to maintain anesthesia. Using aseptic procedures, polyvinyl catheters (inner diameter: 1.3 mm, outer diameter: 2.3 mm) were inserted into the maternal left femoral artery and vein and advanced to the inferior vena cava and descending aorta. Through a midline incision of the abdomen, the pregnant horn of the uterus was exposed, a small hysterotomy performed, and polyvinyl catheters (inner diameter 0.8 mm, outer diameter 1.2 mm) were inserted into a fetal artery and vein. A catheter was also placed into the amniotic cavity. The incisions were sutured in layers, and the catheters were secured and passed through the abdominal wall to the ewe's flank where they were protected by a cloth pouch sewn to the skin. Gentamicin (40 mg), ampicillin (1 g), and metronidazole (50 mg) were administered into the amniotic cavity and maternal vein during surgery as well as on postoperative d 1 through 3.

**Experimental procedures.** Fetal well being was assessed by analyzing fetal arterial blood for pH,  $P_{CO_2}$  and  $P_{O_2}$  (170 pH/Blood Gas Analyzer, Ciba Corning), Hb concentration, and  $O_2$  saturation (OSM2 Hemoximeter, Radiometer Copenhagen).

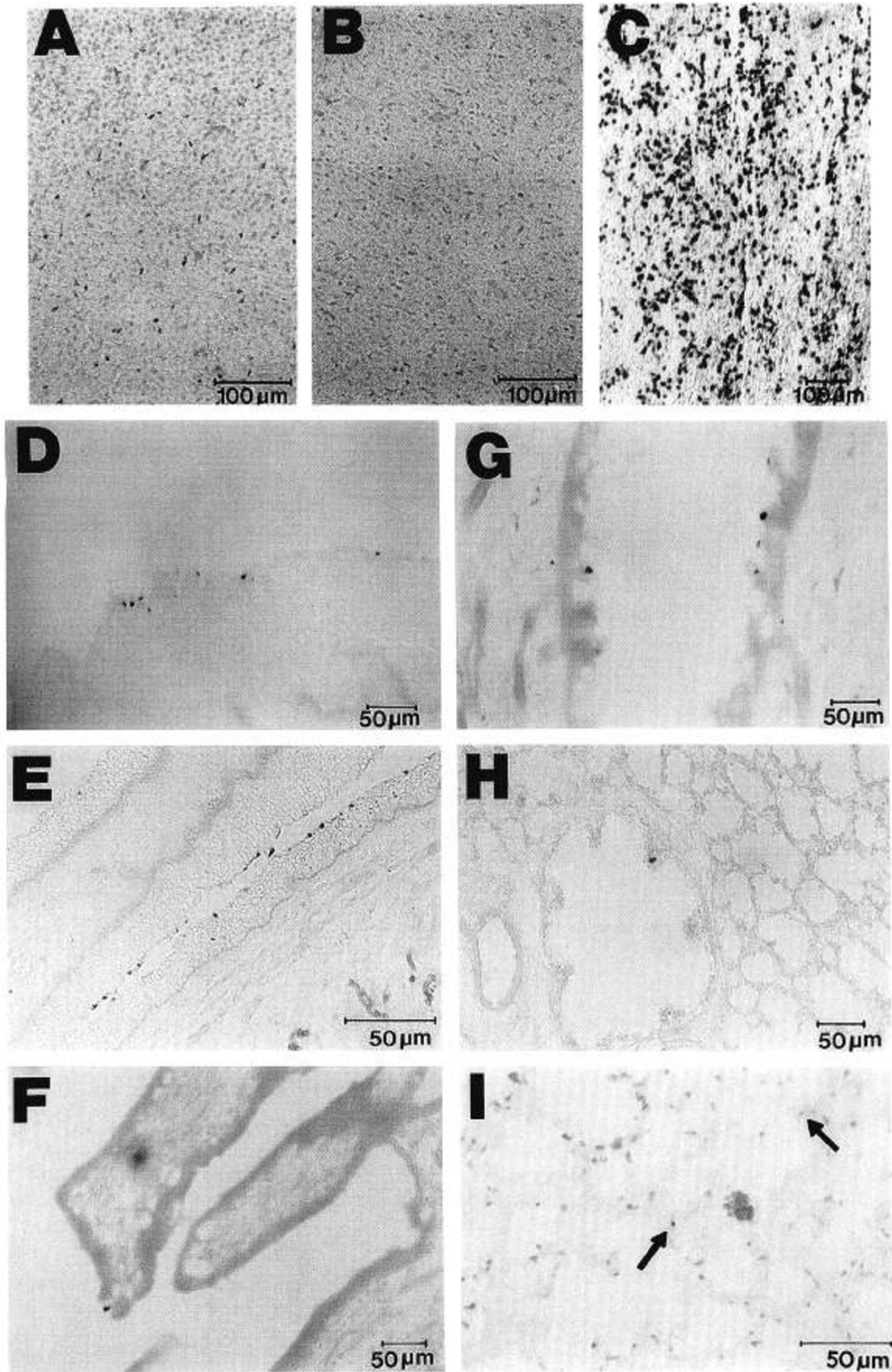
Five to 7 d after surgery,  $1 \times 10^{10}$  pfu ( $n = 2$ ) or  $1.5 \times 10^{11}$  pfu ( $n = 4$ ) of Av1LacZ4 were instilled into the amniotic cavity. Two fetuses received an injection of an equivalent volume of 0.9% saline and served as controls. Two to 3 d after virus or saline administration, amniotic membranes and fetal respiratory and gastrointestinal tract tissues were obtained. The ewes were anesthetized with sodium pentobarbital (30 mg/kg) and secured in a supine position. The fetal head was exposed, and fixative [4% paraformaldehyde in phosphate-buffered 0.9% saline (PBS; 80 mM  $Na_2HPO_4$ , 20 mM  $NaH_2PO_4$ , 150 mM NaCl, pH 7.4)] was instilled into the fetal trachea at a pressure of 30 cm  $H_2O$ . The fixative was also instilled into the esophagus and postpyloric duodenum. At least 15 min later, the larynx, trachea, lungs, esophagus, stomach, and 100 cm of proximal small intestine were isolated, cut into 1  $cm^3$  pieces, and immerse-fixed for 24 h at 4°C. Amniotic membranes were obtained and carefully secured in fixative such that the original geometry was preserved as closely as possible. All tissues except amniotic membranes were immersed in 20% sucrose for 24 h at 4°C, embedded in M1 embedding matrix (Lipshaw, Pittsburgh, PA), and stored at  $-80^\circ C$ .

Frozen tissue sections (12  $\mu m$ ) were obtained from 20 randomly chosen blocks of lung tissue representing all five lobes as well as from all other embedded tissues. One 1.5-cm long piece of tissue was chosen from each 10-cm segment of small intestine distal to the pylorus (11 pieces in total). These tissue sections and five  $1.5 \times 2$  cm of amniotic membrane, randomly chosen from different areas, were stained for bacterial and endogenous  $\beta$ -galactosidase activity. Hematoxylin/eosin staining was performed to evaluate general tissue morphology.

To determine the presence of bacterial  $\beta$ -galactosidase activity, tissue sections and amniotic membrane pieces were placed on glass slides coated with Vectabond (Vector Laboratories Inc., Burlingame, CA) and incubated in an X-gal solution, pH 7.4 [5 mM  $K_3Fe(CN)_6$ , 5 mM  $K_4(CN)_6$ , 2 mM  $MgCl_2$ , 1 g/L X-gal in 50 mL/L *N,N*-dimethylformamide in PBS] for 24 h at 32°C. Tissue sections were then rinsed in 3% DMSO in PBS for 5 min, dehydrated, counterstained with nuclear fast red (amnion) or eosin (all other tissues), coverslipped, and examined microscopically for the presence of blue nuclear staining. In amniotic membrane preparations, the average percentage of positively staining epithelial cells was determined by counts of 10 randomly chosen areas of each of the five membrane samples. Endogenous  $\beta$ -galactosidase activity was examined by incubating tissues in X-gal solution, pH 5.2 (5 mM  $K_3Fe(CN)_6$ , 5 mM  $K_4(CN)_6$ , 2 mM  $MgCl_2$ , 1 g/L X-gal in 50 mL/L *N,N*-dimethylformamide, 107.2 mM  $Na_2HPO_4$ , and 46.4 mM citric acid). Tissues were processed as described above. Additional tissue sections were stained with hematoxylin and eosin.

### Fetal Mice

Four National Institutes of Health Swiss mice were anesthetized (2% Fluothane) on d 15 of gestation. Using sterile procedures, we exposed the uterus through a midline abdominal incision. The fetuses were injected intraamniotically with



**Figure 1.** Histology of fetal sheep tissues. (A) Blue cytoplasmic staining indicates the presence of endogenous  $\beta$ -galactosidase in amniotic membrane of a control animal incubated with X-gal solution, pH 5.2. (B) Absence of blue nuclear staining in amniotic membrane of a control fetus incubated with X-gal solution pH 7.4. (C) Blue nuclear staining indicates *E. coli*-derived  $\beta$ -galactosidase in amniotic membrane of an Av1LacZ4-treated fetal sheep incubated with X-gal

either  $10^9$  pfu of Av1LacZ4 or vehicle (Hanks' balanced salt solution). On d 18 of gestation, the mice were anesthetized (0.25 mg of acepromazine, 0.5 mg of xylazine, and 10 mg of ketamine-HCl intraperitoneally). The uterus was incised, and the fetuses were removed with their membranes intact and analyzed histologically in the same way as fetal sheep tissues with the exception that fixation was performed on whole fetuses.

To determine the dose response of an intraamniotically administered adenoviral vector, seven timed pregnant National Institutes of Health Swiss mice of 15 d of gestation (72 fetuses) were randomly assigned to receive either  $10^5$ ,  $10^6$ ,  $10^7$ ,  $10^8$ , or  $10^9$  pfu of Av1Luc1 or vehicle intraamniotically using the technique described above. Amniotic membranes, fetal respiratory tract organs (trachea, lungs), and gastrointestinal tract organs (esophagus, stomach, 1 cm of proximal small bowel) were isolated. Organs were pooled for each dose group, immersed in 1 mL of lysis buffer (Luciferase Assay System; Promega, Madison, WI), and homogenized. The homogenate was centrifuged at 10,000 rpm at 4°C for 1 min and the supernatant was stored at  $-80^{\circ}\text{C}$ .

Luciferase activity was quantified in triplicate in 20- $\mu\text{L}$  aliquots of supernatant using a luminometric assay (Luciferase Assay System; Promega) performed on a Monolight 2010 luminometer (Analytical Luminescence Laboratories, San Diego, CA). Light emission, which is proportional to enzyme activity, was expressed in means of relative light units and normalized for the total protein content of each sample as determined by the Lowry *et al.* (21) procedure. The interassay coefficient of variation for the luciferase assay was 4%.

All animal procedures in the present studies were approved by the Animal Care and Use Committee of the Children's Hospital Research Foundation, Cincinnati, OH, and followed the guidelines defined in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

## RESULTS

**Fetal sheep.** All fetal sheep were in good health throughout the study. Mean arterial blood pH before virus administration was  $7.41 \pm 0.07$  (mean  $\pm$  SD),  $\text{Po}_2$  and  $\text{Pco}_2$  were  $2.46 \pm 0.43$  and  $7.81 \pm 0.20$  kPa, respectively, the Hb concentration was  $10.86 \pm 0.99$  g/dL, and the blood  $\text{O}_2$ -saturation was  $51.8 \pm 12.5\%$ . None of these values changed significantly after virus injection.

Endogenous  $\beta$ -galactosidase activity was found in all tissues examined by staining with X-gal solution, pH 5.2 (amniotic membrane, Fig. 1A). Endogenous  $\beta$ -galactosidase activity was localized in the cytoplasm and clearly distinguishable from bacterial  $\beta$ -galactosidase activity which was predominantly nuclear. Endogenous  $\beta$ -galactosidase activity was not detectable at pH 7.4 in any tissue (Fig. 1B).

Adenovirus injection into the amniotic cavity resulted in bacterial  $\beta$ -galactosidase gene expression in amniotic mem-

brane epithelial cells. The intensity and distribution of  $\beta$ -galactosidase activity was variable. Amniotic membrane epithelium of three (out of six) treated fetuses expressed *lacZ* (Fig. 1C). Of the five randomly chosen pieces of amniotic membrane of each of these fetuses, two to four stained positive. The level of expression also varied within each of the positive samples. The average percentage of positively staining amnion epithelial cells ranged from 0.8 to 7.4%, but areas in which 40% of cells expressed *lacZ* were identified. In the three fetuses in which no blue nuclear staining was found in amniotic membranes, bacterial  $\beta$ -galactosidase activity was not detected in any other tissues ( $n = 2$ ) or was detected in the gastrointestinal tract only ( $n = 1$ ).

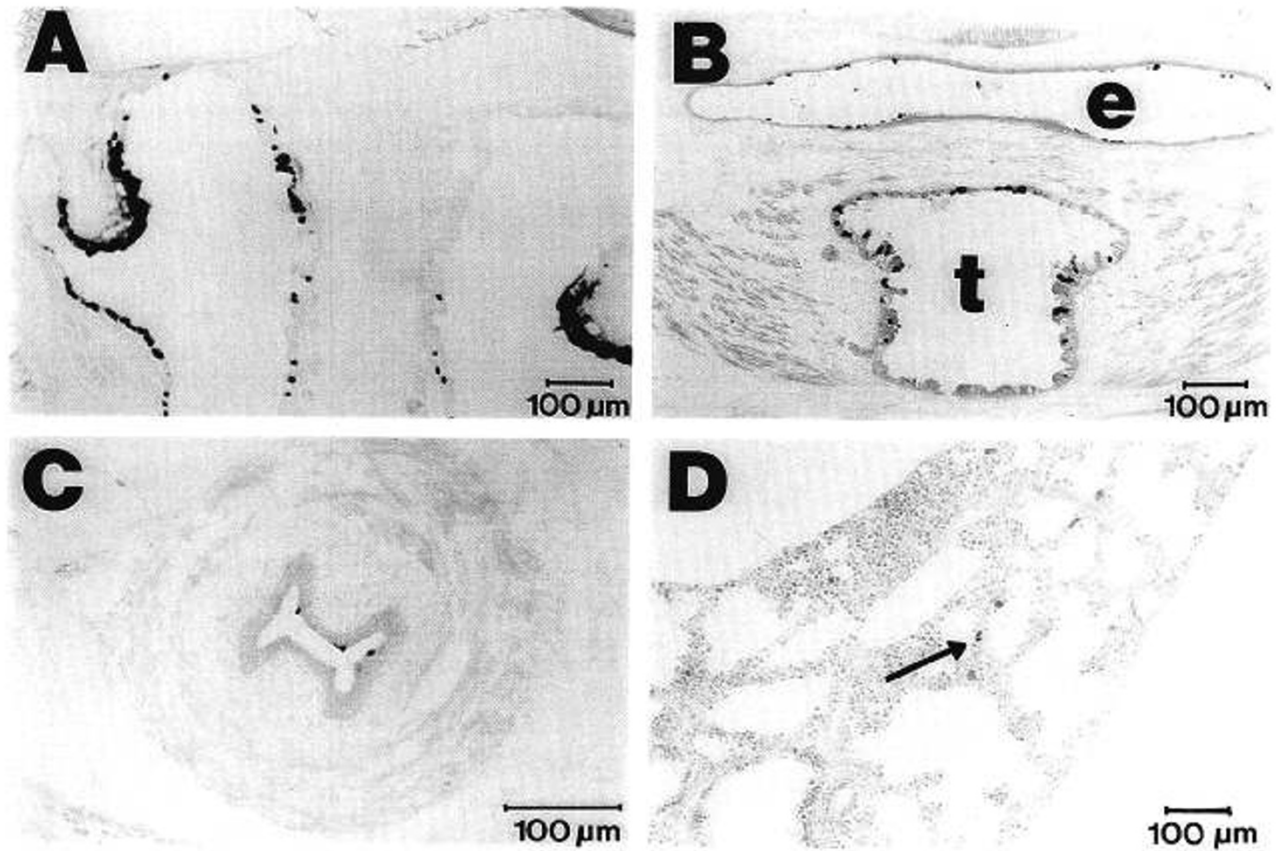
The gastrointestinal tract stained positive for bacterial  $\beta$ -galactosidase. Blue nuclear staining was observed in esophagus and stomach of four fetuses and in the duodenum of one of these fetuses (Fig. 1, D–F). A gradient of  $\beta$ -galactosidase activity was observed only in the esophagus, where blue nuclear staining was greatest in areas close to the pharynx and least in areas close to the esophageal sphincter. No gradient pattern of nuclear blue staining was obvious in gastric epithelium or small intestine, rather a more patchy distribution of  $\beta$ -galactosidase activity was observed. The jejunum did not contain bacterial  $\beta$ -galactosidase in any animal.

The respiratory tract of three fetuses was observed to contain bacterial  $\beta$ -galactosidase activity. The abundance of blue cells in laryngeal epithelium (Fig. 1G) was greater than in tracheal epithelium. Cells staining positively for bacterial  $\beta$ -galactosidase were rare in tracheal epithelium and detected in only two fetuses. The epithelium of a small bronchus and a bronchiolus (Fig. 1H)—both of the same fetus—expressed the transferred *lacZ* gene. Lung tissue of three fetuses expressed bacterial  $\beta$ -galactosidase (Fig. 1I). In two fetuses expression was detected in all lung lobes, and in one fetus expression was found to be confined to the right middle and lower lobe. In these fetuses bacterial  $\beta$ -galactosidase expression was detected in alveolar epithelium and interstitial tissue of alveolar septa. All airway tissues were rich in endogenous  $\beta$ -galactosidase activity.

All tissues analyzed had normal structure and no evidence of any inflammatory response as determined by hematoxylin/eosin staining. No tissues from control fetuses contained nuclear targeted bacterial  $\beta$ -galactosidase activity (data not shown).

**Fetal mice.** Adenoviral vectors were demonstrated capable of transferring *lacZ* and *luc* expression to fetal mouse tissues. Histologic analysis of fetal mice treated with  $10^9$  pfu of Av1LacZ4 revealed expression of bacterial  $\beta$ -galactosidase in amniotic membranes, in the respiratory tract from the nasal cavity to lung tissue, and in the gastrointestinal tract from the oral cavity to the small intestine (Fig. 2). Overall, the density of *lacZ* expressing cells appeared to be higher than in the fetal sheep.

solution, pH 7.4. (A–C, nuclear fast red counterstain.) (D–F) Expression of bacterial  $\beta$ -galactosidase in the gastrointestinal tract; D, esophageal epithelium; E, gastric mucosa; and F, duodenum. (G–I) Expression of bacterial  $\beta$ -galactosidase in the respiratory tract: G, larynx; H, bronchiolus; I, pulmonary alveoli. (D–I, X-gal staining at pH 7.4, eosin counterstain.)



**Figure 2.** Histology of fetal mouse tissues;  $10^9$  pfu of Av1LacZ4 were injected into the amniotic cavity, tissues were stained with X-gal solution, pH 7.4. Blue nuclear staining indicates the presence of *E. coli*-derived  $\beta$ -galactosidase. A, nasal cavity; B, proximal esophagus (e) and trachea (t); C, esophagus at the level of the heart; D, lung. (A-D, eosin counterstain.)

In the dose-response study, all seven maternal animals survived the surgical and injection procedures. One of the dams delivered prematurely on d 17 of gestation. These fetuses were not included in the study. All of the remaining 62 mouse fetuses survived treatment and were analyzed. Luciferase activity in tissue homogenates of Av1Luc1-treated fetal mice was dose-dependent in all tissues examined. Consistent with  $\beta$ -galactosidase abundance, luciferase activity/ $\mu$ g of total protein was significantly higher in amniotic membranes than in gastrointestinal and respiratory tract tissues. A gradient of luciferase activity, decreasing from proximal to distal, was observed in both the gastrointestinal and respiratory tracts (Fig. 3).

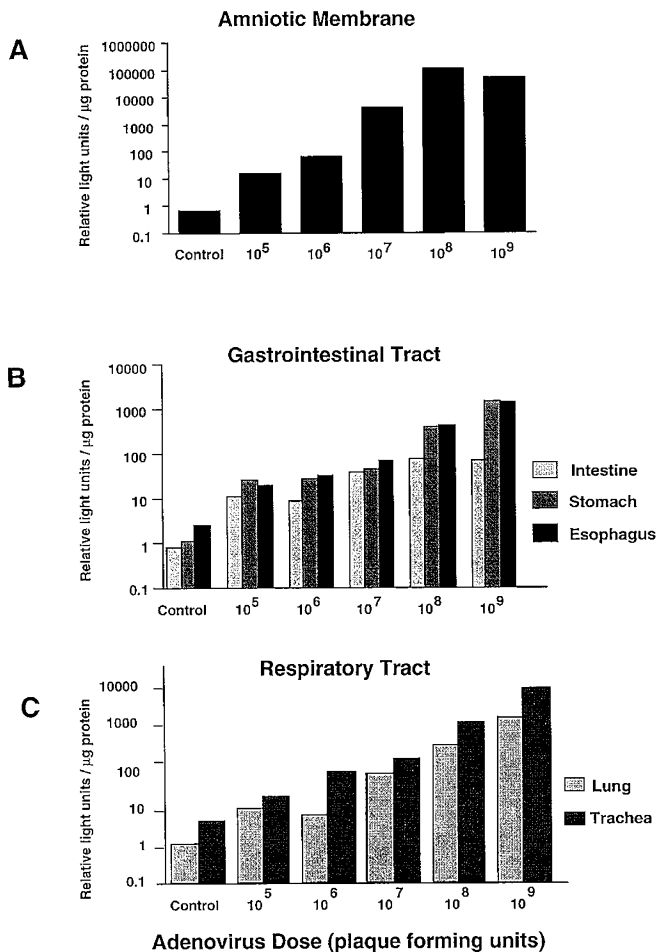
### DISCUSSION

This study demonstrates that gene transfer to fetal tissues is feasible. Administration of a replication-deficient adenoviral gene vector to the amniotic cavity of fetal sheep and mice resulted in expression of the transferred bacterial  $\beta$ -galactosidase and firefly luciferase cDNA in amniotic membranes and gastrointestinal and respiratory tract organs. Abundance of the corresponding products of these genes was greatest in amniotic membranes and significantly less in gastrointestinal and respiratory tract. Expression levels of transferred genes were dose-dependent.

Adenoviral vectors are capable of transducing a variety of epithelial and nonepithelial cells. Replication-deficient human

type 5 adenovirus, which has been demonstrated to be useful for gene transfer *in vivo* to the cotton rat (1, 20), baboons (4), and macaques (22) is an efficient gene vector also in sheep (12, 13, 23) and mouse tissues. Whether its efficiency in various animal models is different from that in humans is not known. In accordance with wild type type 5 human adenovirus, the adenoviral vectors used in these studies can infect respiratory and gastrointestinal tissues. Intraamniotically administered substances diffuse quickly throughout this compartment and even to the most distal parts of the respiratory tract (14–16). Diffusion is probably the main mechanism by which distal airways are exposed, despite the constant outflow of lung fluid into the amniotic cavity. Fetal swallowing of amniotic fluid exposes the surface of the gastrointestinal tract to substances in the amniotic cavity. The results from our studies demonstrate that gene therapy can be initiated before birth by virtue of intraamniotic administration of adenoviral vectors. This approach will be useful in future studies of genetic diseases in mice in which a variety of human diseases has been simulated.

Intraamniotic administration of a replication-deficient adenovirus did not have detectable adverse effects. In the sheep study, all animals completed the procedures. Virus administration did not significantly alter sensitive parameters of fetal well being such as fetal arterial pH,  $P_{CO_2}$ ,  $P_{O_2}$ , hemoglobin concentration, and blood  $O_2$ -saturation over 3 d after treatment. In the fetal mouse study, one premature delivery occurred, but it was most likely due to the surgical procedures and not due to



**Figure 3.** Adenovirus transferred specific gene expression to mouse tissues in a dose-dependent manner. A linear correlation between the dose of Av1Luc1 and luciferase gene expression as determined by luminometric quantitation in pooled fetal mouse tissue homogenates normalized for total protein content was observed. Expression levels in amniotic membranes (A) were significantly higher than in all other tissues. No difference was observed between doses  $10^8$  and  $10^9$  pfu. Luciferase levels detected in gastrointestinal tract (B) and respiratory tract (C) decreased with anatomical distance from the amniotic cavity.

virus-related intrauterine fetal death, because all fetuses of all other dams survived. These preliminary data suggest the absence of short-term adverse effects of intraamniotically administered adenovirus even in relatively high doses used in the mouse study. Long-term effects, however, have not been addressed.

An inflammatory reaction related to treatment with replication-deficient adenovirus has been described by numerous investigators in various organs in a variety of species including fetal sheep (12, 24, 25). Diffuse interstitial thickening, intraluminal neutrophils in the distal airway, and interstitial aggregates of macrophages and lymphocytes were observed in lung tissue of fetal sheep treated intratracheally with Av1LacZ4 (12). However, an inflammatory response was not demonstrated in any organ in the present study. In fetal sheep this observation can be attributed to the fact that tissues were harvested only 3 d after virus administration, probably before the development of an inflammatory response. The amniotic

fluid volume is large relative to the injected volume and diluted the virus concentrate by as much as 1000 times. Furthermore, the low number of cells expressing the transferred gene in organs other than amniotic membrane might not be sufficient to produce a significant inflammatory response. In fetal mice, the absence of an inflammatory response may be related to immunologic immaturity of the fetal mouse that persists into early postnatal life (2).

Bacterial  $\beta$ -galactosidase and luciferase were expressed at various levels in the organs analyzed. Because adenovirus was instilled into the amniotic cavity, it is not surprising that expression levels in amniotic membrane were significantly higher than in any other organ. The variable density of cells expressing bacterial  $\beta$ -galactosidase detected with histologic methods in sheep amniotic membranes may be related to naturally occurring tissue folds creating areas less accessible to the virus. Furthermore, fetal movements made the precise location of the amniotic cavity catheter at the time of virus injection variable. Even distribution of intraamniotically administered substances normally occurs within a short period of time unless a substance is administered directly onto the hairy surface of the fetus, where the viscosity of amniotic fluid is high and mixing is apparently low (Iwamoto HS, unpublished observation). In sheep the efficiency of transfer of the *lacZ* gene to gastrointestinal and respiratory tract appeared to be low. Higher densities of cells expressing bacterial  $\beta$ -galactosidase in fetal mice are due to the relatively higher dose regarding the distribution volume. More sensitive histologic or biochemical methods, however, might be able to detect expression of the transferred gene in ovine cells where it would not be detected by enzyme-histochemistry. The lack of adverse effects in the present study suggests that higher doses resulting in higher levels of expression of the transferred gene in respiratory and gastrointestinal tract might be feasible. Reliable data about the required level of expression of a transferred gene in a certain organ for a given genetic defect to significantly alter the natural course of a genetic disease are not yet available.

The linear dose response in the luciferase studies indicates that adenoviral constructs are reliable vectors for somatic gene transfer. They also support the intraamniotic route of administration as a method to deliver genes to fetal tissues. The efficiency of this approach, however, appears to be low for tissues other than amniotic membrane. The Rous sarcoma virus promoter used in the adenoviral constructs of the present study directed expression of the transferred gene in numerous cell types. In the future, specific promoters could be used to restrict expression of the transferred gene to specific tissues or cells of interest.

The ability to transfer genes to fetal tissues *in utero* could be useful in the correction of a variety of genetic diseases. In addition to a desired permanent correction of a defect, temporary expression of transferred genes could be sufficient to gain time for other treatments to become feasible (e.g. lung transplant in surfactant protein B deficiency). Genes that are developmentally not expressed yet in immature individuals (such as surfactant proteins) or genes the product of which confers protection from adverse effects of a therapeutic agent (e.g. superoxide dismutase in oxygen injury) could also be trans-

ferred. Gene products efficiently expressed in and secreted from amniotic membranes could be absorbed by the fetus and hold a potential for transferring a therapeutic agent to the fetus.

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