

## Full Text



### Stereotactic Injection of Adenoviral Vectors that Target Gene Expression to Specific Pituitary Cell Types: Implications for Gene Therapy

**Author(s):** Lee, Eun Jig M.D., Ph.D.; Thimmapaya, Bayar Ph.D.; Jameson, J. Larry M.D., Ph.D.

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Division of Endocrinology, Metabolism, and Molecular Medicine (EJL, JLJ), and Department of Microbiology/Immunobiology (BT), Northwestern University

**Institution(s):** Medical School, Chicago, Illinois

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#### Abstract

**OBJECTIVE:** Gene therapy is a potentially useful strategy for the treatment of pituitary adenomas or hormone deficiency disorders. We investigated the feasibility of targeting gene expression to specific pituitary cell types *in vivo*, using a combination of stereotactic injection and adenoviral vectors that carry pituitary-specific promoters.

**METHODS:** Recombinant adenoviruses containing the human growth hormone promoter (AdGHGal) or the human glycoprotein hormone [alpha]-subunit promoter (Ad[alpha]Gal) were used to drive expression of the [beta]-galactosidase gene. The expression of [beta]-galactosidase activity in the pituitary was analyzed after the administration of recombinant adenoviruses via the peripheral vein or the carotid artery, or by stereotactic injection into the rat pituitary. Double-label histology was used to evaluate cell-type expression in the pituitary.

**RESULTS:** Intravascular injection of AdGHGal or Ad[alpha]Gal failed to deliver the marker gene to the pituitary. However, direct stereotactic injection of recombinant adenoviral vectors into the pituitary achieved a high level of transgene expression. In addition, immunohistochemical staining revealed selective expression of the AdGHGal or Ad[alpha]Gal transgenes in pituitary cells that normally produce the respective hormones.

**CONCLUSION:** These findings indicate that adenoviral vectors carrying pituitary gland-specific promoters may be useful for targeted gene therapy of pituitary diseases. However, because of low transduction after peripheral administration, stereotactic injection or local administration of viruses at the time of pituitary surgery is probably required for efficient gene expression.

Replication-deficient recombinant adenovirus vectors represent a highly efficient means of transferring genes *in vitro* and *in vivo*, and they are being used in a wide variety of applications in cell culture, experimental animals, and human gene therapy (2, 3, 7). Adenoviral vectors have also been used in gene delivery into central nervous system tissue; recent observations indicate that direct injection results in a high level of gene expression within the brain and spinal cord (4, 12). For most of these applications, the expressed gene is controlled by ubiquitously active viral promoters, such as the cytomegalovirus (CMV) immediate-early promoter/enhancer, the Rous sarcoma virus long terminal repeat, or the adenovirus-type major late promoter. Although these promoters deliver a high level of expression, they do not allow restricted transgene expression in specific cell types (12).

Pituitary cells, because of their characteristic expression of hormone genes, represent an ideal model for developing targeted expression of specific genes with cell-specific promoters. A variety of pituitary cell-type-specific promoters have been identified. These include the promoters of

the major pituitary hormones as well as receptors for hypothalamic releasing hormones, and a number of transcription factors involved in the development of the pituitary gland. Recently, we generated recombinant adenoviruses containing the human growth hormone (GH) and glycoprotein hormone (GPH) [alpha]-subunit promoters linked to transgenes (19). These promoters were active in vitro in infected pituitary cell lines, as revealed by [beta]-galactosidase expression and by toxicity conferred by expression of the herpes virus thymidine kinase gene. In addition, virally mediated expression of the thymidine kinase gene in GH3 cell line tumors carried in nude mice caused rapid regression of the tumors.

These results raise the possibility that recombinant adenoviruses might be used as an adjunctive treatment of pituitary adenomas, or in the replacement of deficient pituitary hormones. However, a major issue in this field is the need to assess the efficiency and specificity of viral transduction and gene expression in vivo. In this study, we addressed two major questions: 1) Can efficient pituitary cell expression be achieved by peripheral injection, or must the viruses be injected directly into the pituitary gland?; and 2) Do pituitary-specific promoters exhibit a restricted pattern of expression in vivo?

## MATERIALS AND METHODS

### Generation of recombinant adenoviral vectors

The recombinant adenoviral vector has a backbone derived from adenovirus Type 5 (Ad5 309/356), in which the E3 regions have been deleted. A cassette containing the recombinant foreign gene was inserted in place of the E1 deletion. The construction of three different adenoviral vectors (AdGHGal, Ad[alpha]Gal, and AdCMVGal) has been described previously (19). All three vectors contain the *Escherichia coli lacZ* gene encoding [beta]-galactosidase, with a preceding nuclear localization signal driven by either the human GH promoter (AdGHGal), the human GPH [alpha]-subunit promoter (Ad[alpha]Gal), or the CMV promoter (AdCMVGal) (Fig. 1). The human GH promoter consists of nucleotides -336 to +58 of the human GH 5'-flanking region, and the human GPH [alpha]-subunit promoter includes nucleotides -846 to +45 of the human [alpha]-subunit 5'-flanking region. The CMV promoter contains 654 base pairs of the immediate-early enhancer region of the CMV gene. AdCMVGal was used as a positive control for expression and detection of [beta]-galactosidase activity. Individual clones of the recombinant adenoviral vectors were purified and titrated by plaque assays. GH-producing pituitary GH3 cells and gonadotropin [alpha]-subunit-producing [alpha]T3 cells were used as positive controls to detect [beta]-galactosidase expression.

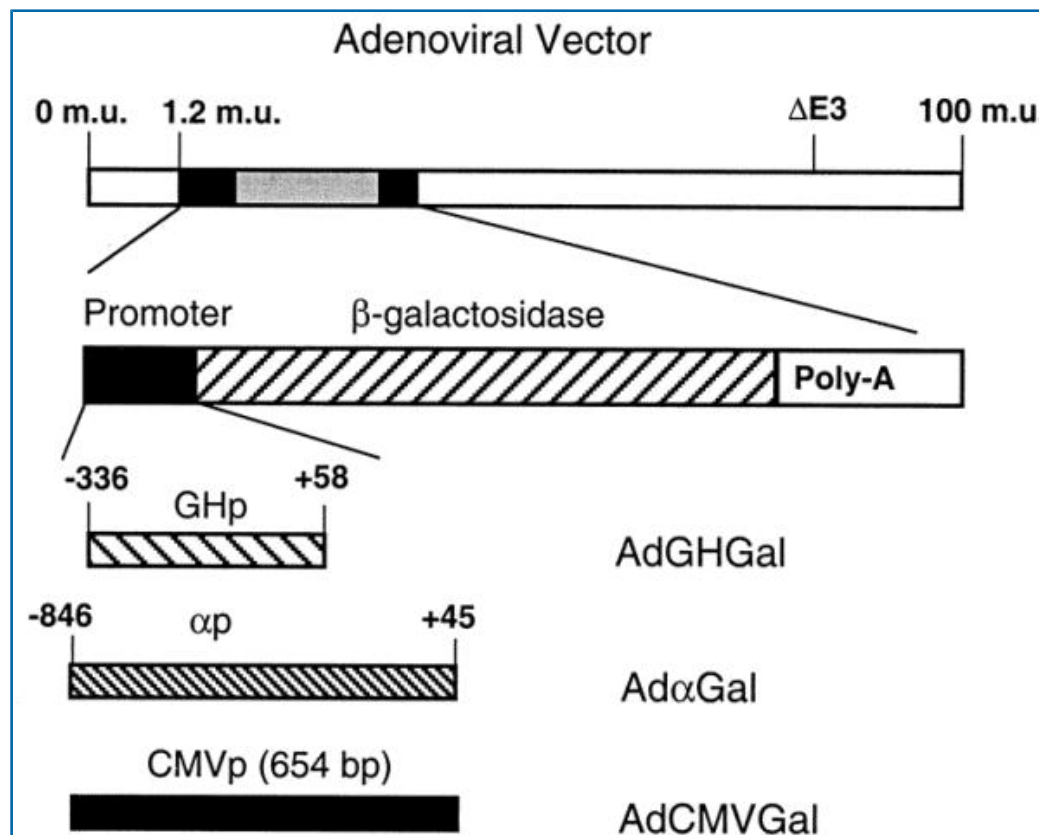


FIGURE 1. Diagrams illustrating recombinant adenoviral vectors. The adenoviral vector contains a backbone derived from adenovirus Type 5 (Ad5 309/356), in which the E1 and E3 regions have been deleted. The genes of interest (*shaded area*) were inserted into the E1 region. Three adenoviral vectors (AdGHGal, Ad[alpha]Gal, and AdCMVGal), each containing different promoters that drive a nuclear-localized form of the [beta]-galactosidase gene, were used in this study. The promoters include the human GH promoter (AdGHGal, *GHp*), the human GPH [alpha]-subunit promoter (Ad[alpha]Gal, *[alpha]p*), and the CMV promoter (AdCMVGal, *CMVp*). *Poly-A*, polyadenylation; *m.u.*, map units; *bp*, base pairs.

Culture of rat pituitary cells and mouse pituitary tissues, and in vitro infection with recombinant adenovirus

Rats (Sprague-Dawley, 200-250 g; Charles River, Wilmington, MA) were killed by CO<sub>2</sub> inhalation and decapitation, and the pituitary glands were

removed under aseptic conditions. The anterior lobes were dissected free from the neural lobes and minced with a surgical blade; pituitary cells were dispersed as described previously (25). Cells were cultured in six-well plates at a density of  $1 \times 10^6$  cells per well and maintained in Dulbecco's modified Eagle's F12 medium, supplemented with 10% fetal bovine serum, penicillin-streptomycin (100 U, 100 g/ml), and amphotericin B (50  $\mu$ g/ml) at 37°C with 5% CO<sub>2</sub>. Thirty-six hours after the cells were plated, infections were performed by the addition of viral solutions (multiplicity of infection [MOI] of 0, 10, 25, and 100 plaque-forming units [PFU]/cell) to cell monolayers, followed by incubation at 37°C for 1 hour, with brief agitation every 15 minutes. After the addition of fresh culture medium, infected cells were returned to the 37°C incubator, and the media were changed 16 hours later. Expression of the [beta]-galactosidase gene was evaluated 4 days after infection.

For cultures of mouse pituitary tissue, 129 Sv/J mice were killed by decapitation, and the pituitary glands were removed. The pituitary glands were washed twice with phosphate-buffered saline, punctured 20 to 30 times with a 25-gauge needle, and incubated in a tube containing adenoviral vectors ( $1 \times 10^9$  PFU) for 1 hour at 37°C. Infected pituitary tissues were cultured in plates precoated with Matrigel (Biocoat; Becton Dickinson, Bedford, MA). The pituitary tissues were removed after 4 days and embedded in optimal cutting temperature medium (OCT) for preparation of frozen sections and evaluation of [beta]-galactosidase gene expression. Animal care and operative procedures were approved by the Animal Care and Use Committee of Northwestern University Medical School and were in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*.

#### Administration of adenoviral vectors through the tail vein and carotid artery

Injections of  $1 \times 10^{10}$  PFU of recombinant adenoviral vectors were given to Sprague-Dawley rats via the tail vein. Rats were killed 5 days after injection, and the pituitary and other organs, including the liver, lung, spleen, heart, and brain, were isolated and embedded in OCT for frozen sections.

For carotid infusion of adenoviral vectors, rats were anesthetized with ketamine (75 mg/kg), xylazine (4 mg/kg), and acepromazine (0.75 mg/kg). The right carotid artery was isolated, a catheter (PE50; Becton Dickinson, Sparks, MD) was inserted, and  $1 \times 10^{10}$  PFU of the recombinant adenoviral vectors were infused at a rate of 15  $\mu$ l/min for 3 hours with an infusion pump. The total infusion volume was 3 ml. Rats were killed 5 days after the infusion, and the pituitary and other organs were isolated and embedded in OCT for frozen sections.

#### Stereotactic injection of adenoviral vectors into the rat pituitary

After induction of anesthesia, rats were placed in a stereotactic frame. After midline incision of the scalp, the vertex area was exposed, and two small openings were made with a dental drill, according to the following coordinates: 2.5 mm rostral to the ear-bar line of the frame and 0.4 mm lateral to the sagittal suture. A stainless steel cannula (30 gauge) was inserted stereotactically through the hole 10.0 mm ventral to the dura to reach the anterior pituitary. The solution containing the adenovirus was infused into both lobes of the pituitary at a rate of 2.0  $\mu$ l/min with a compact infusion pump (Model 975; Harvard Apparatus, Millis, MA). The total infusion volume was 20  $\mu$ l ( $5 \times 10^8$  PFU). The cannula was removed 10 minutes after infusion for perfusion of the adenoviral solution into pituitary tissue. Rats were monitored for 4 to 6 hours after recovery from anesthesia. Animals that exhibited signs of pain or distress were killed immediately. Morbidity or mortality related to the stereotactic injection was uncommon (<5%). Animals were killed 5 days after injection, and the pituitaries were removed and embedded in OCT. Frozen sections were used for X-gal staining, and paraffin blocks were used for immunohistochemistry. Other major organs were also removed and processed for the detection of [beta]-galactosidase gene expression.

#### Quantitation of [beta]-galactosidase activity

Triplicate wells of infected primary pituitary cells were used to measure [beta]-galactosidase activity, with the use of *o*-nitrophenyl [beta]-D-galactopyranoside as a substrate (Sigma Chemical Co., St. Louis, MO). Culture media were aspirated, cell lysis solution was added, and lysates were mixed with the substrate solution and incubated at 37°C for 2 hours. The reaction was stopped with 100  $\mu$ l of 1 mol/L Na<sub>2</sub>CO<sub>3</sub>. Absorption was measured at 405 nm, and [beta]-galactosidase activity was calculated using a standard curve.

For measurement of mouse pituitary [beta]-galactosidase content, each pituitary gland was placed in 150  $\mu$ l of lysate solution. After mechanical dispersion with a tissue grinder, the samples were centrifuged at 12,000  $\times g$  for 5 minutes, and the supernatant was used to assess the [beta]-galactosidase activity as described above. Six pituitary glands were prepared for each viral construct.

#### Histology and immunohistochemistry

Cultured cells were fixed with 1.0% glutaraldehyde for 10 minutes, washed with phosphate-buffered saline (pH 7.4), and incubated with X-gal substrate solution (10.0 mmol/L potassium ferricyanide, 10.0 mmol/L potassium ferrocyanide, 1 mmol/L MgCl<sub>2</sub>, 0.2% Nonidet P-40, and 0.1% X-gal in phosphate-buffered saline) at 37°C for 2 hours. For frozen tissues, 10- $\mu$ m sections were cut and fixed in 4% paraformaldehyde in phosphate-buffered saline for 5 minutes and stained for [beta]-galactosidase activity with X-gal as described previously (5). Immunohistochemistry was also performed to exclude the presence of endogenous [beta]-galactosidase activity using rabbit polyclonal anti-[beta]-galactosidase (Clontech Laboratories, Inc., Palo Alto, CA). Liver tissues from rats given an injection with or without AdCMVGal were used as controls for [beta]-galactosidase expression.

The cell specificity of [beta]-galactosidase expression was assessed by an X-gal enzymatic assay and then immunohistochemistry for individual pituitary hormones. Pituitaries were fixed for 1 hour in 4% paraformaldehyde in pH 7.2 sodium phosphate buffer and incubated at room temperature overnight in X-gal solution (0.01% X-gal). After 3 hours of fixation in buffered formaldehyde, the samples were embedded in paraffin and 5- $\mu$ m sections were prepared. Immunohistochemical analyses were performed with the Histostain-Plus kit (Zymed, San Francisco, CA). After deparaffinization, the specimens were treated with 3% hydrogen peroxide in absolute methanol and preincubated with serum blocking solution

(Zymed). Sections were incubated with primary antibodies specific for pituitary hormones: rabbit anti-human GH (prediluted; Zymed), rabbit anti-human adrenocorticotrophic hormone (ACTH, prediluted; Zymed), mouse anti-human follicle-stimulating hormone (FSH) [beta] (1:250; National Hormone and Pituitary Program, National Institute of Diabetes, Digestive and Kidney Disease [NIDDK], Rockville, MD), rabbit anti-rat luteinizing hormone (LH) [beta] (1:250; NIDDK), rabbit anti-rat prolactin (PRL, 1:500; NIDDK), rabbit anti-rat GPH [alpha]-subunit (1:250; NIDDK), and rabbit anti-rat thyroid-stimulating hormone (TSH) [beta] (1:500; NIDDK). Incubation with primary antibodies was performed for 1 hour at room temperature (GH, ACTH, FSH[beta], and LH[beta]) or overnight at 4°C (PRL, GPH [alpha]-subunit, and TSH[beta]). After washing with triethanolamine-buffered saline/0.025% Tween, staining was performed with biotinylated secondary antibodies and streptavidin-peroxidase (Zymed) according to the manufacturer's protocol. Diaminobenzidine tetrahydrochloride (Zymed) was used as a chromogen.

## RESULTS

### Gene transfer efficiency of AdGHGal, Ad[alpha]Gal, and AdCMVGal in vitro

Infection of primary rat pituitary cells with AdCMVGal was used to test gene transduction efficiency. As shown in [Figure 2 B](#), 95 to 100% of rat primary pituitary cells expressed [beta]-galactosidase 4 days after infection with 10 PFU per cell of AdCMVGal. The cells expressing [beta]-galactosidase included endocrine cells (round morphology) as well as nonendocrine fibroblasts and folliculostellate cells, reflecting the ubiquitously active nature of the CMV promoter. In a previous study of pituitary tumor cell lines ([19](#)), 95 to 100% of GH3 or [alpha]T3 cells expressed [beta]-galactosidase 4 to 5 days after infection with 10 PFU per cell of AdGHGal or Ad[alpha]Gal. Therefore, 10 PFU per cell of the adenoviral vectors were used as the lowest dose in primary pituitary cell cultures. With the GH promoter-driven adenovirus, AdGHGal (MOI of 10 PFU/cell), approximately 10% of primary pituitary cells were positively stained 4 days after infection ([Fig. 2 C](#)). With the GH promoter, the pituitary cells expressing [beta]-galactosidase appeared to consist exclusively of round endocrine cells. The percentage of cells expressing [beta]-galactosidase reached a plateau, with an MOI of 10 PFU per cell; infection with a greater amount of AdGHGal did not increase the number of cells expressing [beta]-galactosidase. With the [alpha]-promoter-driven adenovirus, Ad[alpha]Gal, 10 to 15% of pituitary cells were positively stained at 4 days, and the positive cells were also predominantly round endocrine cells ([Fig. 2 D](#)). [beta]-Galactosidase activity of primary pituitary cells infected with AdGHGal or Ad[alpha]Gal was less than 1% of that seen with AdCMVGal, reflecting the broader expression and greater activity of the CMV promoter ([Fig. 3 A](#)).

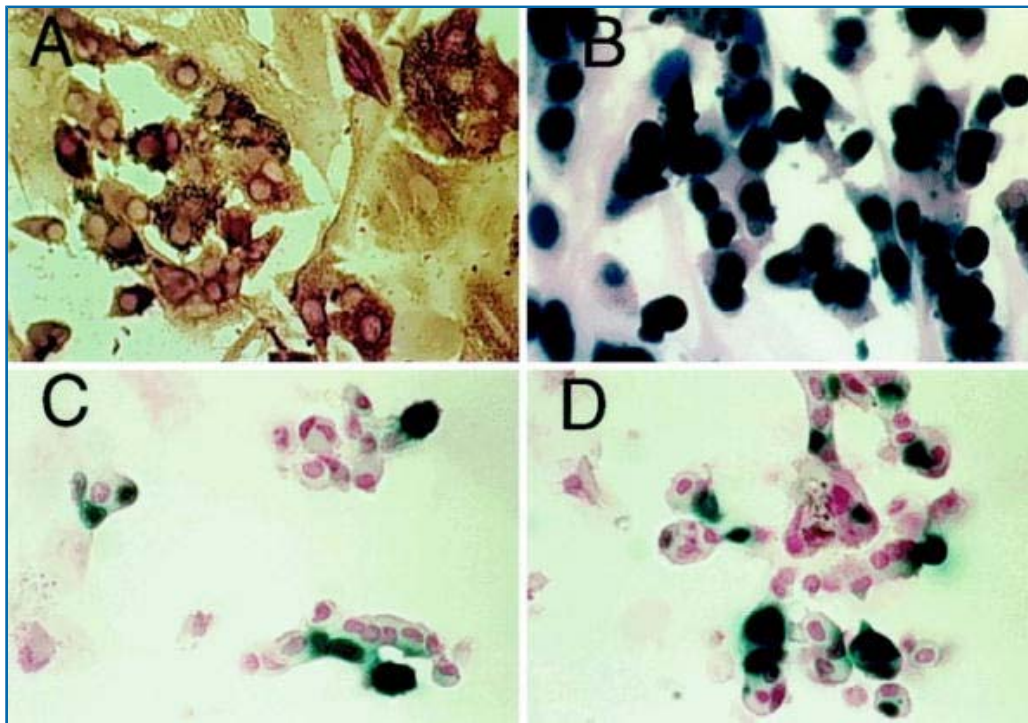


FIGURE 2. Photomicrographs revealing expression of [beta]-galactosidase in primary rat pituitary cells infected with recombinant adenoviral vectors (MOI of 10 PFU/cell). Four days after infection, cells were fixed and incubated with X-gal substrate solution for visualization of [beta]-galactosidase expression. *A*, no virus (original magnification,  $\times 630$ ); *B*, AdCMVGal (original magnification,  $\times 630$ ); *C*, AdGHGal (original magnification,  $\times 400$ ); *D*, Ad[alpha]Gal (original magnification,  $\times 400$ ).

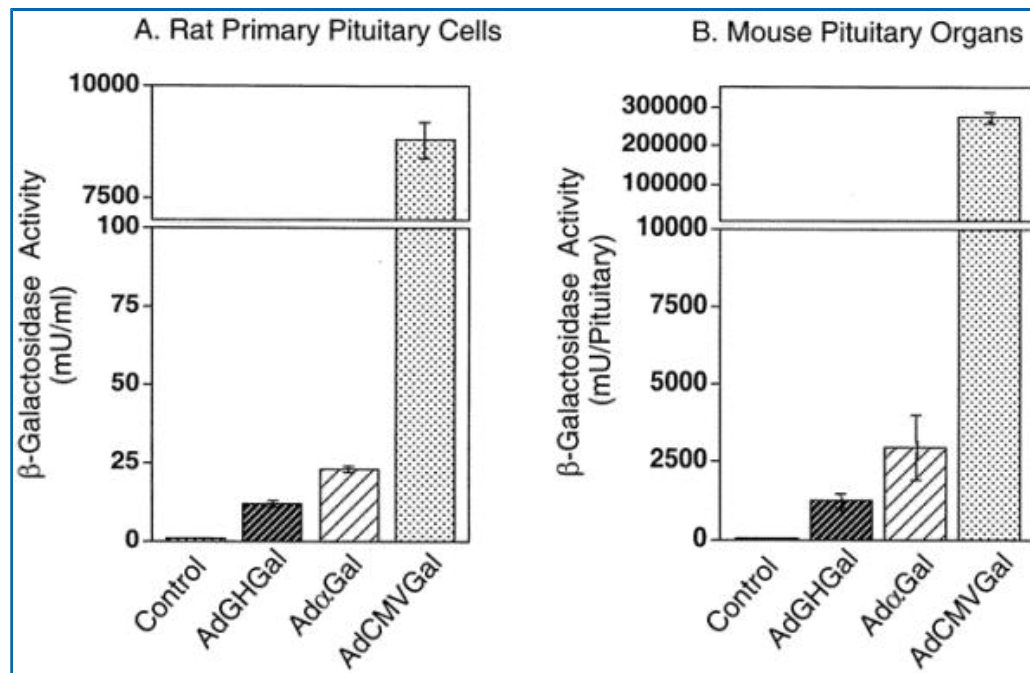


FIGURE 3. Histograms showing quantitation of [beta]-galactosidase activity in (A) rat primary pituitary cells and (B) mouse pituitary glands infected with recombinant adenoviral vectors. The [beta]-galactosidase activity was measured 4 days after infection. Results are expressed as the mean  $\pm$  standard deviation (*bars*).

Explants of whole mouse pituitary glands were prepared to test the ability of the adenoviral vectors to penetrate into tissues and to serve as controls when the adenoviral vectors were injected into the pituitary gland *in vivo*. A high level of [beta]-galactosidase expression was seen in the AdCMVGal-infected mouse pituitary, and expression was mainly localized to cells near the needle tract (Fig. 4 A). With the AdGHGal or Ad[alpha]Gal vectors, the level [beta]-galactosidase expression was lower than that with the CMV promoter-driven vector, although the same amount of virus was used. Positively stained cells were scattered throughout the pituitaries infected with AdGHGal or Ad[alpha]Gal (Fig. 4, B and C). The staining with AdGHGal was slightly lower than that with Ad[alpha]Gal. Quantitative assays revealed that the activity of AdGHGal or Ad[alpha]Gal was approximately 1% of that seen with AdCMVGal (Fig. 3B).

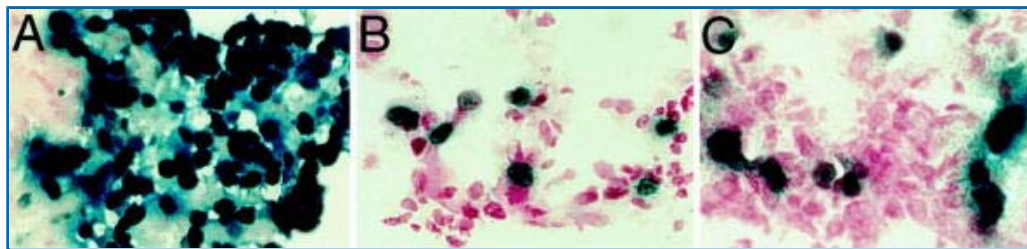


FIGURE 4. Photomicrographs revealing expression of [beta]-galactosidase in mouse pituitary gland explants infected with recombinant adenoviral vectors. Five days after infection ( $1 \times 10^9$  PFU), pituitary glands were embedded in OCT and frozen sections were prepared. Slides were incubated with X-gal substrate solution to visualize expression of the [beta]-galactosidase gene. A, AdCMVGal (original magnification,  $\times 400$ ); B, AdGHGal (original magnification,  $\times 400$ ); C, Ad[alpha]Gal (original magnification,  $\times 400$ ).

#### Expression of the [beta]-galactosidase gene after peripheral injection or carotid infusion of adenoviral vectors

Adenoviral vectors ( $1 \times 10^{10}$  PFU) were injected into the tail vein of adult Sprague-Dawley rats to assess expression *in vivo*. Sections of the pituitary gland and several other major organs were stained for [beta]-galactosidase activity as an index of the specificity of the pituitary promoters. A high level of [beta]-galactosidase gene expression was observed throughout the livers of AdCMVGal-injected rats (Fig. 5 A). Less intense [beta]-galactosidase staining was seen in the glomeruli of kidneys. Scattered expression was observed in the spleen and the heart, with very low expression in the lung, and none in the brain. Similar results have been documented in another study (11). In contrast, in AdGHGal- or Ad[alpha]Gal-injected rats, [beta]-galactosidase gene expression was not detected in the livers (Fig. 5B) or in the other major organs tested. Although AdCMVGal was highly expressed in liver and other tissues, only a few pituitary cells were positive for [beta]-galactosidase expression in AdCMVGal-injected rats (Fig. 5C). No positive pituitary cells were detected after intravenous injection of AdGHGal or Ad[alpha]Gal (not shown). These data suggest that the transfer efficiency of the adenoviral vector to the pituitary gland is very low after intravenous administration. On the other hand, because the tropism of adenoviral vectors for the liver is strong, the absence of expression of AdGHGal or Ad[alpha]Gal in the liver indicates that the GH promoters and [alpha]-promoters restrict transgene expression.

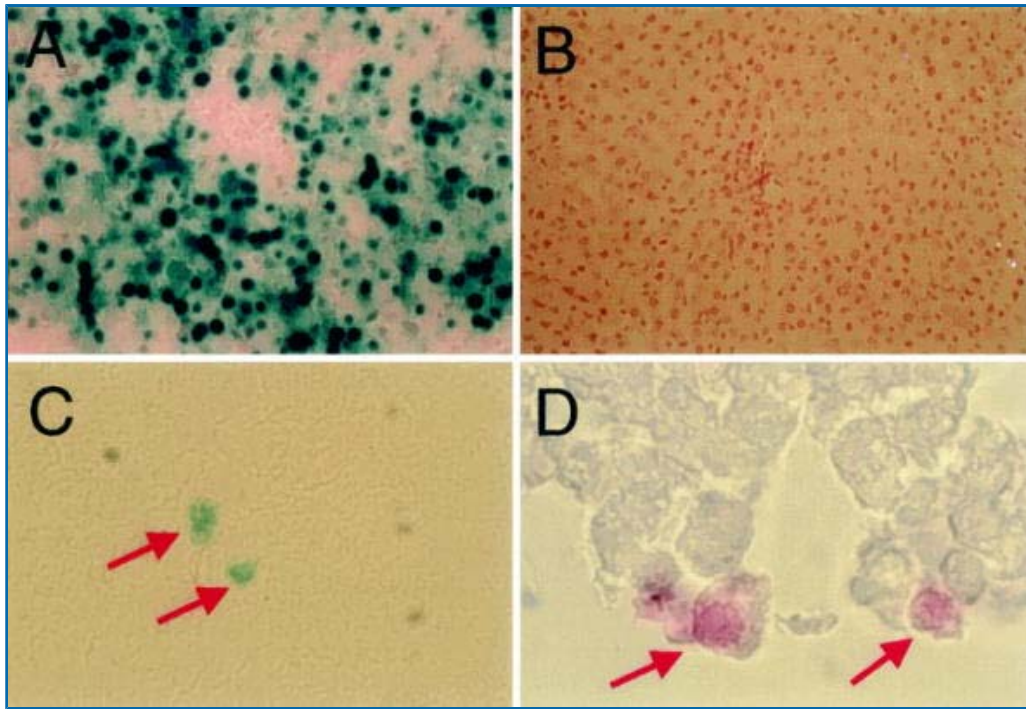


FIGURE 5. Photomicrographs demonstrating expression of the [beta]-galactosidase gene after intravascular administration of recombinant adenoviral vectors. Adult Sprague-Dawley rats received injections of the indicated adenoviral vectors ( $1 \times 10^{10}$  PFU) through the tail vein (A and C) or via the carotid artery (B and D). The pituitary and liver were removed 5 days after injection and examined using X-gal solution (A-C) or immunohistochemistry (D). The sections show liver (A, original magnification,  $\times 200$ ) and pituitary (C, original magnification,  $\times 400$ ) from rats given an injection of AdCMVGal through the tail vein, and liver (B, original magnification,  $\times 200$ ) and pituitary (D, original magnification,  $\times 630$ ) from rats given an infusion of Ad[alpha]Gal through the carotid artery.

In an attempt to deliver a higher concentration of viruses to the pituitary gland, intracarotid arterial infusion of adenoviral vectors was performed. However, expression of the [beta]-galactosidase gene was observed throughout the livers and other peripheral tissues, similar to the results observed with tail vein injection of AdCMVGal in rats (not shown). Immunohistochemistry for [beta]-galactosidase revealed an occasional positive pituitary cell in 5- to 10- $\mu$ m sections of the pituitary after carotid infusion with AdGHGal or Ad[alpha]Gal (Fig. 5D). Thus, the vascular route, whether by the peripheral vein or by the carotid artery, does not provide a practical means to deliver adenoviruses to the pituitary.

#### Expression of the [beta]-galactosidase gene after stereotactic injection of adenoviral vectors into the rat pituitary gland

Adenoviral vectors ( $5 \times 10^8$  PFU) were injected directly into the pituitary gland with a stereotactic device. After 5 days, histological sections of these pituitaries were stained for [beta]-galactosidase activity. As shown in Figure 6, A and B, dense blue staining was observed around the injection site of AdCMVGal. Less intense and more scattered staining was seen after pituitary injection with the same amount of AdGHGal (Fig. 6C) or Ad[alpha]Gal vectors (Fig. 6D).

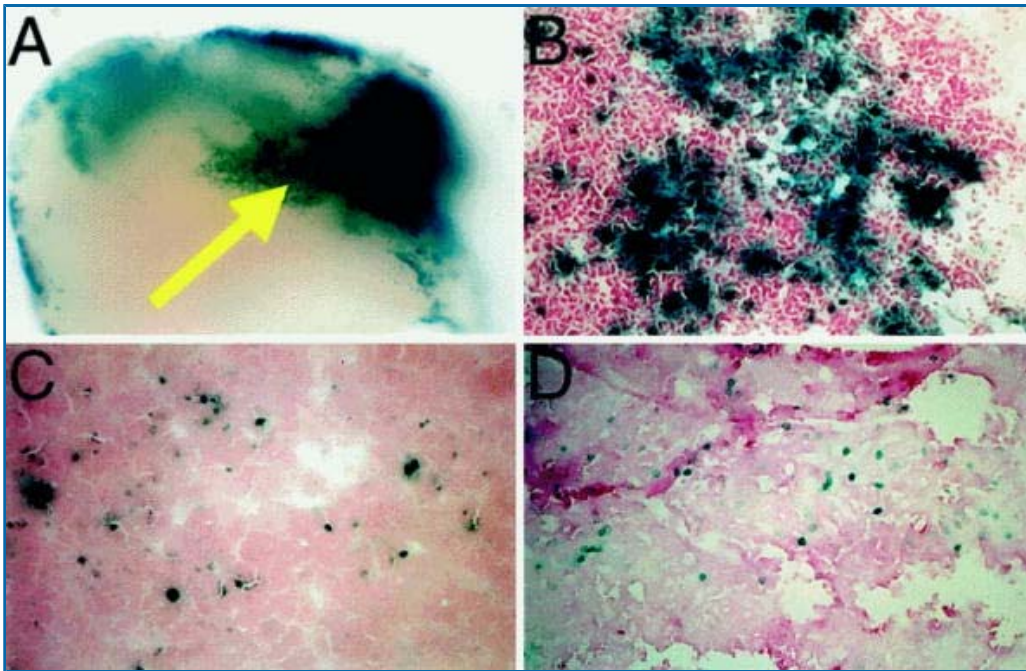


FIGURE 6. Photomicrographs showing expression of the [beta]-galactosidase gene in the pituitary after direct injection of recombinant adenoviral vectors. An injection of  $5 \times 10^8$  PFU of the indicated adenoviral vectors was administered stereotactically into the pituitary gland of adult Sprague-Dawley rats. Pituitaries were removed 5 days after injection and stained in X-gal solution. Low-magnification (original magnification,  $\times 40$ ) section shows AdCMVGal-injected pituitaries (lateral wing, *A*); high-magnification (original magnification,  $\times 200$ ) sections show AdCMVGal-injected (*B*), AdGHGal-injected (*C*), or Ad[alpha]Gal-injected (*D*) pituitaries. The injection site is indicated (*A*, *arrow*).

Immunohistochemical staining was performed in the paraffin-embedded pituitary after injection of AdGHGal or Ad[alpha]Gal, to determine whether the expression of [beta]-galactosidase was specific for different hormone-producing cell types. In the AdGHGal-injected pituitary, most of the cells expressing [beta]-galactosidase were somatotropes (GH), and expression was not seen in corticotropes (ACTH), gonadotropes (LH[beta] and FSH[beta]), or thyrotropes (TSH[beta], not shown) (Fig. 7). However, AdGHGal transgene expression was detected in a single PRL-producing lactotrope cell (not shown). Ad[alpha]Gal transgene expression was detected in pituitary cells that stained positively for the [alpha]-subunit, which is common to the GPHs (Fig. 8). Immunostaining for LH[beta], FSH[beta], and TSH[beta] revealed that these cells were gonadotropes or thyrotropes (Fig. 8). Ad[alpha]Gal transgene expression was also detected in one somatotrope (GH, not shown), but not in corticotropes or lactotropes (Fig. 8).

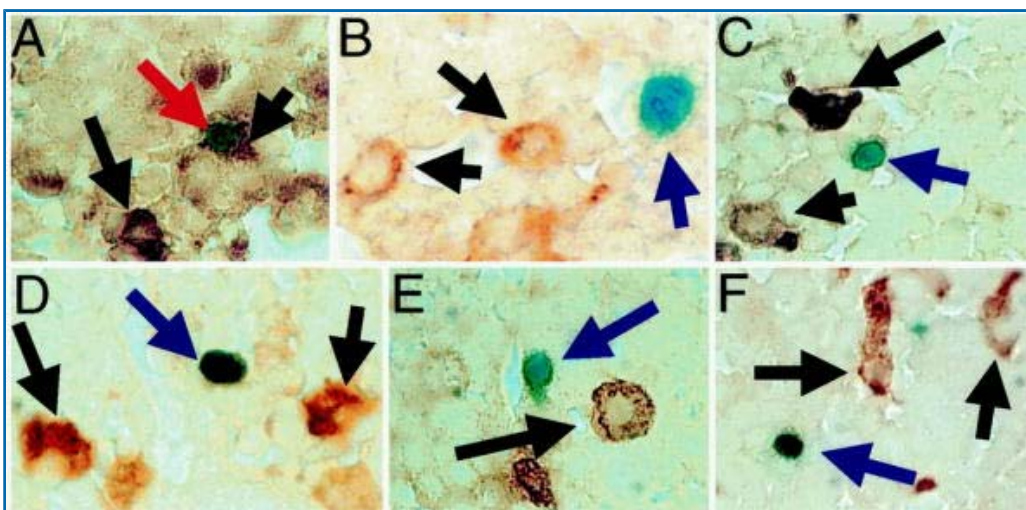


FIGURE 7. Photomicrographs revealing colocalization of pituitary hormones with the AdGHGal transgene. Paraffin sections of [beta]-galactosidase-stained pituitaries after injection with AdGHGal were immunostained with hormone-specific antibodies. Cells with blue nuclei (*blue arrows*) express the AdGHGal transgene. Immunostaining for individual pituitary hormones (brown cytoplasm) is denoted by *black arrows*. Costaining with [beta]-galactosidase and GH is indicated by a *red arrow*. *A*, GH; *B*, PRL; *C*, ACTH; *D*, LH[beta]; *E*, FSH[beta]; *F*, GPH [alpha]-subunit (*A-F*, original magnification,  $\times 1000$ ).

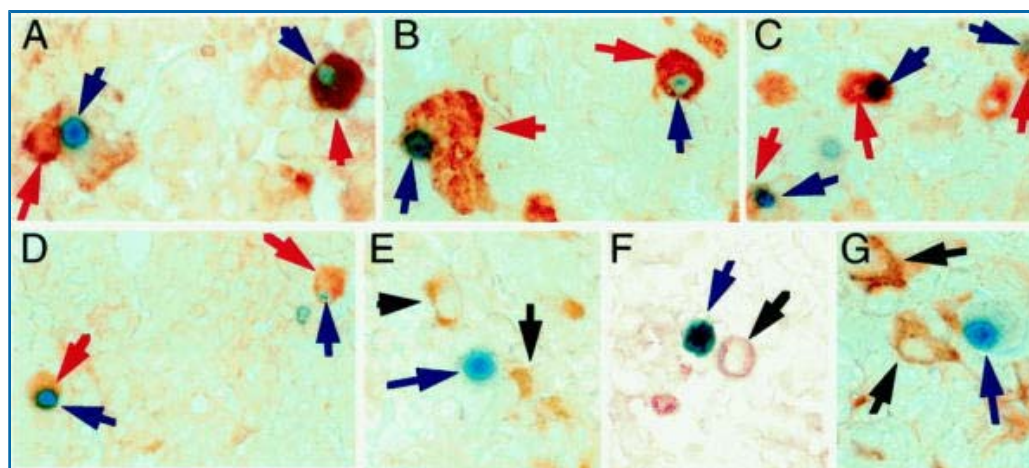


FIGURE 8. Photomicrographs showing colocalization of pituitary hormones with the Ad[alpha]Gal transgene. Paraffin sections of [beta]-galactosidase-stained pituitaries after injection with Ad[alpha]Gal were immunostained with hormone-specific antibodies. Cells with blue nuclei (*blue arrows*) express the Ad[alpha]Gal transgene. Immunostaining for individual pituitary hormones (brown cytoplasm) is denoted by *black arrows*. Costaining with X-gal and GH is indicated by *red arrows*. A, GPH [alpha]-subunit; B, LH[beta]; C, FSH[beta]; D, TSH[beta]; E, GH; F, PRL; G, ACTH (A-F, original magnification,  $\times 1000$ ).

## DISCUSSION

We previously demonstrated that adenoviruses containing the human GH promoters or [alpha]-promoters were capable of conferring cytotoxicity to tumor cell lines when linked to a toxic gene, such as thymidine kinase (19). In the present study, we show that these adenoviruses can efficiently transfer genes to pituitary cells *in vivo* when injected under stereotactic guidance. Together, these studies suggest a potential strategy for the treatment of pituitary diseases *in vivo*. In the case of human pituitary tumors, this type of microinjection could be performed at the time of transsphenoidal surgery. For other pituitary diseases, such as hormone deficiencies, there are greater challenges, including the need to infect renewable progenitor cells and the need to achieve regulated expression. Nevertheless, this study takes an important step toward the goal of gene transfer into individual pituitary hormone-producing cells.

We chose adenoviral vectors for this early phase of research on pituitary gene therapy for several reasons. Adenoviruses have a large genome (~36 kilobases [kb]), which provides the potential to carry relatively large, or multiple, foreign genes. They are easy to propagate and manipulate, and they have a broad host range *in vitro* and *in vivo*. Because adenoviruses do not integrate into the host cell genome, the foreign genes delivered by adenoviral vectors are expressed epichromosomally, and therefore have low genotoxicity for host cells (2, 8, 11).

Despite these advantages, there are several limitations for the use of adenoviruses in the clinical setting. These include cytotoxicity (particularly hepatotoxicity), induction of immune responses, and the potential for recombination *in vivo* (13). We did not see inflammatory changes at the sites of adenoviral injection, but these might have been apparent if analyses had been performed at later times after injection (6). Tissue tropism of the adenovirus is another limitation, particularly when one wishes to achieve tissue-specific effects and to minimize toxicity (12). As shown in this study and others, intravascular administration of these vectors results in a high level of gene transfer to the liver, but other targets, such as the pituitary gland, are not infected efficiently. Several approaches can be used in an attempt to circumvent this problem. Direct administration of adenoviral vectors into a specific organ or tissue can result in efficient infection and a high level of transgene expression (1, 21). In our study, injection into the carotid artery was not sufficient to convey preferential expression in the pituitary gland, presumably because of rapid dilution of the virus and entry into the general circulation. Although it remains possible that better vascular access could improve viral delivery, this is likely to be technically difficult and variable in its efficacy. On the other hand, stereotactic injection directly into the pituitary gland resulted in selective expression in the pituitary, with a reduced level of infection of other organs, such as the liver. However, with a ubiquitous promoter, such as CMV, this injection method does not allow selective expression in specific cell types. For this reason, we used an additional strategy for achieving cell-specific expression, with highly regulated hormone promoters to target expression of the transgene (14). The combination of stereotactic injection and pituitary-specific promoters was capable of restricting transgene expression to specific pituitary cell types.

The choice of promoter fragments for these studies was based in part on known functional sequences involved in expression and cell specificity, but also on practical limitations involving the amount of deoxyribonucleic acid that can be inserted into the modified adenoviral genome. In this study, we used 0.4 kb of the human GH promoter and 0.9 kb of the human [alpha]-subunit promoter. Although these promoters are sufficient to express the [beta]-galactosidase gene *in vitro* and *in vivo*, there was some evidence of leaky expression *in vivo*. For example, we identified a PRL-producing lactotrope that expressed the AdGHGal transgene. This finding may reflect the common embryonic lineage of lactotropes and somatotropes from mammosomatotropes (10). It is also known that a subset of normal pituitary cells produces both GH and PRL (9). This result is also in agreement with findings in transgenic animals and in transient gene expression studies of transfected cell lines (23). In our primary pituitary cell cultures, the percentage of cells expressing AdGHGal was approximately 10 to 15%, which is lower than expected, as the relative proportion of somatotrope cells in the pituitary is as much as 40 to 50%. Quantitative activity was also lower compared with Ad[alpha]Gal activity. These findings suggest that additional sequences may be needed to achieve greater activity of the human GH promoter.

The expression and regulation of the [alpha]-subunit promoter have been studied extensively in transgenic mice, revealing strong expression in gonadotrope and thyrotrope cells with the use of promoter sequences between 4.6 and 0.48 kb (15, 16, 22). Because it is of interest to target the various types of GPH-producing cells ([alpha]-subunit, TSH, LH, FSH) (18), we used -846 to +45 base pairs of the human [alpha]-promoter sequence (20). In addition to its predominant expression in gonadotropes and thyrotropes, we also observed rare Ad[alpha]Gal expression in somatotropes. This finding is not entirely unexpected. In a previous study of these vectors in tumor cell lines (19), we found substantial overlap in the expression of the GH promoters and [alpha]-subunit promoters. However, promoter activity appears to be more specific for their respective pituitary cell types in vivo, perhaps because the individual pituitary cells are more fully differentiated.

Although cell-specific expression is a desirable feature of these vectors as a means to target a selected population of pituitary cells, equally important is the finding that these promoters restrict expression in tissues, such as liver, which are infected with very high efficiency. This feature should help minimize toxicity from viruses designed to carry suicide genes, such as thymidine kinase. Although direct injection of adenoviral vectors into the pituitary may be feasible, it would be desirable to ultimately modify the tissue tropism of adenoviral vectors by changing their surface charge (24) or by performing genetic modifications (17, 26). Alteration of the natural tropism of the adenovirus by modification of the adenoviral fiber coat protein has been used successfully to retarget adenoviral vectors to endothelial, smooth muscle, fibroblast, and macrophage cells (27). Thus, modifications of the virus itself may represent an alternative strategy for restricting adenoviral transgene expression to specific cell lineages, and for avoiding damage to normal tissues.

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#### COMMENTS

The promise of gene therapy for the treatment for human disease at this time is still just a promise. Two problems have yet to be adequately overcome for its widespread application to human disease: vector delivery and vector specificity. The authors address the second question in this study. They made adenoviral vectors with cell-type-specific promoters and demonstrated that direct stereotactic injection of these adenoviral vectors into the pituitary gland results in the expression pattern expected from the promoter used. The question of vector delivery remains an important issue.

The authors themselves comment that the adenovirus is effective only in cells near the needle tract; thus, without a problematically large number of injections, one would expect that we would be unable to sufficiently transfect the pituitary macroadenoma with an adenoviral construct

carrying a gene directed against the tumor cells. Similarly, the use of this technique for hormone replacement is problematical, placing the growth hormone gene behind the growth hormone promoter. In a patient with growth hormone deficiency, which may not allow for normal control of growth hormone levels, would such a gene in a normal thyrotropin-expressing cell be expected to release growth hormone appropriately? The problems of gene delivery in gene therapy remain substantial.

Corey Raffel

Rochester, Minnesota

Advances in our understanding of eukaryotic transcriptional regulation have delineated promoter sequences that confer tissue specificity. Integrating these sequences into the construction of gene therapy vectors offers the promise of targeting individual cell types. This strategy has intriguing clinical possibilities for cell-specific diseases, such as pituitary tumors.

In this article, the authors describe a variation of tissue-specific gene therapy. An adenoviral vector was constructed expressing the [beta]-galactosidase gene downstream of one of three promoters: 1) a cytomegalovirus promoter, which provides nonselective high levels of transcriptional activation; 2) the human growth hormone promoter, which provides specificity for somatotrope cells; or 3) the human glycoprotein [alpha]-subunit promoter, which provides specificity for gonadotrope and thyrotrope cells. Successful transfection into rat pituitary glands was achieved with stereotactic injection of the adenoviruses, and specificity was confirmed with immunohistochemistry. Additionally, nonspecific expression was shown by: 1) the human glycoprotein [alpha]-subunit promoter construct in somatotropes, and 2) the human growth hormone promoter construct in lactotropes.

The limitations of applying this approach in humans are well stated. As with other forms of gene therapy, adequate delivery is a major obstacle. Although the authors had success with local injection, intravascular injection failed to deliver a meaningful marker gene to the pituitary. Additional limitations include the attenuation of gene therapy efficacy from an immune response to the adenoviral vectors in human subjects.

The authors have provided a good beginning for investigating the in vivo specificity of constructs targeting pituitary cells. We hope that future studies will include further analysis of the sequences conferring in vivo specificity.

Andrew T. Parsa

Jeffrey N. Bruce

New York, New York

The authors have studied the effects of peripherally administered and stereotactically injected adenoviral vectors on the expression of a transgene within pituitary cell types. Several different promoters were used to drive expression of the transgene, and it was determined that the cytomegalovirus promoter led to the strongest expression within pituitary cells after stereotactic administration.

It is indeed unfortunate, but perhaps not unexpected, that the intravascularly delivered virus had such poor concentration within the pituitary gland. I say this because of the known absence of a blood-brain barrier in this region of the brain. Still, local administration of viral vectors is a viable option for gene therapy and will continue to fall within the domain of the neurosurgeon. The authors achieved accurate targeting of viruses in their rodent model system.

Dewey et al. (1) have recently described chronic inflammatory changes in the brain after adenoviral gene delivery. It is encouraging that such changes were not found by Lee et al.

James T. Rutka

Toronto, Ontario, Canada

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Key words: Adenovirus; [beta]-Galactosidase; Glycoprotein hormone; Growth hormone; Pituitary; Stereotactic injection