PEGylated Adenoviruses for Gene Delivery to the Intestinal Epithelium by the Oral Route

Xuan Cheng,[1] Xin Ming,[1] and Maria A. Croyle[1,2,3]

Received March 2, 2003; accepted May 20, 2003

Purpose. Adenoviruses are being developed for diseases of the gastrointestinal tract. Several *in vitro* assays were used to predict stability of PEGylated adenovirus along the GI tract and determine *in vivo* gene transfer after oral administration.

Methods. Recombinant adenovirus was modified with monomethoxypoly(ethylene) glycols activated by cyanuric chloride, succinimidyl succinate, and tresyl chloride. Transduction efficiency was assessed on Caco-2 cells. *In vitro* stability of viruses in simulated gastric fluid, pancreatic fluid, and bile was assessed by serial dilution on 293 cells. Transduction efficiency *in vivo* was determined by oral administration of 1×10^{12} particles of unmodified or PEGylated virus to fasted Sprague-Dawley rats.

Results. Titers of unmodified virus declined to undetectable levels after 40 min in simulated gastric fluid while the infectious titer of the modified vectors did not change for 3 h. Similar results were seen with simulated pancreatic fluid. PEGylation also enhanced adenoviral transduction efficiency in Caco-2 cells by a factor of 20. PEGylation enhanced adenovirus transduction efficiency 10- to 40-fold *in vivo* in intestinal segments that do not express significant amounts of adenovirus receptors (jejunum, colon) with transgene expression located in the crypt regions.

Conclusions. PEGylated adenoviruses are suitable gene delivery vehicles for oral administration.

KEY WORDS: adenovirus; PEGylation; intestine; gene therapy; colon.

INTRODUCTION

Colorectal cancer is one of the leading causes of cancer mortality in Western countries. Despite advances in surgical technique, chemotherapy, and radiotherapy, the overall survival rate has remained around 40 to 60% for the past 20 years (1). In recent years, gene therapy has been proposed as an alternative treatment of cancers of the gastrointestinal tract, and several clinical trials have been initiated (2). Results from these studies indicate that several significant issues must be addressed before gene therapy can be applied in standard clinical settings. These include (a) improving the efficiency of gene transfer, (b) targeting of gene transfer to tumor sites and avoiding toxicity to normal tissues, (c) administration of vectors in a convenient, noninvasive manner, and (d) mitigation of the host immune response against the gene delivery vector.

Adenovirus is a suitable gene delivery vector for cancer because it is capable of producing high levels of gene expression rapidly and transiently and has been used in several clinical gene therapy protocols for this purpose (3,4). Even though the gastrointestinal (GI) tract represents one of the largest and most readily accessible targets for a variety of gene delivery and vaccination strategies, gene transfer to the GI tract with viral vectors has been somewhat difficult. Many viral receptors play a role in tight junction formation along the intestinal tract and are unavailable for virus binding and internalization (5,6). Formulation of adenovirus in mucolytics and dispersing agents to enhance cellular contact and nonspecific internalization of viral particles significantly improved viral transduction of differentiated intestinal epithelial cells *in vitro* and *in vivo* (7,8). Pretreatment with medicinal agents to reduce intestinal motility and distension of isolated intestinal segments with large fluid loads have also improved adenovirus transduction efficiency *in vivo* (9,10).

Despite these improvements, oral administration of adenoviral vectors in the context of gene delivery to the intestine has not been actively pursued. In order to protect the virus from the oppressive environment of the upper GI tract, the transduction efficiency of adenoviral vectors has been assessed only after surgical instillation into isolated portions of the intestine (5–10). Several groups have shown that recombinant adenoviral vectors are unstable at acidic pH, and a significant fraction of the dose could be lost on contact with stomach contents (11,12). Proteolytic and digestive enzymes present in the intestinal tract may continue to inactivate the virus because viral capsid proteins are susceptible to digestion *in vitro* (13). In addition, the presence of bile salts in bile could induce aggregation of adenoviral particles, rendering them uninfectious (14).

We have previously found that covalent attachment of activated monomethoxypoly(ethylene) glycols (MPEGs) to free lysine groups on the protein capsids of adenoviral vectors enhances virus stability under stressed storage conditions (15). We have also found that the transduction efficiency of viruses modified in this manner is not significantly compromised (15,16). From this information, we thought that PEGylated adenovirus would be a suitable test candidate for oral gene delivery. This report describes our initial efforts to develop a noninvasive method for gene delivery to the intestine. We assessed the transduction efficiency of PEGylated adenovirus in monolayers of differentiated intestinal epithelial cells. We then systematically assessed the stability of the modified virus *in vitro* in preparations that simulate biologic fluids encountered along the GI tract. As a final test, we administered unmodified and PEGylated viruses orally to immunocompetent animals and assessed the level of gene expression along the entire gastrointestinal tract.

MATERIALS AND METHODS

Production of Conjugated Adenoviral Vectors

First-generation adenovirus-expressing β-galactosidase under the control of a CMV promoter was amplified in 293 cells (ATCC CRL-1573) from secondary lysates by banding twice on CsCl gradients according to established methods (5). Protein content of each preparation was determined by a microplate assay with Bio-Rad DC protein assay reagents and bovine serum albumin as a standard.

Three types of activated monomethoxypoly(ethylene)

[1] College of Pharmacy, Division of Pharmaceutics, The University of Texas at Austin, Austin, Texas 78712.
[2] Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, Texas 78712.
[3] To whom correspondence should be addressed. (e-mail: macroyle@mail.utexas.edu)

0724-8741/03/0900-1444/0 © 2003 Plenum Publishing Corporation

glycol (MPEG, MW 5000) were used in this study: tresyl-MPEG (TMPEG), succinimidyl succinate MPEG (SSPEG), and cyanuric chloride MPEG (CCPEG). All were obtained from Sigma Chemicals (St. Louis, MO). Conjugation reactions were performed as described previously for 2 h at 25°C with gentle stirring (15). Ten micrograms of PEG was added for each microgram of protein present in each preparation. Reactions were stopped by addition of 10× L-lysine (Sigma Chemicals) with respect to the amount of PEG added. Unreacted PEG, excess lysine, and reaction byproducts were eliminated by buffer exchange over a Sephadex G-50 column equilibrated with 10 mM potassium buffered saline (KPBS) pH 7.4. Fractions containing virus were identified by absorbance at 260 nm, and concentrations determined according to the method of Maizel *et al.* (17). A fluorescamine assay was used to confirm a consistent coupling efficiency of approximately 70 to 80% for each lot of virus used in these studies (15).

Plaque Assays

Viral preparations were serially diluted in DMEM supplemented with 2% fetal bovine serum (FBS, BioWhittaker, Walkersville, MD) and 0.25 ml of each dilution placed on monolayers of 293 cells for 2 h at 37°C. Cells were overlaid with 0.8% agarose (SeaPlaque, BioWhittaker Molecular Applications, Rockland, ME), in Modified Eagle's Medium (Gibco Life Technologies, Grand Island, NY) containing 2% FBS and 10 mM $MgCl_2$ according to established protocols (18). Plaque-forming units (pfus) were calculated according to the following formula:

$$\text{pfu/ml} = \text{average no. of plaques} \times \text{dilution factor} \times 4.$$

The particle to pfu ratio of PEGylated preparations used in the studies outlined below was consistently lower than the unmodified virus by a factor of 4. The average particle to pfu ratio for unmodified preparations was 450 ± 50:1, and that of the PEGylated preparations was 112 ± 20:1.

Assay for Detection of Replication-Competent Adenovirus

A two-cell-line bioassay was performed on each viral preparation to determine the presence of replication-competent adenopvirus (RCA) according to an established protocol (19). One RCA event was detected for every 3×10^{12} virus particles tested.

In Vitro Transduction Studies

Caco-2 cells (ATCC HTB37, passages 53–60) were seeded at a density of 1×10^4 cells/well in either 12-well culture dishes or 6-well plates equipped with porous polycarbonate cell culture inserts with a pore size of 0.4 μm and a surface area of 4.7 cm^2 (Costar Transwell, Cambridge, MA). Medium [Dulbecco's Modified Eagle's Medium (Cellgro, Mediatech, Herndon, VA) containing 10% FBS, 1% nonessential amino acids (Gibco Life Technologies, Grand Island, NY), 1 mM sodium pyruvate (Life Technologies), 1% L-glutamine (Gibco), and penicillin (100 U/ml) and streptomycin (100 μg/ml) (Mediatech)] was changed every other day until monolayers became fully differentiated (15 days after seeding). Virus was added to monolayers at a concentration of 50 viral particles/cell. After incubation at 37°C for 2 h, the virus preparation was removed, replaced with 2 ml maintenance medium, and the infection allowed to continue for 48 h. TEER measurements were taken from cells seeded on Transwell inserts by an epithelial voltohmeter with a special "chopstick" electrode designed for this purpose (World Precision Instruments, Sarasota, FL). Transgene expression was detected by X-gal staining and visual inspection of positive cells. Cells were stained for a maximum of 4 h to minimize detection of endogenous β-galactosidase activity.

Receptor-Blocking Studies

Caco-2 cells were seeded in 12-well plates as described, and infections performed 3 days after seeding. Virus was at a concentration of 50 virus particles/cell. β-Galactosidase expression was assessed 48 h after infection by X-gal staining. Anti-coxsackievirus and adenovirus receptor (CAR) antibody (1:50 dilution, Upstate Biotechnology, Waltham, MA) or 1.7 mM GRGDSP peptide (CNbiosciences, Inc., La Jolla, CA) to block α_{v3} receptors in culture medium containing 2% FBS was added to monolayers for 1 h at 4°C before addition of virus according to established methods (5,20). Interaction with heparan sulfate proteoglycans was blocked by incubating virus in DMEM containing 10 μg/ml heparin (sodium salt from porcine intestine, Sigma Chemicals, St. Louis, MO) and 0.1% FBS for 1 h at 37°C before addition to monolayers according to established methods (21). For triple blocking studies, virus incubated with heparin was added to cells pretreated with blocking reagents for both CAR and integrin receptors as described above.

Stability Studies

All viral preparations used in stability studies were at a concentration of 1×10^{12} virus particles/ml. At each time point, 50-μl aliquots were taken from each preparation and added to DMEM containing 2% FBS to assess viral titer by limiting dilution/lac-forming assays as described below.

Gastric Acid

Simulated Gastric Fluid, USP (7 ml 1 N HCl, 2 g NaCl, and 3.2 g pepsin/liter, pH 1.2) was purchased from Sigma Chemicals (St. Louis, MO). One milliliter of virus was added to 4 ml of simulated gastric fluid, based on the physiology of the rat (22). This mixture was placed at 37°C for a period of 3 h. Samples were taken every 10 min for 1 h, then every 30 min for the remaining 2-h period.

Pancreatic Fluid

Simulated pancreatic fluid (20% trypsin, 0.5% phospholipase A, 16% chymotrypsin, 5% amylase, 11% carboxypeptidase A, 3.1% elastase in a 140 mM sodium bicarbonate buffer, pH 8.5) was prepared with reagents from Sigma Chemicals according to descriptions of pancreatic secretions of the rat (22). One milliliter of virus was mixed with 3 ml of simulated pancreatic fluid, based on rat physiology. The mixture was incubated at 37°C for a period of 3 h. Samples were taken every 5 min for 20 min then every 10 min for the remaining 2-h period.

Bile

Bile was collected by cannulation of the bile duct of one male Sprague-Dawley rat (350 g) according to established methods (23). Virus was added to bile at a ratio of 1:2 (volume virus: volume bile) according to published rat bile secretion rates (22). These samples were incubated at 37°C for a period of 30 min. Samples were taken every 10 min and assessed for virus titer.

Limiting Dilution/Lac-Forming Assays

The term lac-forming unit (lfu) describes the amount of virus present in a preparation that can infect cells and induce expression of the *E. coli* β-galactosidase gene at levels sufficient for visual identification of blue reaction product resulting from enzymatic cleavage of the chromogenic substrate, X-gal. Samples of virus were serially diluted in DMEM supplemented with 2% FBS and 0.2 ml of each dilution placed on monolayers of 293 cells for 2 h at 37°C. Two milliliters of complete medium was added to each well, and the infection was allowed to progress for 20 h at 37°C. Cells were stained for β-galactosidase expression as described previously (11). Lac-forming units were calculated using the following formula:

$$\text{lfu/ml} = z \times a \times d$$

z = 845 a magnification constant.

a = The average number of blue lac$^+$ cells from triplicate wells.

d = The dilution of the sample.

Oral Administration of Adenovirus

All procedures were approved by the Institutional Animal Care and Use Committee of The University of Texas at Austin. Male Sprague-Dawley rats (10 weeks old, Harlan Sprague Dawley, Indianapolis, IN) were fasted for 24 h before vector administration with free access to water. One milliliter of virus (1×10^{12} particles/ml) was administered to each animal via a tuberculin syringe affixed to a 3-inch 18-gauge curved feeding needle (Popper & Sons, Inc., New Hyde Park, NY) placed deep into the esophagus. A separate group was dosed in a similar manner with 1 ml potassium phosphate-buffered saline as a vehicle control. Twenty-four hours after administration, rats were euthanized with sodium pentobarbital (Sigma Chemicals, St. Louis, MO), and specific tissues of the gastrointestinal tract (esophagus, stomach, duodenum, ileum, jejunum, colon, and liver) were harvested and washed twice in cold PBS. Tissue was split open longitudinally and rapidly immersed in disposable peel-away molds containing OCT (Optimal Cutting Temperature) compound (Sakura Finetek USA, Torrance, CA). Frozen tissues were stored at –80°C until sectioned.

Additional tissue samples were placed in cold DMEM on ice and homogenized in 1ml lysis buffer (provided with the Beta-Gal ELISA kit; Roche Applied Science, Indianapolis, IN) as described previously (16). Tissues were homogenized using a Brinkman polytron. Extracts were centrifuged at 3500 $\times$ *g* for 10 min, and protein concentration of cleared supernatants determined by a microplate assay with Bio-Rad DC protein assay reagents and bovine serum albumin as a standard. Extracts were quick-frozen in a dry ice/ethanol bath and stored at –80°C until assayed. β-Galactosidase concentrations were determined with an enzyme-linked immunosorbent assay (ELISA) kit (Roche Applied Science, Indianapolis, IN) according to manufacturer's instructions.

X-gal Histochemistry

Frozen sections (6–8 μm) were fixed in 0.5% glutaraldehyde and stained for β-galactosidase expression as described (24). Sections were incubated in the stain for no longer than 6 h to minimize detection of endogenous β-galactosidase activity, counterstained with eosin (Fisher Diagnostics, Middletown, VA), observed with a MicrosOptics IV900 microscope and photographed using a Nikon Coolpix 4500 digital camera and Nikon View software.

RESULTS

Effect of PEGylation on Adenovirus Transduction Efficiency in Differentiated Enterocytes *in Vitro*

We have found previously that PEGylation did not significantly compromise adenovirus transduction efficiency *in vitro* and *in vivo* in several target tissues (15,16,24). In order to determine if this technology could produce similar results in the intestinal tract, the transduction efficiency of the modified viruses was assessed in differentiated Caco-2 cells. Approximately 0.5% of the cells were transduced by the unmodified virus (Fig. 1A). Most of the positive cells were located near raised domed regions of the monolayer (data not shown). Modification of adenoviral capsids with TMPEG significantly increased transduction efficiency 16-fold so that 8.0 ± 0.8% of the cells expressed the transgene (Student's *t* test, $p \leq 0.001$). Viral preparations modified by CCPEG and SSPEG effectively transduced 9.4 ± 2.2% and 10.2 ± 0.4% of the differentiated enterocyte population, respectively (Fig. 1A).

Transepithelial electrical resistance (TEER), a measure of monolayer integrity and an indirect assessment of viral infection (25), was assessed in monolayers seeded on Transwell culture inserts after application of each viral preparation (Fig. 1B). Monolayer integrity was significantly compromised by the most effective preparations (CCPEG and SSPEG), as they reduced electrical resistance by 1000 and 650 $\Omega \times \text{cm}^2$ respectively over a 3-h treatment period (Student's *t* test, $p \leq 0.001$). The TMPEG preparation, however, reduced electrical resistance by only 400 $\Omega \times \text{cm}^2$, approximately half of that seen with unmodified virus (250 $\Omega \times \text{cm}^2$).

In Vitro Assessment of the Stability of PEGylated Adenovirus in Components of the Gastrointestinal Tract

Gastric Fluid

Simulated Gastric Fluid USP is routinely used in quality control testing to assess the release profiles of various oral pharmaceutical dosage forms (26). Because this represents the medium that adenovirus, formulated as a liquid preparation, would initially come in contact with after oral administration, we chose this medium to first assess the physical stability of the modified viruses. At 37°C, titer of unmodified virus dropped by 2 logs from 8×10^{10} lfu/ml to 7.9×10^8 lfu/ml

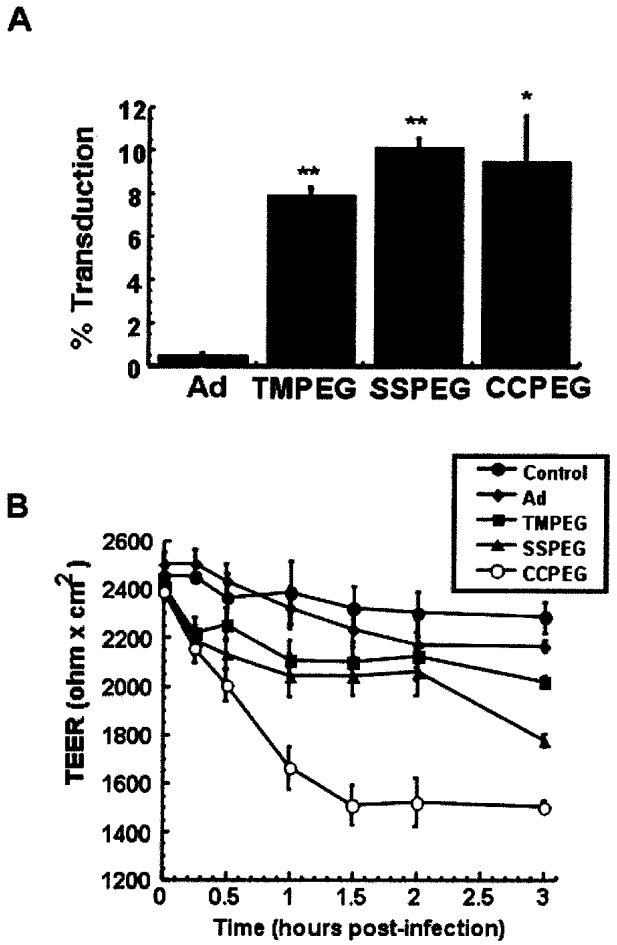

Fig. 1. A. PEGylation enhances adenoviral transduction efficiency in differentiated enterocytes. Adenoviral preparations were added to Caco-2 cells (passage 53) at a concentration of 50 virus particles/cell. Percent transduction is the number of transduced cells relative to the total cellular population. $*p \leq 0.05$, $**p \leq 0.001$, Student's t test. B. PEGylated adenoviral vectors significantly affect transepithelial electrical resistances (TEER) of differentiated monolayers. TEER, a measure of monolayer integrity, was assessed in differentiated Caco-2 cells seeded on Transwell culture inserts. TEER values were determined by subtracting measured TEER values from the intrinsic resistance of the membrane. Data are reported as measured resistance × surface area of the membrane. In both panels, error bars represent the standard deviation of the data (n = 6).

after 20 min and to undetectable levels within 40 min in this medium (Fig. 2). The TMPEG preparation demonstrated an initial drop of 1 log in titer from 5.8×10^{10} to 6.0×10^{9} lfu/ml after the first 20 min in the acidic medium. This preparation did not experience an additional drop in titer until 90 min later, when it fell to 1.0×10^{7} lfu/ml. The CCPEG preparation experienced an initial drop in titer of 0.5 log but did not experience additional loss of titer for the remainder of the study period. The SSPEG preparation seemed to be the most acid-resistant preparation, as negligible loss in titer was detected throughout the entire study period (one-way ANOVA with a Bonferonni/Dunn *post-hoc* analysis, $p = 0.05$).

Pancreatic Fluid

As the virus enters the upper duodenum, it will come in contact with pancreatic fluid, a mixture of digestive enzymes

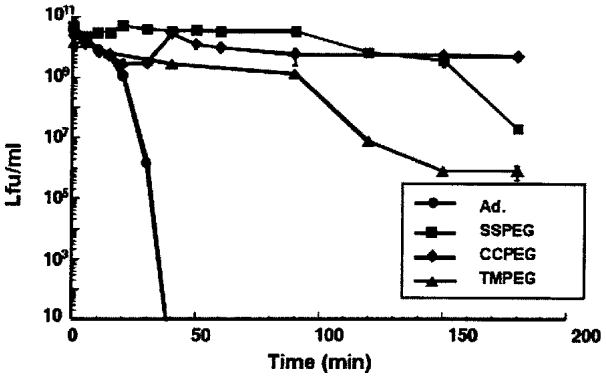

Fig. 2. PEGylated adenovirus is stable in gastric fluid. One milliliter of each viral preparation was added to 4 ml of simulated gastric fluid and incubated at 37°C. Data are the result of two separate experiments (n = 3 each), and error bars represent the standard deviation of the data.

buffered above physiologic pH to neutralize contents as they leave the stomach. After incubation in simulated pancreatic fluid at 37°C for 20 min, titer of the unmodified virus dropped from 7.5×10^{10} lfu/ml to 1.3×10^{10} lfu/ml (Fig. 3). Titer dropped an additional log to 1.5×10^{9} lfu/ml after 50 min. Further loss of titer could not be detected after this time for the remainder of the study. The CCPEG preparation dropped from 7.5×10^{10} lfu/ml to 3.7×10^{10} lfu/ml after the first 20-min incubation period. Titer continued to decline at a steady rate to 1.7×10^{9} lfu/ml after 60 min, after which no additional loss of titer could be observed. Both the SSPEG and the TMPEG preparations experienced negligible losses in titer for a period of 30 min in the digestive medium (one-way ANOVA with a

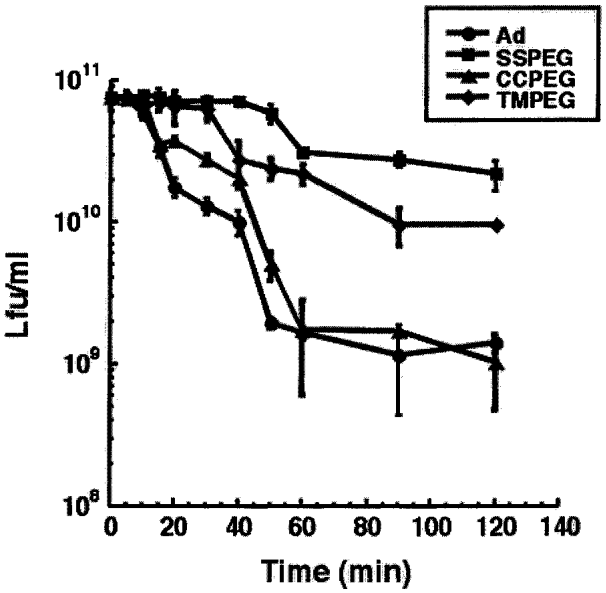

Fig. 3. PEGylation protects adenovirus against degradation from pancreatic enzymes. Simulated pancreatic fluid was prepared as described in Materials and Methods and added to viral preparations (1×10^{12} particles/ml) in a ratio of 1:3 (virus:fluid), based on rat physiology (22). Infectious titer (lfu/ml) was determined by limiting dilution, infection of 293 cells, and X-gal staining. Data are the result of two separate experiments (n = 3), and error bars represent the standard deviation of the data.

Bonferonni/Dunn *post-hoc* analysis, $p = 0.06$). Titer of the TMPEG preparation dropped to 2.7×10^{10} lfu/ml after 40 min and again to 9.7×10^{9} lfu/ml at the end of the 2-h incubation period. The SSPEG virus was clearly the most stable preparation in the pancreatic digestive medium, as infectious titer continued to remain high for 50 min, after which it suffered a drop to 2.8×10^{10} lfu/ml. Thus, this preparation was selected for additional study of its stability in the gastrointestinal environment and subsequent *in vivo* testing.

Bile

In order to determine if bile secretion would hinder the adenoviral transduction efficiency, unmodified adenovirus or SSPEG virus was incubated with freshly isolated bile for a period of 30 min. Transduction efficiency of both viruses was unaffected by the presence of bile (one-way ANOVA with a Bonferonni/Dunn *post-hoc* analysis, $p = 0.07$, data not shown).

Effect of PEGylation on Adenovirus-Mediated Transduction of the Gastrointestinal Tract *in Vivo*

In order to determine how these results from *in vitro* stability studies translate to an *in vivo* situation, fasted male Sprague-Dawley rats were given 1 ml of either unmodified or SSPEG virus orally. Rats dosed in a similar manner with 1 ml potassium phosphate-buffered saline served as a vehicle control. Twenty-four hours after administration, animals were euthanized, and gene expression was assessed along the entire gastrointestinal tract.

Transgene expression was relatively low in the esophagus and stomach as unmodified adenovirus produced 14.5 ± 11.6 and 7.4 ± 1.8 pg β-galactosidase/mg protein in each of these tissues, respectively (Fig. 4). The PEGylated preparation, however, improved transgene expression fourfold in the esophagus (64.3 ± 0.3 pg/mg protein) and produced a 10-fold increase in expression in the stomach (75.4 ± 20.7 pg/mg protein). Of all the tissues analyzed, the duodenum was the region where the unmodified adenovirus produced the greatest level of transgene expression (31.7 ± 20.2 pg β-galactosidase/mg protein). This was not significantly different from that observed in animals treated with the PEGylated preparation (88.2 ± 40.8 pg β-galactosidase/mg protein, Student's *t* test, $p \leq 0.05$).

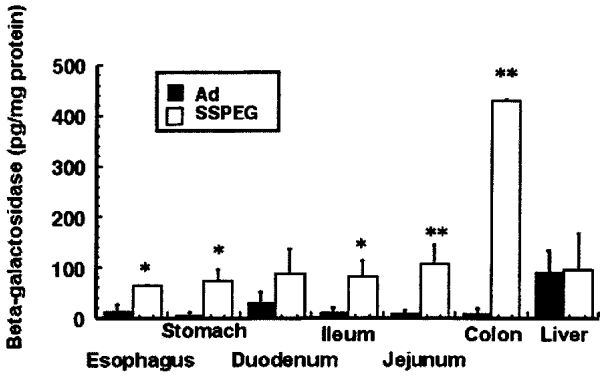

Fig. 4. PEGylation enhances transduction efficiency in parts of the gastrointestinal tract after a single oral dose of an adenoviral vector. Groups (n = 4) of male Sprague-Dawley rats were dosed with 1 ml (1×10^{12} particles/ml) of either unmodified Ad (Ad), PEGylated Ad (SSPEG), or phosphate-buffered saline via a tuberculin syringe attached to a feeding needle. Twenty-four hours after administration, animals were necropsied, and gene expression was assessed in target organs by a β-galactosidase ELISA assay. Transgene expression could not be detected in any tissues from animals treated with a bolus of potassium phosphate buffered saline. Data are the result of two separate experiments, and error bars represent the standard error of the mean of the data. *$p \leq 0.05$, **$p \leq 0.001$, Student's *t* test. Results marked statistically significant were compared against transgene expression obtained from animals treated with the unmodified virus.

On inspection of tissue sections, it is clear that the PEGylated virus transduced cells deep in submucosal structures (Fig 5I) with gene expression located in crypt epithelial cells of Brunner's glands (inset). In contrast, the unmodified virus transduced cells in the lamina propria of the superficial mucosa (Fig. 5E). Additional studies must be done to confirm the identity of the cell populations transduced by adenovirus in these samples because the lamina propria consists of a mixture of various cell types (fibroblasts, macrophages, lymphocytes, endothelial cells, etc.), many of which may be unsuitable for adenoviral gene delivery strategies.

The PEGylated preparation also increased transduction efficiency in the ileum eightfold (83.1 ± 15.2 pg β-galactosidase/mg protein). Histologic assessment of sections from animals treated with the PEGylated virus revealed that transgene expression was located in cells in the lamina propria (Fig. 5J). β-Galactosidase transgene expression could not be detected in any ileal sections obtained from animals treated with the unmodified virus (Fig. 5F).

The PEGylated preparation also enhanced gene expression by a factor of 10 in the jejunum with a β-galactosidase level of 109.2 ± 34.8 pg/mg protein, whereas the unmodified preparation produced 11.0 ± 3.9 pg/mg in this tissue. Sections from animals treated with adenovirus conjugated to SSPEG revealed that transgene expression was located in cells of the lamina propria (Fig. 5K). Transgene expression could not be detected in sections from animals treated with the unmodified virus (Fig. 5G).

The colon of animals treated with the PEGylated viruses had β-galactosidase levels of 430.2 ± 15.8 pg/mg protein. This was the highest level of gene expression detected throughout the entire gastrointestinal tract and was 40 times higher that that seen with the unmodified vector (11.0 ± 7.6 pg/mg protein). Histologic assessment of this area has revealed that gene expression was located in the crypt epithelial cells (Fig. 5L). Gene expression could not be detected in sections of colon from animals treated with the unmodified virus (Fig. 5H). Each preparation tested produced similar levels of gene expression in the liver (approximately 95 pg β-galactosidase/mg protein). β-Galactosidase gene expression could not be detected either by ELISA assay (data not shown) or histochemical staining in any of the tissues from animals treated with a bolus of potassium phosphate-buffered saline (Fig. 5A–D).

DISCUSSION

This manuscript summarizes our initial efforts to determine if conjugation of poly(ethylene) glycol to the capsid proteins of a recombinant adenovirus would offer significant advantage for gene delivery to the GI tract, a target tissue in which gene expression has been rather unremarkable. In the

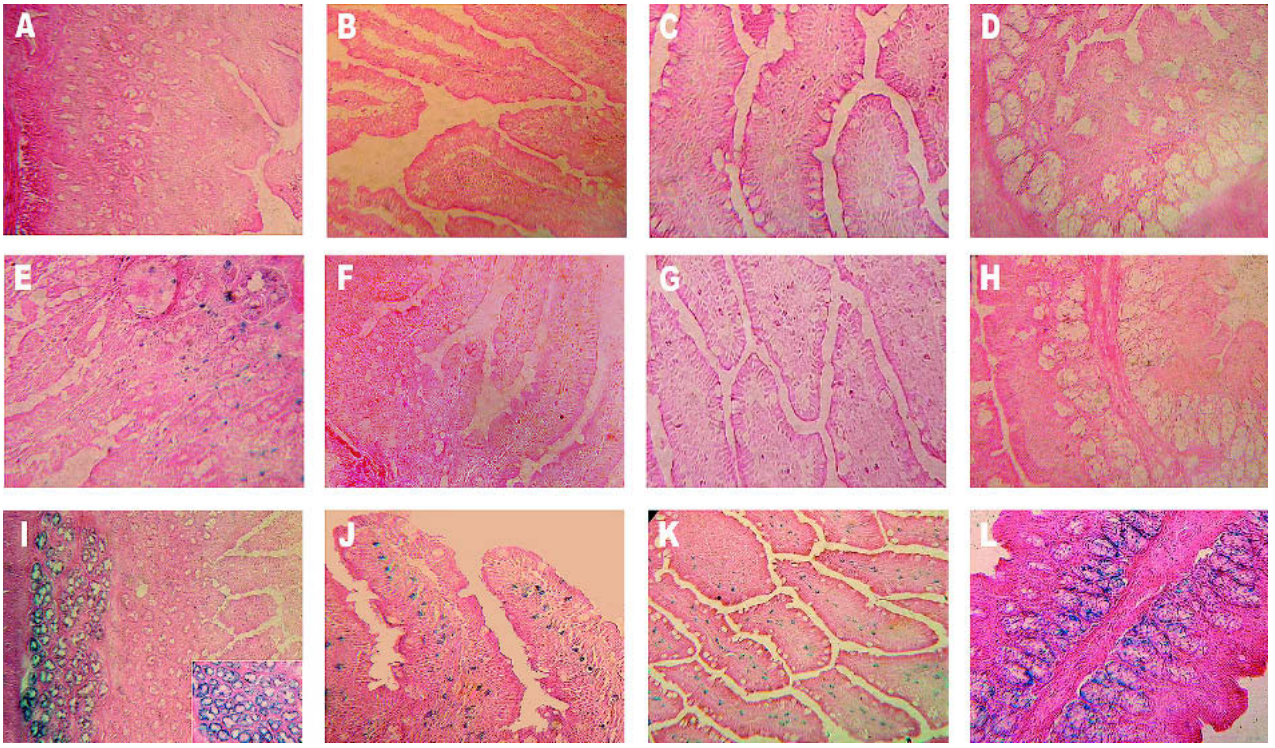

Fig. 5. Gene expression of PEGylated adenovirus along the gastrointestinal tract after a single oral dose. Fasted male Sprague-Dawley rats were dosed orally with either unmodified first-generation recombinant adenoviruses containing the *E. coli* β-galactosidase gene (panels E–H), virus conjugated with SSPEG (panels I–L), or phosphate-buffered saline (panels A–D). Animals were necropsied 24 h after vector administration, and duodenum (first column), ileum (second column), jejunum (third column), and colon (fourth column) were evaluated for lacZ expression by X-gal histochemistry. Morphologic assessment was performed from serial sections of three rats for each treatment group. PEGylated virus produced significant levels of gene expression throughout the entire gastrointestinal tract (panels I–L), with the heaviest area of gene expression located in the colon (panel L). Cells expressing the transgene could be detected only in the duodenum of animals dosed with the unmodified virus (panel E). Gene expression was not observed in the ileum, jejunum, or colon of each animal in this treatment group (panels F–H). Magnification: panels A and I, 100×; panels B–H and J–L, 240×.

studies outlined here, we found that conjugation of monomethoxypoly(ethylene) glycol to adenoviral capsid proteins produced a marked increase in transduction efficiency in the intestine *in vitro* and *in vivo* (Figs. 1, 4, and 5). Although this effect was seen in previous studies of gene delivery to the lung and liver, the reason for this effect was quite perplexing. This modification reduces the negative surface charge of the virus, increases its partition coefficient, and prevents aggregation of virions (15). Even though these characteristics could promote contact with cellular targets, they cannot solely account for the marked transduction efficiency in differentiated enterocytes, especially because PEG is located on fiber, hexon, and penton proteins responsible for virus binding and internalization (unpublished data).

In an effort to determine if PEGylated virus is capable of entering cells in a receptor-independent manner, we treated undifferentiated Caco-2 cells with agents to block receptors associated with adenovirus binding and internalization: the coxsackievirus and adenovirus receptor (CAR), α_v integrins and heparan sulfate proteoglycans (HSPGs) (27) (Fig. 6). Blocking of CAR, α_v integrins, and HSPGs reduced the infectivity of the unmodified virus by 58.4, 67.5, and 75.6%, respectively. When all three receptors were blocked, infectivity of this virus was reduced by 97.8%. The infectivity of the SSPEG virus was unaffected when CAR and α_v integrins were blocked; however, treatment of the virus to prevent in-

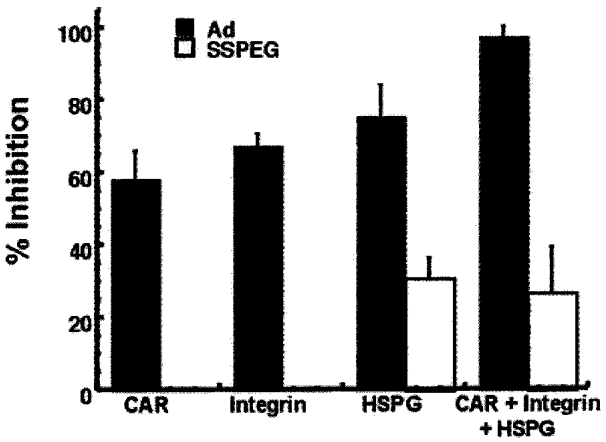

Fig. 6. PEGylation alters affinity of adenovirus for viral receptors on enterocytes. Undifferentiated Caco-2 cells (passage 60) were treated with agents to block receptors associated with adenovirus binding and internalization: the coxsackievirus and adenovirus receptor (CAR), α_v integrins (Integrin) and heparan sulfate proteoglycans (HSPGs). Cells were treated with each reagent at 4°C for 1 h before addition of virus at a concentration of 50 virus particles/cell. Percentage inhibition is the difference between the number of cells transduced in the absence of treatment and the number of transduced cells from a given treatment group. Data reflect the average values from six replicates, and error bars represent the standard deviation of the data.

teraction with HSPGs reduced infectivity by 30.9%. Blocking all three receptors had a similar effect on this virus (Student's *t* test, $p = 0.6$). This suggests that the PEGylated virus enters enterocytes partially through HPSGs. It is important to note that under our conjugation conditions, PEG occupies only 70 to 80% of the available sites on virus capsid proteins. Reactions in which PEG occupied all available sites have significantly compromised the ability of the virus to infect cellular targets (28). Our modification appears to maintain virus infectivity but changes the specificity of the virus and allows it to enter cells in a receptor-independent manner or by another receptor not yet associated with adenovirus infection. Additional studies are under way to explain why the PEGylated virus could transduce differentiated cells and areas of the intestinal tract where expression of these receptors is sparse because of their role in tight junction formation (5,6,29).

We thought that enhanced transduction efficiency alone would not be sufficient for efficient gene transfer to the intestinal epithelium via the oral route. The vector would be exposed to abrupt changes in pH, proteolytic enzymes and other biologic secretions which could inactivate the virus and impede effective gene transfer. *In vitro* testing of the PEGylated preparations in simulated gastric fluid (pH 1.2) revealed that the modified vectors were resistant to this effect while the unmodified virus was inactivated within 40 min (Fig. 2). However, our *in vivo* data from fasted rats revealed that a certain portion of the original dose of unmodified virus did survive the acidic environment of the stomach and transduced segments of the duodenum at levels quantitatively similar to that seen with the PEGylated vector (Fig. 4). This could be because the gastric pH of the rat is 3.5, higher than that of the human (30). However, this virus failed to transduce segments of the lower GI tract. *In vitro* stability of the unmodified virus was significantly compromised in the presence of simulated pancreatic fluid and suggests that the virus could have been degraded by the time it reached the ileum, jejunum, and colon, making transduction of these areas impossible (Fig. 3).

Another significant barrier to effective viral-mediated gene transfer to the GI tract is the presence of the gut-associated lymphoid tissue (GALT) (31). Foreman *et al.* found that areas containing Peyer's patches had a 10-fold higher level of gene expression than areas void of these vesicles (8). We harvested tissue that did not contain Peyer's patches. We did notice that at the time of necropsy intestinal segments of animals dosed with the unmodified virus were spotted with prominent areas of inflamed Peyer's patches. This phenomenon was not observed in those given the PEGylated vector. A significant portion of the unmodified virus could have been deposited in these lymphatic vesicles while the PEGylated preparation avoided these areas. The immunologic effect of oral administration of PEGylated preparations in the GI tract is not clear at this time but is also currently under investigation in our laboratory.

One of the most significant barriers to effective gene expression in the gastrointestinal tract is the short half-life of intestinal epithelial cells (48–72 h). We chose to assess gene expression *in vivo* 24 h after administration, when we thought the maximal number of transduced cells would be present along the GI tract. Although we did not assess gene expression beyond this time point and, perhaps, did not assess peak transgene expression in any of the tissues studied, it is important to note that the levels of β-galactosidase transgene expression that we observed are comparable to those reported by others using similar methods to assess transgene expression in the rat model 48 h after surgical placement of the virus in the GI tract (8–10). The limited life span of intestinal epithelial cells also dictates that readministration of virus would be required for long-term therapy. The PEGylated virus transduced crypt epithelial cells deep in the submucosal regions of the intestine. These areas are the site of regeneration of cells that line the GI tract and suggest that PEGylation of other viral vectors capable of inducing stable transduction of dividing cells may be suitable for certain applications. This also suggests that these viruses are capable of penetrating deep into the submucoa and may be able to effectively disperse and deliver cytotoxic genes to tumor masses. Currently, studies are under way to assess gene expression for a longer period of time to determine when readministration may be necessary and if significant levels of gene expression can be achieved after a series of oral doses of PEGylated adenovirus.

The purpose of this project was to assess the ability of PEGylated adenovirus to withstand the harsh environment of the GI tract and transduce the intestinal epithelium after a single oral dose in fasted Sprague-Dawley rats. This is the first report in which the stability of a recombinant viral vector was assessed by standard *in vitro* techniques commonly used to predict the absorption profiles of traditional oral dosage forms. This is also the first time the virus was not surgically inserted into a specific area of the intestine but was administered as an oral bolus. The PEGylated preparation produced transgene expression along the entire length of GI tract from the esophagus to the colon. This pattern of gene expression may not be appropriate for many gene therapy applications, especially if cytotoxic transgenes must be expressed. Thus, further efforts must be made to limit transgene expression to specific areas of the intestine. The use of heterofunctional PEG molecules would allow us to physically target the virus to specific regions of the intestine (32). Tissue specific and conditional promoters may also assist us in targeting specific regions of the intestine (33). In addition, pH-sensitive polymeric coatings could be used for regional release in the intestine over time (34). These technologies are currently under study in our laboratories for several applications involving intestinal gene delivery.

ACKNOWLEDGMENTS

We would like to acknowledge Dr. Wallace Baze, D.V.M. of The University of Texas M. D. Anderson Cancer Center at Science Park, Bastrop, Texas for assistance with analysis of tissue histology. We also thank Dr. John Richburg of The University of Texas at Austin Center for Cellular and Molecular Toxicology for use of laboratory facilities to process histologic samples. This work was supported by a Summer Research Assignment award from The University of Texas at Austin Vice President for Research (MAC).

REFERENCES

1. L. A. Ries, P. A. Wingo, D. S. Miller, H. L. Howe, H. K. Weir, H. M. Rosenberg, S. W. Vernon, K. Cronin, and B. K. Edwards. The annual report to the nation on the status of cancer, 1973–1997, with a special section on colorectal cancer. *Cancer* **88**:2398–2424 (2000).
2. M. J. Chen, G. A. Chung-Faye, P. F. Searle, L. S. Young, and

D. J. Kerr. Gene therapy for colorectal cancer: therapeutic potential. *BioDrugs* **15**:357–367 (2001).
3. T. Reid, E. Galanis, J. Abbruzzese, D. Sze, J. Andrews, L. Romel, M. Hatfield, J. Rubin, and D. Kirn. Intra-arterial administration of a replication-selective adenovirus (dl1520) in patients with colorectal carcinoma metastatic to the liver: a phase I trial. *Gene Ther.* **8**:1618–1626 (2001).
4. M. W. Sung, H.-C. Yeh, S. N. Thung, M. E. Schwartz, J. P. Mandeli, S.-H. Chen, and S. L. Woo. Intratumoral adenovirus-mediated suicide gene transfer for hepatic metastases from colorectal adenocarcinoma: Results of a Phase I clinical trial. *Molec. Ther.* **4**:182–191 (2001).
5. M. A. Croyle, E. Walter, S. Janich, B. J. Roessler, and G. L. Amidon. Role of integrin expression in adenovirus-mediated gene delivery to the intestinal epithelium. *Hum. Gene Ther.* **9**: 561–573 (1998).
6. C. J. Cohen, J. T. C. Shieh, R. J. Pickles, T. Okegawa, J.-T. Hsieh, and J. M. Bergelson. The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. *Proc. Natl. Acad. Sci. USA* **98**:15191–15196 (2001).
7. M. A. Croyle, B. J. Roessler, C. P. Hsu, R. Sun, and G. L. Amidon. Beta-cyclodextrins enhance adenoviral-mediated gene delivery to the intestine. *Pharm. Res.* **15**:1348–1355 (1998).
8. P. K. Foreman, M. J. Wainwright, B. Alicke, I. Kovesdi, T. J. Wickham, J. G. Smith, S. Meier-Davis, J. A. Fix, P. Daddona, P. Gardner, and M. T. F. Huang. Adenovirus-mediated transduction of intestinal cells *in vivo*. *Hum. Gene Ther.* **9**:1313–1321 (1998).
9. D. Y. Cheng, J. K. Kolls, D. Lei, and R. A. Noel. *In vivo* and *in vitro* gene transfer and expression in rat intestinal epithelial cells by E1-deleted adenoviral vector. *Hum. Gene Ther.* **8**:755–764 (1997).
10. J. W. Sandberg, C. Lau, M. Jacomino, M. Finegold, and S. J. Henning. Improving access to intestinal stem cells as a step toward intestinal gene transfer. *Hum. Gene Ther.* **5**:323–329 (1994).
11. M. A. Croyle, B. J. Roessler, B. J. Davidson, J. M. Hilfinger, and G. L. Amidon. Factors that influence stability of recombinant adenoviral preparations for human gene therapy. *Pharm. Dev. Technol.* **3**:373–383 (1998).
12. D. K. Hoganson, J. C. Ma, L. Asato, M. Ong, M. A. Printz, B. G. Huyghe, B. A. Sosnowski, and M. J. D'Andrea. Development of a stable adenoviral vector formulation. *Bioprocessing* **1**:43–48 (2002).
13. G. Vellekamp, F. W. Porter, S. Sutjipto, C. Cutler, L. Bondoc, Y. H. Liu, D. Wylie, S. Cannon-Carlson, J. T. Tang, A. Frei, M. Voloch, and S. Zhuang. Empty capsids in column-purified recombinant adenovirus preparations. *Hum. Gene Ther.* **12**:1923–1936 (2001).
14. X. Xie, C. E. Forsmark, and J. Y. N. Lau. Effect of bile and pancreatic juice on adenoviral-mediated gene delivery: implications on the feasibility of gene delivery through ERCP. *Dig. Dis. Sci.* **45**:230–236 (2000).
15. M. A. Croyle, Q. C. Yu, and J. M. Wilson. Development of a rapid method for the PEGylation of adenoviruses with enhanced transduction and improved stability under harsh storage conditions. *Hum. Gene Ther.* **11**:1721–1730 (2000).
16. M. A. Croyle, N. Chirmule, Y. Zhang, and J. M. Wilson. "Stealth" adenoviruses blunt cell mediated and humoral immune responses against the vector and allow for significant gene expression upon re-administration in the lung. *J. Virol.* **75**:4792–4801 (2001).
17. J. V. Maizel, Jr., D. O. White, and M. D. Scharff. The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. *Virology* **36**:115–125 (1968).
18. F. L. Graham and A. J. van der Eb. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**:456–467 (1973).
19. P. Murakami, E. Pungor, J. Files, L. Do, R. Van Rijnsoever, R. Vogels, A. Bout, and M. McCaman. A single short stretch of homology between adenoviral vector and packaging cell line can give rise to cytopathic effect-inducing, helper-dependent E1-positive particles. *Hum. Gene Ther.* **13**:909–920 (2002).
20. J. M. Bergelson, J. A. Cunningham, G. Droguett, E. A. Kurt-Jones, A. Krithivas, J. S. Hong, M. S. Horwitz, R. L. Crowell, and R. W. Finberg. Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. *Science* **275**:1320–1323 (1997).
21. M. C. Dechecchi, P. Melotti, A. Bonizzato, M. Santacatterina, M. Chilosi, and G. Cabrini. Heparan sulfate glycosaminoglycans are receptors sufficient to mediate the initial binding of adenovirus types 2 and 5. *J. Virol.* **75**:8772–8780 (2001).
22. P. E. Sharp and M. C. La Regina. *The Laboratory Rat* (G. J. Krinke, ed.), Academic Press, San Diego, California (2000).
23. H. B. Waynforth and P. A. Flecknell. *Experimental and Surgical Techniques in the Rat.* Academic Press, San Diego, California (1992).
24. M. A. Croyle, N. Chirmule, Y. Zhang, and J. M. Wilson. PEGylation of first-generation recombinant adenoviruses achieves significant gene expression upon intravenous re-administration. *Hum. Gene Ther*. 2002:1887–1900 (2002).
25. R. W. Walters, P. Freimuth, T. O. Moninger, I. Ganske, J. Zabner, and M. J. Welsh. Adenovirus fiber disrupts CAR-mediated intercellular adhesion allowing virus escape. *Cell* **110**:789–799 (2002).
26. H. M. Abdou. *Dissolution, Bioavailability and Bioequivalence*, Mack, Easton, Pennsylvania (1989).
27. J. M. Bergelson. Receptors mediating adenovirus attachment and internalization. *Biochem. Pharmacol.* **37**:975–979 (1999).
28. C. O'Riordan, A. Lachapelle, C. Delgado, V. Parkes, S. C. Wadsworth, A. E. Smith, and G. E. Francis. PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody *in vitro* and *in vivo*. *Hum. Gene Ther.* **10**:1349–1358 (1999).
29. M. Beltig. Heparan sulfate proteoglycan as a plasma membrane carrier. *Trends Biochem. Sci.* **28**:145–151 (2003).
30. T. T. Karali. Comparison of the gastrointestinal anatomy, physiology and biochemistry of humans and commonly used laboratory animals. *Biopharm. Drug Dispos.* **16**:351–380 (1995).
31. P. Brandzaeg. Overview of the mucosal immune system. *Curr. Top. Microbiol. Immunol.* **146**:13–28 (1989).
32. H. Romanczuk, C. E. Galer, J. Zabner, G. Barsomian, S. C. Wadsworth, and C. R. O'Riordan. Modification of an adenoviral vector with biologically selected peptides: a novel strategy for gene delivery to cells of choice. *Hum. Gene Ther.* **10**:2615–2626 (1999).
33. S. M. Cohn, T. C. Simon, K. A. Roth, E. H. Birkenmeier, and J. I. Gordon. Use of transgenic mice to map *cis*-acting elements in the intestinal fatty acid binding protein gene (fabpi) that control its cell lineage specific and regional patterns of expression along the duodenal-colonic and crypt-villus axes of the gut epithelium. *J. Cell Biol.* **119**:27–44 (1992).
34. A. Rubenstein. Approaches and opportunities in colon-specific drug delivery. *Crit. Rev. Ther. Drug Carrier Syst.* **12**:101–149 (1995).