

Cell Type-Specific Adenoviral Transgene Expression in the Intact Ovine Pituitary Gland after Stereotaxic Delivery: An *in Vivo* System for Long-Term Multiple Parameter Evaluation of Human Pituitary Gene Therapy*

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ABSTRACT

Ablative therapies for pituitary tumors commonly cause irreversible damage to normal pituitary cells. Toxin gene therapy should therefore ideally be targeted to specific cell types to avoid collateral cell damage. To evaluate cell-type-specific adenoviral gene transfer in the intact pituitary gland we have used stereotaxic transcranial delivery of recombinant adenoviruses in the sheep with continuous assessment of endocrine function. Adenoviral β -galactosidase expression was driven either by the human cytomegalovirus (hCMV) promoter or the human PRL gene promoter. The hCMV promoter directed adenoviral β -galactosidase expression in all pituitary cell

types, but the PRL promoter restricted this exclusively to lactotropic cells, indicating that this promoter conferred appropriate cell type specificity in the context of adenoviral transduction *in vivo*. Serial measurements of plasma hormones showed no disruption of endocrine function over 7 days after intrapituitary injection. In summary, this work shows cell type-specific expression of an adenoviral transgene in the mixed cell population of the intact pituitary gland *in vivo* in a large animal model and indicates that stereotaxic intrapituitary delivery does not disrupt normal endocrine function. (*Endocrinology* 142: 795–801, 2001)

THE ANTERIOR PITUITARY gland contains a mixed cell population of endocrine cells (lactotropic, somatotropic, thyrotropic, corticotropic, and gonadotropic cells, secreting PRL, GH, TSH, corticotropin, and gonadotropins, respectively) together with folliculo-stellate and endothelial cells. Thus, a series of diverse endocrine functions depends on a complex organization of seven different cell types that are closely intermingled. Pituitary adenomas comprise clonal expansions of one of these cell types (1). Thus, any therapy designed to achieve permanent ablation of a pituitary tumor risks collateral damage to the other normal cells, and existing ablative therapies commonly result in a series of irreversible hormonal deficiencies that require life-long treatment (2, 3). Surgical therapy has variable success according to tumor type and size, and the best results are generally disappointing (4, 5).

The development of specific ablation therapy through expression of toxin genes can now be contemplated be-

cause of the growth in knowledge of the mechanisms of cell type specificity of hormone gene expression in the pituitary (1, 6). The anterior pituitary gland is an attractive model system in which to study this approach to cell type targeting *in vivo*, as the cell types can be readily monitored structurally by immunocytochemistry, and functionally by serial measurements of their secreted hormone products in peripheral blood.

Recombinant adenoviruses have become increasingly used as effective tools for gene transfer and are under intensive investigation in human gene therapy protocols. Previous reports have confirmed their efficacy *in vitro* using cultured pituitary cells (7, 8), *in vivo* in pituitary tumors propagated in nude mice (9), and in the intact rat pituitary after estrogen/sulpiride administration (10, 11). Further development of such a strategy for potential human therapy requires substantial validation using suitable *in vivo* systems, in which normal pituitary function should not be disrupted.

The aims of this study were therefore to evaluate cell type-specific adenoviral gene transfer in a large animal pituitary gland as a model of potential human pituitary gene therapy. We used stereotaxic transcranial injection of recombinant adenoviruses into the sheep pituitary gland *in vivo* and measured effects on anterior pituitary gland function using serial hormone measurements over 7 days. Adenoviral β -galactosidase expression was driven either by the human cytomegalovirus (hCMV) promoter or the human PRL

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(hPRL) promoter in an effort to achieve lactotroph-specific expression.

Materials and Methods

Recombinant adenovirus vector construction

The recombinant adenovirus vectors were based on adenovirus type 5, in which the E1 and part of the E3 regions were deleted. In one of these, RAD-CMV- β -gal (also termed RAD-35) the β -galactosidase gene is driven by the short immediate-early hCMV (sMIEhCMV) promoter inserted in place of the E1 deletion, as previously described (7). In the other vector (RAD-hPRL- β -gal) the promoter element comprised a -4429/+14 fragment from the pituitary-specific promoter of the human PRL gene (11-14), constructed as previously described (11).

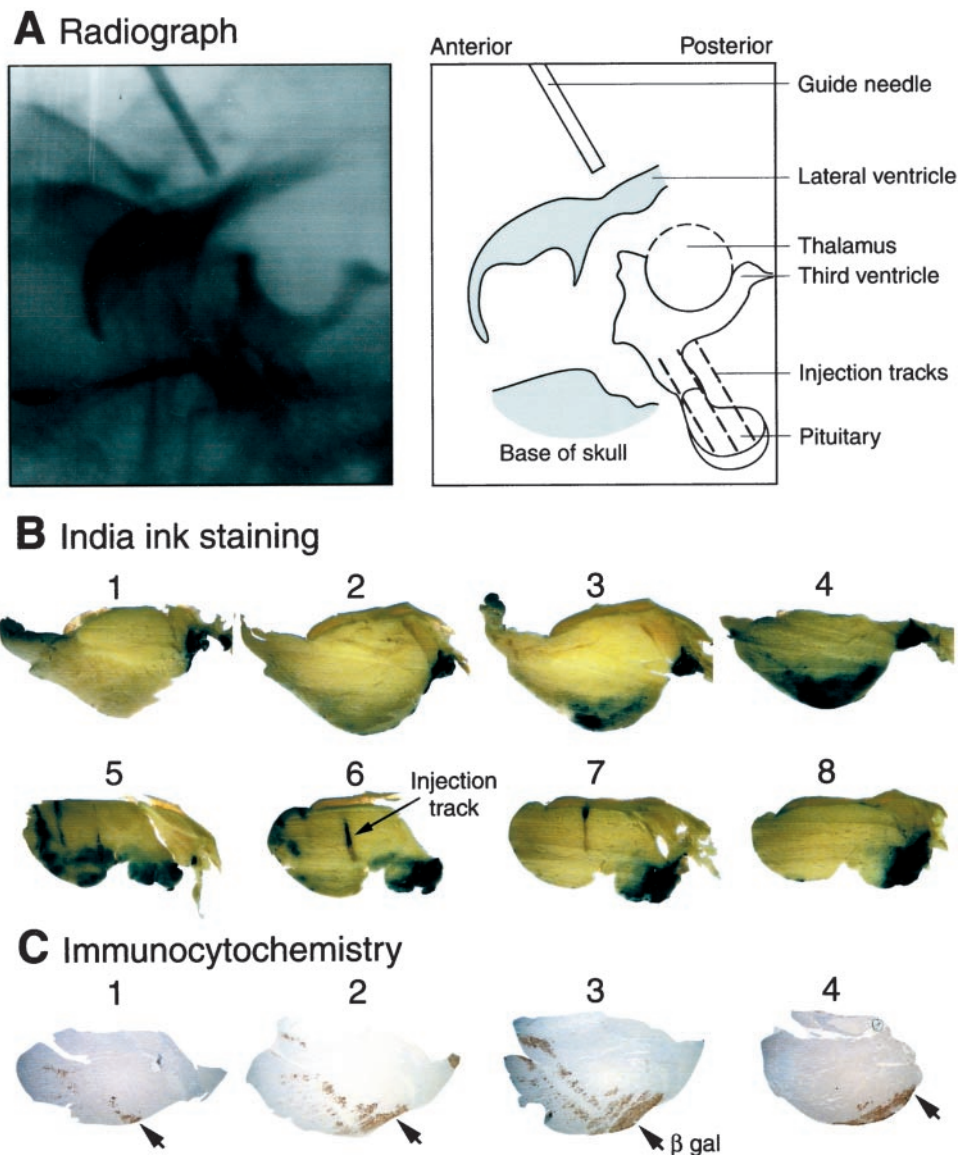
Animals and stereotaxic pituitary injection

Anestrous Suffolk ewes (2-3 yr old, 35-45 kg) were anesthetized with thiopentone and maintained with fluothane and nitrous oxide during the surgical procedure. Using a stereotaxic frame (14), a burr-hole was made in the skull 2 cm anterior to the bregma. A spinal needle was inserted 2 mm lateral to the midline angled cranio-caudally 30° from the vertical and lowered until its tip reached the lateral ventricle, judged by

free flow of cerebrospinal fluid (CSF). One milliliter of radioopaque dye (Omnipaque, Birmingham, UK) was instilled into the CSF, and a lateral radiograph was taken 30 sec later (75 kV, 50 mA, 0.4 sec) to allow visualization of the cerebral ventricles (Fig. 1A). The location of the pituitary gland was deduced from the positions of the infundibular and mammillary recesses of the third ventricle (15). A fine-bore metal cannula was inserted through the spinal needle and lowered to the base of the pituitary fossa. It was withdrawn 2 mm, and 250 μ l viral vector were injected into the pituitary gland at each of three levels. The cannula and guide needle were left in place for 1 min, then both were completely withdrawn and reintroduced 2 mm anteriorly, and the procedure was repeated. A third injection procedure was carried out posterior to the initial injection site. A total of nine injection sites were used to inject virus, using a total volume of approximately 2.2 ml, usually taking 50-60 min. The procedure was initially validated using injection of India ink (Fig. 1B), confirming accurate targeting of injectate within the pituitary gland. Virus suspensions of RAD-hPRL- β -Gal or RAD-hCMV- β -Gal were prepared at a concentration that delivered approximately 1.5×10^8 plaque-forming units/site, giving a total of approximately 14×10^8 plaque-forming units injected into the gland.

Blood samples were taken daily 28 and 4 h before surgery and then daily via an indwelling jugular catheter. Samples were analyzed for hormones by RIA and for routine hematology. Animals were killed 4-7

FIG. 1. Stereotaxic injection of pituitary gland. A, Lateral radiograph and diagram showing radioopaque dye in lateral and third ventricles and the position of the guide needle to direct injection into the pituitary gland. B, Low power photomicrograph of sequential thick sagittal sections (200 μ m) through the right lobe of an India ink-injected pituitary gland, with the infundibulum located anteriorly to the upper left, oriented as in the radiograph. Note the distribution of ink along needle tracks and at the base of the gland. C, Distribution of adenoviral transgene expression within the pituitary. Low power photomicrograph of β -galactosidase immunostaining in sections from one lobe of a sheep anterior pituitary gland injected with RAD-CMV- β gal. The adenoviral transgene expression (stained brown) in the injected gland is distributed along two visible needle tracks and at the base of the gland (arrowed). Sections were 5 μ m thick and taken at 150- μ m intervals, visualized with diaminobenzidine and lightly counterstained with hematoxylin (magnification, $\times 4$). The infundibulum is to the upper left of each section, oriented as in A.



days after virus injection with an overdose of iv pentobarbitone. The dura overlying the pituitary gland was examined for puncture holes, and the pituitary glands were dissected, divided sagittally into thirds, and placed into Bouin's fixative within 5 min of death. All experimental procedures were conducted in accordance with the Home Office Animals (Scientific Procedures) Act 1986 of the United Kingdom.

Immunocytochemistry

Sections of each pituitary gland were cut after preparation exactly as described previously (16). Initially, β -galactosidase staining was assessed at 50- μ m intervals to determine the general distribution of expression within the whole pituitary gland. For this initial single staining screen, sections were treated with hydrogen peroxide in methanol to block endogenous peroxidase activity and microwaved in sodium citrate buffer, pH 6.0, before staining with monoclonal anti- β -galactosidase antibody (1:200; Promega Corp., Madison, WI) with visualization using diaminobenzidine (DAKO Corp.).

Regions of the pituitary glands identified in this way as containing large numbers of β -galactosidase-positive cells were then subjected to dual immunofluorescence for β -galactosidase and pituitary hormones (16) using the following rabbit polyclonal antibodies: 1) PRL, ASMcN-R51, 1:2500; 2) LH, ASMcN-R23, 1:100; 3) FSH, M91, 1:100; 4) GH, 1:500, (NIDDK, NIH); 5) TSH, 1:100, (Dr. J. G. Pierce); and 6) ACTH, 1:300. Sections were treated with H_2O_2 in methanol, microwaved as described above, and incubated with anti- β -galactosidase antibody (1:50) in blocking buffer (normal goat serum, 10% in Tris-buffered saline) overnight at 4 C in a humidity chamber. Second antibody (goat antimouse Ig biotinylated, DAKO Corp.) was added, sections were washed, and avidin and biotinylated horseradish peroxidase complex (DAKO Corp.) were added for 30 min at room temperature. The β -galactosidase signal was amplified using a tyramide step, and sections were incubated with avidin-fluorescein isothiocyanate conjugate (Sigma, St. Louis, MO). Anti-hormone antibodies were then added at the dilutions indicated above and incubated overnight at 4 C. The hormone signal was visualized using goat antirabbit tetramethylrhodamine isothiocyanate conjugate (Sigma). Dual immunofluorescence images were obtained using an Olympus Corp. Provis fluorescence microscope (New Hyde Park, NY). For each hormone, 40–300 cells were identified in each of two sections taken from two widely separated regions of each pituitary gland to ensure representative estimates of transgene expression in different cell types. A mean result for each animal was generated from these four sections to provide an overall mean for each group of five animals.

Hormone assays and hematology

Plasma concentrations of PRL (17), LH and FSH (18), TSH (19), and GH and cortisol (20) were measured by RIAs as described previously. All samples were measured in one assay, with sensitivities of 0.5 ng (PRL, LH), 0.1 ng (FSH, GH), 0.2 ng (TSH), and 1 ng (cortisol) per ml plasma and intraassay coefficients of variation less than 8% for all assays. Routine hematology was analyzed by the Diagnostic Services Clinical Laboratories, Royal (Dick) Veterinary School (Edinburgh, UK).

Results

Initial studies in two sheep using India ink confirmed that the stereotaxic approach targeted the pituitary gland accurately, with a satisfactory distribution of dye within the gland after slow injection (Fig. 1B). Ten sheep were studied after injection of RAD-hCMV- β -gal ($n = 5$), or RAD-hPRL- β -gal ($n = 5$). All animals recovered uneventfully, and showed normal behavior thereafter. During removal of pituitary glands at the end of the experimental period, small blood clots were found in the CSF in relation to the needle track in two animals that had been apparently well, but in all the others the CSF was free of blood-staining.

The overall distribution of adenovirally mediated β -galactosidase expression in the pituitary gland was assessed in animals injected with RAD-hCMV- β -gal, and an example

is shown in Fig. 1C. Intense staining was seen in relation to the needle tracks and at the base of the pituitary, with stronger expression in the animals injected with RAD-CMV- β -gal than in those injected with RAD-hPRL- β -gal.

Dual immunofluorescence staining for β -galactosidase with each of the six pituitary hormones (Fig. 2) showed that in animals injected with RAD-hCMV- β -gal, β -galactosidase staining was colocalized with hormone staining in all six endocrine cell types (Fig. 2, *left panels*) and also in S-100-staining folliculo-stellate cells (data not shown). Transgene expression was detected in varying proportions of all six endocrine cell types, and this was quantitated in multiple sections of the pituitary glands. As the β -galactosidase expression was regional, sections were chosen for quantitation from β -galactosidase-positive areas that also contained sufficient numbers of cells that were positive for each of the six hormones, and percentages of the β -galactosidase-positive cells that expressed each of the pituitary hormones were calculated. Of the β -galactosidase-positive cells, 29–92% of cells coexpressed PRL in the five animals studied (mean, $69 \pm 28\%$); of the other hormones, coexpression with β -galactosidase varied from 8% (ACTH) to 33% (TSH) (Fig. 3A). In contrast, in animals injected with the RAD-hPRL- β -gal vector, $93.0 \pm 3.9\%$ of cells staining for β -galactosidase coexpressed PRL, 4–5% stained for LH or FSH, and less than 2% stained for GH, TSH, or ACTH (Fig. 2, *right panels*, and Fig. 3B). It should be noted that because of the regional clustering of different cell types within different areas of the anterior pituitary gland, the apparent prevalence of the different endocrine cell types was overestimated by this method, but the approach was used to avoid selection bias and to ensure that adequate numbers of each endocrine cell type were assessed to allow genuine quantitation.

Measurement of plasma hormones (Fig. 4) showed transient rises in cortisol in all animals and in PRL in five of nine animals, immediately after surgery, an expected stress response after a general anesthetic. Other hormones showed no abnormal responses. In particular, normal fluctuations were noted in LH and GH secretion, and there was no persistent abnormal elevation of PRL that might have suggested pituitary stalk disruption.

Routine hematological testing 7 days after injection shortly before sacrifice showed normal red and white cell indexes in all animals tested, except in one animal that showed post-mortem evidence of some bleeding into the CSF and had a mildly raised neutrophil count (8.09×10^9 /liter; reference range, $0.4–5.0 \times 10^9$ /liter).

Discussion

This report demonstrates adenoviral transgene expression in the intact eutopic sheep pituitary gland *in vivo* for at least 7 days and shows that stereotaxic delivery is reliable, accurate, and safe in a large animal. Furthermore, by quantitation of the six endocrine cell types expressing the transgene, we have shown that the PRL gene promoter confers appropriate cell type specificity to transgene expression *in vivo* in the context of a recombinant adenoviral vector. Most important, in terms of potential clinical application, we found no del-

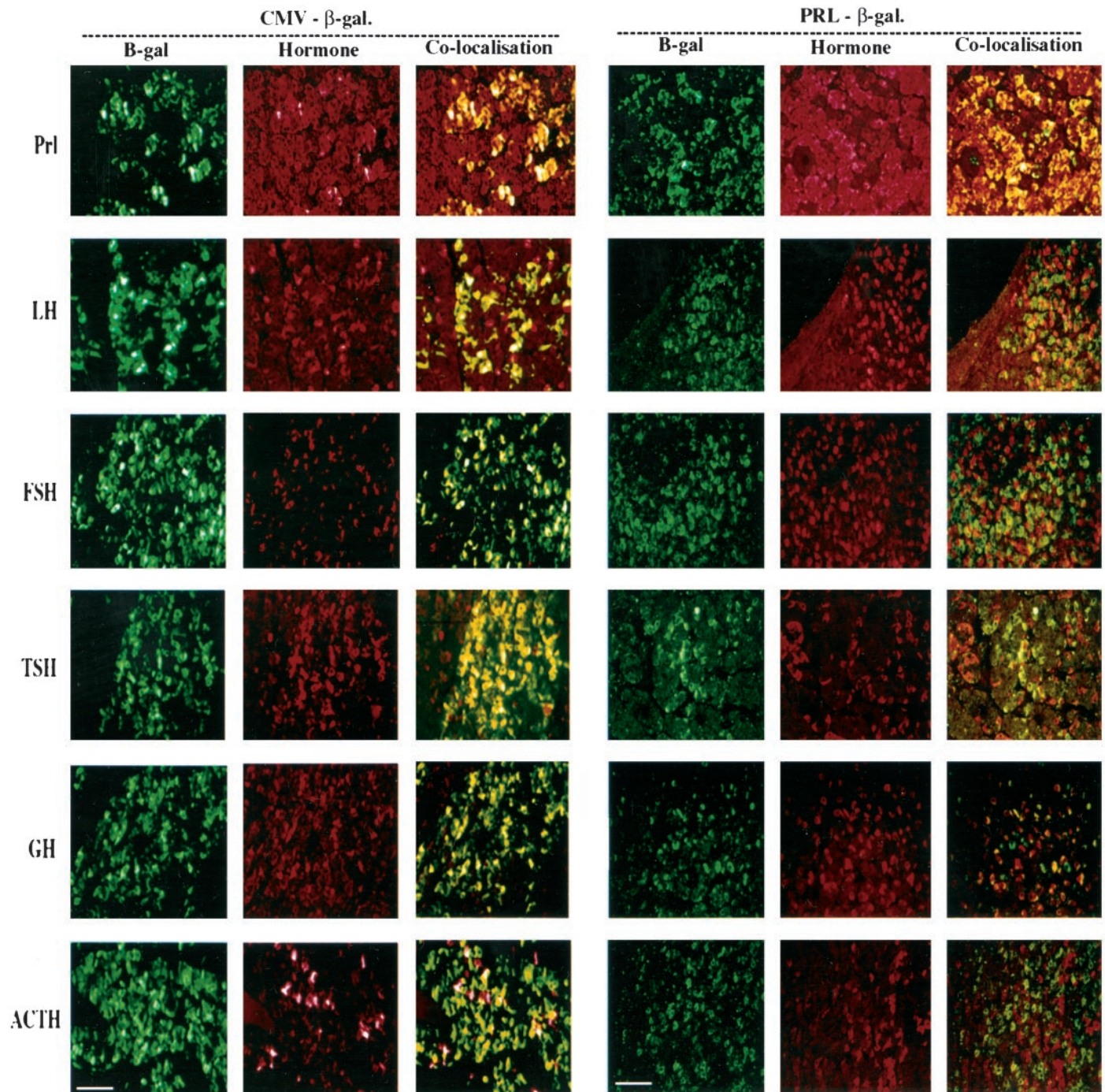


FIG. 2. Cell type specificity of adenoviral transgene expression. *Left panels*, Representative dual immunofluorescence staining of pituitary sections from animals treated with RAD-CMV- β gal injection. β -Galactosidase staining is shown in green (*left panels*), hormone staining in red (*center panels*), and overlay plots show colocalization of β -galactosidase with hormone as yellow (*right panels*). β -Galactosidase expression colocalized with (top to bottom) PRL, LH, FSH, TSH, GH, and ACTH, indicating expression of the viral transgene in all endocrine cell types. All images were taken using a $\times 40$ magnification lens. *White bar*, 50 μ m. *Right panels*, Representative dual immunofluorescence staining of pituitary sections from animals treated with RAD-hPRL- β gal injection. Panels are presented as in left-hand series, and overlay plots show colocalization of β -galactosidase only with PRL (*top right panel*) and not with LH, FSH, GH, TSH, or ACTH. All images were taken using a $\times 40$ magnification lens. *White bar*, 50 μ m.

eterious effect of adenoviral injection on endocrine function of the pituitary gland.

A large animal model is essential for evaluation of this potential human application of endocrine gene therapy, as multiparameter longitudinal assessment is needed to deter-

mine the effects of adenoviral gene transduction on pituitary function. The ability to take serial blood samples to track hormonal changes over days or weeks is severely limited in small animals such as rats or mice. We selected the sheep as a suitable model because the pituitary gland is of similar size,

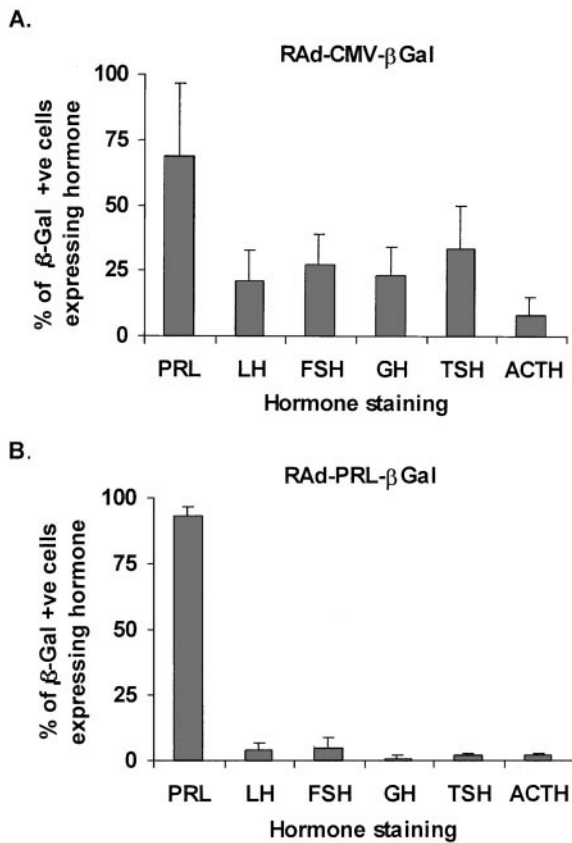


FIG. 3. Quantitative analysis of cell type specificity of adenoviral transgene expression. A, Cell type specificity of RAD-CMV- β gal transgene expression. The histogram shows the percentage of β -galactosidase-positive cells that costained for each of the six pituitary hormones. Data shown are the mean \pm SD ($n = 5$ animals, derived from four separate pituitary sections in each case, with 40–200 cells counted/section). B, Cell type specificity of RAD-hPRL- β gal transgene expression. The histogram shows the percentage of β -galactosidase-positive cells that costained for each of the six pituitary hormones. Data shown are means \pm SD ($n = 5$ animals, derived from four separate pituitary sections, with 40–100 cells counted/section).

configuration, and accessibility to the human pituitary and has been the subject of careful immunohistochemical evaluation (16). In addition we have previous experience in longitudinal evaluation of pituitary hormone secretion (19). The sheep pituitary is 10–12 mm in antero-posterior diameter, and the results reported here indicate that a local injection of recombinant adenovirus can achieve effective transgene expression across substantial regions of the gland. This suggests that direct adenoviral injection of a pituitary tumor at transsphenoidal surgery may in principle be able to achieve adequate expression of desired suicide genes for future potential tumor therapy applications, as discussed below.

A key point of this study was to employ transcriptional targeting of transgene expression to a single cell type within a mixed cell population by exploiting the known cell type specificity of PRL gene expression. The human PRL gene promoter was used to generate the recombinant adenovirus vector RAD-hPRL- β -gal in view of potential future therapeutic applications (11, 21). This gene promoter has been extensively studied *in vitro*, and the promoter fragment used here confers appropriate hormonal regulation onto reporter

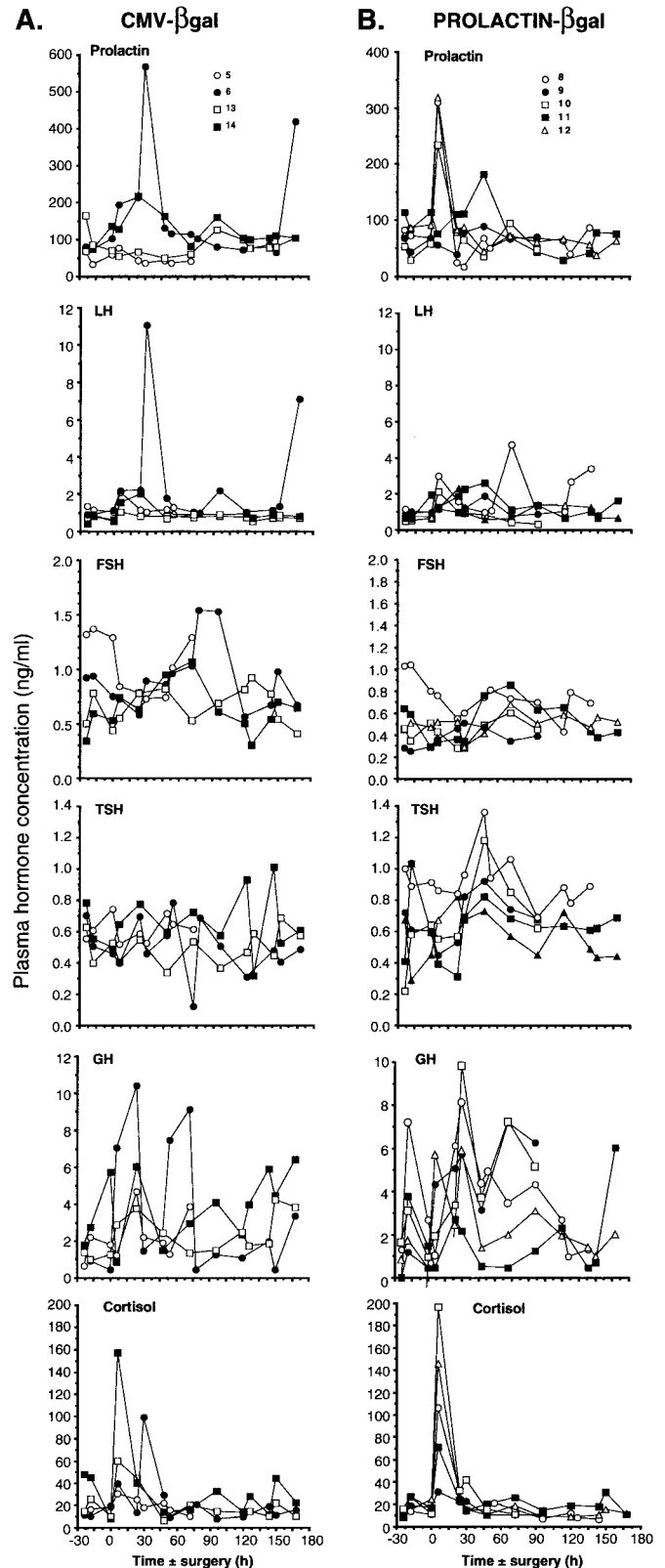


FIG. 4. Pituitary function before and after adenovirus injection. Plasma levels of the pituitary hormones PRL, LH, FSH, TSH, and GH and plasma cortisol plotted before (–28 and –4 h) and then daily for 7 days after RAD injection at 0 h. Four animals injected with RAD-hCMV- β -gal are shown in the left panels, and five injected with RAD-hPRL- β -gal are shown in the right panels.

gene expression in pituitary cells in both transient and stable transfection systems (12–14, 22). The present study confirms that the PRL promoter in the context of a recombinant adenovirus is activated specifically in lactotrophic cells *in vivo*, with minimal reporter gene transcription in other cell types. In other words, although the adenovirus vectors are capable of infecting all of the cell types found in the pituitary (Refs. 7 and 11 and the present data), the PRL promoter restricts the activation of adenoviral transgene expression effectively to the lactotroph cell population. The RAD-hPRL- β -gal vector has also been used in primary cultures of rat anterior pituitary cells and in the rat pituitary gland *in vivo*, with similar restriction of transgene expression mainly to lactotrophic cells (11). The slightly higher than expected transgene expression from this vector in gonadotrophic cells may be explained by the intimate relation between lactotrophic and gonadotrophic cells *in vivo* (16), which could result in overestimation of apparent transgene expression in gonadotrophs. Recent work (23) has used the GH gene promoter to achieve similar restriction of transgene expression to the rat pituitary gland *in vivo*.

An important goal in this study was to assess the effect of adenoviral injection on the endocrine function of the normal pituitary gland in a large animal model, and the size of the sheep allowed us to track circulating plasma hormone levels. Single time point analysis 10 days after transcranial or transauricular injection of the rat pituitary indicated little or no major change in circulating hormone levels (10, 11). In the present, more extensive, longitudinal evaluation, we were able to monitor pituitary function serially over 7 days. We found a transient rise in plasma cortisol and PRL, a well recognized feature of an anesthetic stress response in sheep (19). In the following 7 days hormone levels and secretory patterns remained normal, however, with no evidence to suggest disruption of pituitary function. It is encouraging that even injection of relatively large volumes of virus into a tightly organized tissue appears to cause no adverse endocrine or other systemic effects.

If adenoviral gene transfer into the normal eutopic pituitary gland can be confirmed to be safe and effective over the longer term, without disrupting pituitary function as our data suggest, it may be possible to consider the potential for ablative therapy for pituitary tumors using suicide or cytotoxic genes. Although pituitary tumors are not usually lethal in themselves, their treatment remains unsatisfactory, and ablative therapy frequently damages residual pituitary function. Pituitary tumors have the advantage of well understood cell biology, and they are a suitable target for further development of adenoviral gene transfer approaches (21, 24). Future developments are likely to include more heavily deleted ("gutless") adenovirus vectors (see Ref. 24 for review), which will enable use of combinations of tissue-specific promoters together with regulatable transcription factors to obtain highly controlled transgene expression (25–27). Extensive validation will be required to establish their safety and efficacy in different circumstances (28, 29), and the system presented in this report will be valuable in this effort.

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