

Switching On and Off Transgene Expression within Lactotrophic Cells in the Anterior Pituitary Gland *in Vivo**

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ABSTRACT

To further develop our understanding of anterior pituitary (AP) function and to aid the development of gene therapy strategies for the treatment of pituitary diseases, adenovirus (Ad)-mediated gene transfer to the AP gland will be a useful tool. Although successful widespread gene transfer within the AP has been achieved using first generation Ads the ability to control transgene expression would be very beneficial when studying AP regulatory functions and delivering a potentially therapeutic gene into the AP gland. A dual adenoviral vector system encoding for cell type-specific and regulatable transcription units was developed to achieve transcriptionally targeted transgenesis within specific cell populations in the adult AP gland. To achieve regulatable transgene expression within predetermined AP cells, the tetracycline-responsive transcriptional elements have been engineered to be under the control of human, lactotroph-specific PRL (hPRL) promoter elements within a dual adenoviral vector system.

The inducibility, cell type specificity, and levels of transgene expression were characterized *in vitro* and *in vivo* and compared with the strong ubiquitous β -actin/human cytomegalovirus (CAG) promoter. Inducible expression of the marker gene β -galactosidase under the control of the hPRL promoter was restricted to lactotrophic tumor cell lines and lactotrophic cells within primary AP cultures. Lactotroph cell type specificity and inducible transgene expression were also observed within the AP gland *in vivo*, and this could be switched on or off. Administration of doxycycline abrogated transgene expression both *in vitro* and *in vivo*. Our results also provide evidence that an excess of *trans*-activator is needed to achieve maximal transgene expression. Our data indicate that combined transcriptional and inducible transgenesis can be achieved using adenoviral vectors that allow spatial and temporal restriction of transgene expression within the adult AP gland *in vivo*. (*Endocrinology* **142**: 2521–2532, 2001)

WITH THE RECENT emergence of the sequence for the human genome and the inevitable wealth of DNA sequences that will be gathered from it, the use of genetic manipulation as a tool in uncovering the molecular basis of many physiological processes within the anterior pituitary (AP) gland will become apparent. The use of knockout and transgenic technologies has already been extremely useful in elucidating roles for many gene products. It is now well established that these approaches have their drawbacks, *i.e.* although the

introduction or deletion of a particular gene may be useful in indicating a particular role *in vivo*, the compensatory changes that are likely to occur during development may alter the animal's phenotype. This could, in turn, give a blighted view of what may occur in wild-type animals. With this in mind, we sought to develop a system in which an animal could develop normally and then have a particular gene switched on and/or restricted to a designated cell type. Such a cell type-specific and regulatable gene transfer system would also be useful when developing potential strategies for therapeutic treatment of pituitary diseases. The use of gene delivery techniques to treat pituitary diseases has been proposed (1–4), but to enable safe and efficient treatment, controlled gene expression would be advantageous. Furthermore, for the treatment of a pituitary tumor, such as an invasive macroprolactinoma or an ACTH-secreting adenoma, it would be desirable to restrict the expression of a toxic gene product to a particular hormone-expressing cell population to prevent adverse cytotoxicity. Also, for the treatment of chronic long-term pituitary disorders, such as GH deficiency, it would be advantageous to regulate transgene expression so that therapeutic, and not detrimental, levels of gene product are produced. A cell type-specific and regulatable gene delivery system will allow us not only to predetermine the cells in which the transgene is expressed, but also to very tightly control, using small molecules such as antibiotics, the levels of transgene expression.

Received October 5, 2000.

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* This work was supported by funds from the Molecular Medicine and Gene Therapy Unit receives from the Wellcome Trust (UK), Biotechnology and Biological Sciences Research Council (BBSRC) (UK), the Royal Society, Parkinson's Disease Society (UK), and EU-Biomed program (Contracts BMH4-CT98–3277, BMH4-CT98–0297, and QLK3-CT-1999–00364); a joint BBSRC-AstraZeneca CASE studentship (to J.R.S.-A.); and a BBSRC Ph.D. studentship (to D.S.).

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Recently, *in situ* transduction of the AP gland has been shown using recombinant adenoviral vectors (RAd) after transauricular (5) or transcranial stereotaxic delivery (6–8). No cytotoxicity or loss of AP hormonal functions is apparent after vector delivery (7), indicating that RAd would be a useful tool for studying gene function within the AP gland and could be further developed for gene therapy applications.

Targeted transgene expression to predetermined cell types after adenovirus-mediated delivery to the AP gland could be achieved through two alternative methods. The retargeting of RAd so that they bind to a specific membrane receptor can be achieved, and this would enable viral targeting to a given cell population (9). An alternative to direct viral targeting is the use of cell type-specific promoters that limit transgene expression to a particular cell type. RAd driving transgene expression from cell type-specific promoters have been successfully used (6–8, 10–12).

To generate a system in which transgene expression can be regulated, we used an inducible transcription system, *i.e.* the tetracycline-dependent regulatory (tet) system (13). Gene expression using variations of this system can be switched on or off by the presence of tetracycline, enabling controlled transgene expression during any given time period. In the tet off system a synthetic tetracycline-dependent *trans*-activator (tTA) is able to bind to and activate transcription from a tetracycline-responsive element (TRE) promoter in the absence, but not the presence, of tetracycline. In the tet on system a mutated tetracycline-dependent transactivator (rtTA) is able to bind to and activate transcription from the TRE promoter in the presence, but not the absence, of tetracycline. Although when used in transgenic mouse lines regulation from the tet system can be variable (14), its use within RAd has provided successful regulation of transgene expression (15–18).

To test whether both cell type-specific and regulatable transient transgene expression could be attained in the AP gland *in vivo*, a dual adenoviral vector system was developed. The lactotroph-specific human PRL (hPRL) promoter (bp +14 to –4429) drove the expression of a nuclear localized tTA [tTA(nls)] from one RAd, and the TRE promoter drove expression of the β -galactosidase reporter from another RAd. The cell type specificity and regulatable transgene expression of this system were assessed in cell lines, primary AP cultures, and the AP gland *in situ*.

Our data demonstrate that the hPRL promoter can be combined with the tet off system within RAd to produce combined cell type-specific and regulatable transgene expression in lactotroph tumor cells, lactotroph cells within primary AP cultures, and *in vivo*, within the AP gland *in situ*. This system should provide an alternative to knockout or transgenic animals for studies of molecular and biochemical mechanisms within the AP gland. It should also prove useful for the development of therapeutic treatments within the AP gland, such as the treatment of hypopituitarism, that rely upon the controlled pulsatile release of AP hormones.

Materials and Methods

Cell lines and culture conditions

Hamster kidney fibroblast BHK 21 CL13 (IZS) cells were purchased from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). CNS-1 cells (a rat glioma cell line) were provided by

Dr. W. Hickey (Dartmouth Medical School, Lebanon, NH). GH₃ cells (a rat PRL/GH-secreting cell line) were provided by Dr. S. Cockle (Department of Biochemistry and Physiology, University of Reading, Reading, UK). AtT20 D16V cells (a murine ACTH-secreting cell line) were described previously (19). Human embryonic kidney 293 cells were obtained from Microbix Biosystems, Inc. (Toronto, Canada). Cells were grown as previously described (5, 6). Buffalo rat anterior pituitary cultures were prepared and cultured as described previously (20).

Construction of recombinant adenoviruses

Previously the full-length rat PRL promoter containing proximal (+33 to –422 bp) and distal (–1500 to –1800 bp) positive regulatory elements with 90% homology to the hPRL promoter (21) was shown to restrict expression of nerve growth factor, transforming growth factor- α , or hGH to lactotrophic (22, 23) and mammosomatotrophic (24) cells in transgenic mice. As it was shown that the distal and proximal elements of the rat promoter were essential for strong lactotroph-specific expression, we decided to use a 4.4-kb hPRL promoter fragment (+14 to –4429 bp) that contains the proximal (–40 to –250 bp), the distal (–1300 to –1750 bp), and a portion of the super distal (–3500 to –5000 bp) elements of the hPRL promoter (21). The 4.4-kb hPRL fragment of the hPRL promoter, the tTA(nls) open reading frame (15) from pCANtTA (Dr. H. Hamada, Department of Molecular Biotherapy Research, Cancer Institute, Tokyo, Japan), and the simian virus 40 polyadenylation signal were cloned as an *Xba*I fragment into p Δ E1sp1A (Microbix Biosystems, Inc.) to create the p Δ E1-hPrl-tTA(nls) shuttle vector. The CAG-tTA(nls) expression cassette from the plasmid pCANtTA, containing the CAG (chicken β -actin/hCMV) promoter, was inserted as a *Clal*/BglIII fragment into p Δ E1sp1A to create the p Δ E1-CAG-tTA(nls) shuttle vector. The TRE- β -galactosidase cassette from pTRE- β -galactosidase (CLONTECH Laboratories, Inc., Palo Alto, CA) was inserted as an *Xho*I/*Hind*III fragment into p Δ E1sp1A to create the p Δ E1-TRE- β -galactosidase shuttle vector. RAd-CAG-tTA(nls), RAd-hPRL-tTA(nls), and RAd-TRE- β -galactosidase were generated by homologous recombination in 293 cells of the respective shuttle vector with either pJM17 or pBHG10 (Microbix Biosystems, Inc.). Characterization, production of high titer stocks, purification by double cesium chloride density gradient separation, and titration of viruses were carried out as previously described (25, 26). Stocks were found to be free of replication competent adenovirus using a supernatant rescue assay able to detect 1 replication competent virus within 10⁹ recombinant viruses (27). Adenovirus preparations were ascertained to be endotoxin (lipopolysaccharide) free, according to the criteria of Cotten *et al.* (28), using the E-TOXATE assay (Sigma-Aldrich Corp., Dorset, UK).

Quantitative *in vitro* analysis of β -galactosidase activity

Cells were plated in 48-well plates at a density of 1×10^4 cells/well 1 day before infection in the presence or absence of the tetracycline analog doxycycline (500 ng/ml). Primary rat AP cultures were incubated for 7 days before infection, and doxycycline (500 ng/ml) was administered 24 h before infection. Cell lines were infected with virus combinations at ratios of 10:1 and 1:1, with appropriate multiplicities of infection (MOIs) of 500:50 or 50:50, whereas primary rat AP cultures were infected with virus combinations at a ratio of 10:1 with a respective MOI of 500:50. Cells and primary rat AP cultures were infected with virus at constant MOIs for each experiment and at levels that do not cause cytotoxicity, as determined previously (data not shown). On the day of infection cells were counted and infected at appropriate MOI values to produce ratios of tTA(nls):TRE of 10:1 [MOI of 500 (5×10^6 infectious units (iu) to MOI of 50 (5×10^5) of each vector] or a ratio of ETA (nls):TRE of 1:1 [MOI of 50 (5×10^5 iu) to MOI of 50 (5×10^5) of each vector], with virus combinations of RAd-CAG-tTA(nls):RAd-TRE- β -galactosidase or RAd-hPRL-tTA(nls):RAd-TRE- β -galactosidase. Infected cells were left for 48 h after infection, after which cell lysates were assessed for relative β -galactosidase activity as described previously (6, 26). $n = 4$ for each group, and each experiment was repeated at least twice.

Delivery of RAd to the anterior pituitary gland *in vivo*

Male 8-week-old Buffalo rats were house-bred at the University of Manchester Biological Safety Unit. All animals had free access to food

and water and were kept under a 12-h light, 12-h dark cycle with constant housing temperature and humidity. Experiments were conducted according to the United Kingdom Animal (Scientific Procedures) Act of 1986. One day before surgery rats were given water containing 1% sucrose in the absence or presence of doxycycline (2 mg/ml). The procedure for stereotaxic delivery of RAds to the AP gland *in vivo* has been described in detail previously (4). Briefly male 8-week-old Buffalo rats were anesthetized with halothane, placed in a stereotaxic frame, and injected six times in the AP gland (three sites per lobe) with a total dose of 1×10^8 iu virus. The specified dose was chosen because it has previously been shown to produce high levels of gene transfer in the AP gland without causing cytotoxicity (7). A volume of 1 μ l was injected per site over a period of 1 min. Animals were then given 10 ml saline, sc, and allowed to recover. After 3 days animals were given a lethal injection of pentobarbitone and perfused transcardially with Tyrode's solution (132 mM NaCl, 1.9 mM CaCl₂, 0.32 mM NaH₂PO₄, 5.56 mM glucose, 11.6 mM NaHCO₃, and 2.68 mM KCl), and pituitary glands were removed and postfixed by placing them in 4% paraformaldehyde solution. Pituitaries were then paraffin-embedded, cut into 5- μ m sections using a microtome, and placed onto 3-aminopropyltriethoxysilane-coated slides.

In the experiments in which expression of β -galactosidase was switched on and off, male 8-week-old Buffalo rats were given water containing 1% sucrose in the absence or presence of doxycycline (2 mg/ml) 1 day before surgery. Four days after surgery animals initially given water with doxycycline were given water without doxycycline, and animals that had initially been given water without doxycycline were given water with doxycycline. Groups of animals were then killed 11 and 18 days after surgery.

Fluorescence immunohistochemical detection of β -galactosidase and hormones

Protocols for fluorescent immunohistochemical detection within primary pituitary cultures and paraffin-embedded pituitary sections have been described in detail previously (5, 6, 20). The primary antibody for detecting β -galactosidase was a polyclonal rabbit anti- β -galactosidase (1:500) provided by Dr. R. Goya (University of La Plata School of Medicine, La Plata, Argentina). The primary antibodies to hormones were polyclonal: guinea pig antirat PRL (1:100), guinea pig antihuman GH (1:100), and guinea pig antirat α -LH (1:100) provided by Dr. A. F. Parlow and the NIDDK National Hormone and Pituitary Program (Bethesda, MD). Secondary antibodies were goat antiguinea pig fluorescein isothiocyanate (1:100) and goat antirabbit tetramethyl rhodamine isothiocyanate (1:100) from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Fluorescence images were taken using an Olympus Corp. Vanox-T microscope (New Hyde Park, NY) with BP490:O515 and BP545:O590 exciter:barrier filter combinations for fluorescein isothiocyanate and tetramethyl rhodamine isothiocyanate, respectively.

Statistical analysis

In vitro experimental results were analyzed using Student's two-tailed *t* test.

Results

Generation of *Rad-CAG-tTA(nls)*, *Rad-hPRL-tTA(nls)*, and *Rad-TRE- β -galactosidase*

To achieve lactotroph-specific and regulatable transgene expression, two kinds of RAds were generated containing either the tTA or TRE elements from the previously described tetracycline regulatory system (13) (Fig. 1). RAds driving expression of a nuclear localized transactivator from the ubiquitous chicken β -actin/human cytomegalovirus (hCMV) fusion (CAG) promoter [Rad-CAG-tTA(nls)] or the lactotroph-specific hPRL promoter [Rad-hPRL-tTA(nls)] were generated (Fig. 1, A and B). A RAD with the TRE driving expression of the β -galactosidase reporter gene (Rad-TRE- β -galactosidase) was also generated (Fig. 1C). The presence of the expression cassettes within each RAD was confirmed by Southern blotting

*Hind*III-digested viral DNA using specific probes for each expression cassette. Bands of the expected size from each viral DNA digest were positive for the respective expression cassettes (Fig. 1, A–C).

Quantitative analysis of cell type-specific and regulatable transgene expression in tumor cell lines *in vitro*

To assess whether cell type-specific and regulatable expression could be achieved from the dual adenovirus system, *in vitro* analysis was carried out in various tumor cell lines, *i.e.* GH₃ cells, which synthesize PRL and GH; AtT20 cells, which synthesize POMC; BHK cells; and CNS-1 cells, using two different ratios of *trans*-activator to response element. RAds driving expression of tTA(nls) from both the lactotroph-specific hPRL and pancellular CAG promoter were tested. BHK, CNS-1, AtT20, and GH₃ cells were infected with Rad-CAG-tTA(nls):Rad-TRE- β -galactosidase or Rad-hPRL-tTA(nls):Rad-TRE- β -galactosidase virus combinations at tTA(nls):TRE ratios of 10:1 and 1:1 in the absence or presence of doxycycline, after which the β -galactosidase activity was quantitated. The ratios used relate to the number of infectious units and not to the number of tTA(nls) molecules to TRE elements, as the level of tTA(nls) is dependent on both the cell type(s) infected and the promoter (CAG or hPRL) used. When BHK, AtT20, CNS-1, and GH₃ cells were infected with Rad-CAG-tTA(nls):Rad-TRE- β -galactosidase at a ratio of 1:1 in the absence of doxycycline, specific β -galactosidase activity was induced to 74.8 ± 6.5 , 0.007 ± 0.002 , 77.9 ± 6.1 , and 65.2 ± 3.9 U/ μ g protein, respectively (Fig. 2). It must also be noted that the two independent infectious events needed per cell from our dual adenovirus system coupled to the fact that AtT20s are not very receptive to adenovirus infection mean that AtT20 cells show comparatively low levels of β -galactosidase expression. Similar results were obtained when using a single adenovirus vector expressing β -galactosidase (6). When BHK, AtT20, CNS-1, and GH₃ cells were infected with Rad-CAG-tTA(nls):Rad-TRE- β -galactosidase at a ratio of 10:1 in the absence of doxycycline, specific β -galactosidase activity was induced to 103 ± 8.1 , 0.017 ± 0.001 , 94.8 ± 2.2 , and 40.4 ± 2.6 U/ μ g protein, respectively (Fig. 2). When BHK, CNS-1, and AtT20 cells were infected with Rad-hPRL-tTA(nls):Rad-TRE- β -galactosidase at ratios of 1:1 or 10:1 in the absence of doxycycline, no induction of specific β -galactosidase activity was seen (Fig. 2). When GH₃ cells were infected with Rad-hPRL-tTA(nls):Rad-TRE- β -galactosidase at a ratio of 1:1 in the absence of doxycycline, specific β -galactosidase activity was induced to 1.7 ± 0.06 U/ μ g protein (Fig. 2). When GH₃ cells were infected with Rad-hPRL-tTA(nls):Rad-TRE- β -galactosidase at a ratio of 10:1 in the absence of doxycycline, specific β -galactosidase activity was induced to 6.4 ± 0.1 U/ μ g protein (Fig. 2). Negligible basal levels of specific β -galactosidase activity were seen in all groups in the presence of doxycycline.

Lactotroph-specific and regulatable transgene expression in primary anterior pituitary cultures

To assess the cell type-specific and regulatable transgene expression attainable within primary AP cultures with the dual RAD system, we analyzed the cell types in which β -galactosidase expression is seen and compared it with the expression of specific hormones, *i.e.* ACTH, PRL, LH, FSH,

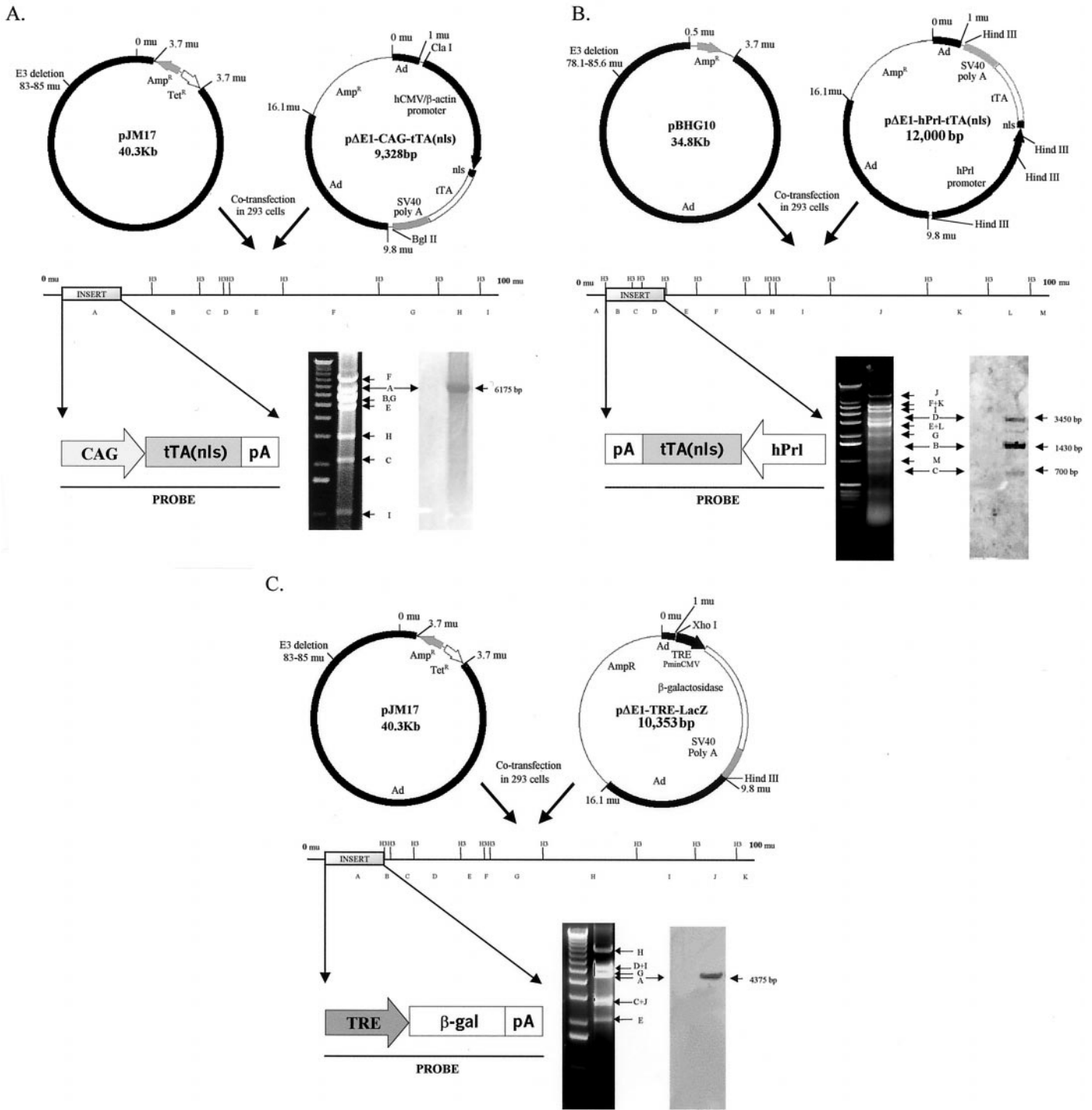


FIG. 1. Generation and characterization of Rad-hPRL-tTA(nls), Rad-CAG-tTA(nls), and Rad-TRE-β-galactosidase. Each virus was generated through cotransfection in 293 cells of the respective shuttle vector with either pJM17 or pBHG10 (A–C). Homologous regions of Ad 5 genome (■) recombine to produce a recombinant genome with an insertion in E1. A schematic representation of each linear recombinant genome is shown below along with the respective insert and the region used to make a probe for Southern blotting. Each recombinant genome digested with *Hind*III is also shown alongside the respective Southern blot, indicating the presence of each expression cassette. Viral genome bands positive for the respective expression cassettes are indicated along with their respective sizes.

TSH, and GH, using double immunofluorescence techniques. Primary AP cultures were infected with Rad-CAG-tTA(nls):Rad-TRE-β-galactosidase or Rad-hPRL-tTA(nls):Rad-TRE-β-galactosidase virus combinations at a tTA(nls):

TRE ratio of 10:1 in the absence or presence of doxycycline. Double immunofluorescence labeling was then carried out for β-galactosidase and the specific AP hormones. Infection with Rad-CAG-tTA(nls):Rad-TRE-β-galactosidase resulted

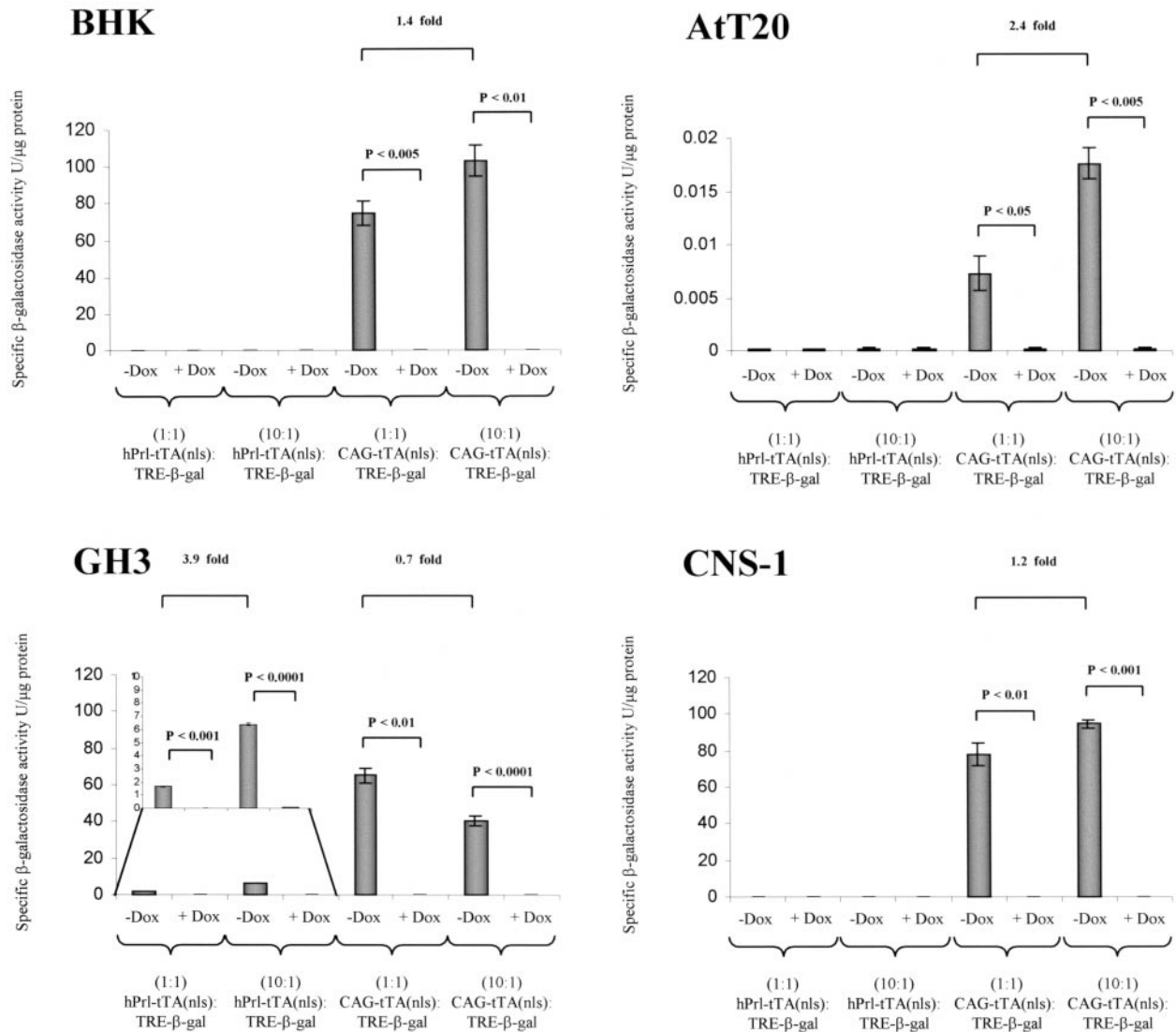


FIG. 2. Quantitative analysis of β -galactosidase expression in BHK, AtT20, GH₃, and CNS-1 cells after infection with RAd-CAG-tTA(nls):RAd-TRE- β -galactosidase or RAd-hPRL-tTA(nls):RAd-TRE- β -galactosidase at tTA(nls):TRE ratios of 10:1 and 1:1 in the absence and presence of doxycycline. Error bars represent the SEM ($n = 4$). The fold difference between the 1:1 and 10:1 ratios is indicated along with the fold difference between the hPRL and CAG RAds where relevant.

in expression of β -galactosidase in AP cells expressing PRL, GH, LH (Fig. 3A and Table 1), FSH, TSH, or ACTH (data not shown) in the absence, but not in the presence, of doxycycline. Infection with RAd-hPRL-tTA(nls):RAd-TRE- β -galactosidase resulted in expression of β -galactosidase in fewer cells, in the absence, but not the presence, of doxycycline. Expression was restricted mainly to lactotrophic (PRL-expressing) cells and a subpopulation of cells expressing GH that are likely to be mammosomatotrophs and also express PRL (Fig. 3B and Table 1). Of the total β -galactosidase-positive cell population, about 87% were lactotrophs and 13% were mammosomatotrophs. This cell type specificity achieved with RAd-hPRL-tTA(nls):RAd-TRE- β -galactosidase explains the lower number of cells expressing β -galactosidase compared with RAd-CAG-tTA(nls):RAd-TRE- β -galactosidase and is expected, because PRL-positive cells typically make up approximately 35% of primary anterior

pituitary cultures. No β -galactosidase expression was seen in cells expressing the other AP hormones, *i.e.* LH (Fig. 3B) or FSH, TSH, or ACTH (data not shown), in either the presence or absence of doxycycline.

Quantitative analysis of cell type-specific and regulatable transgene expression in primary AP cells

To assess the levels of inducible transgene expression that could be achieved in a mixed AP cell culture *in vitro*, primary AP cultures were infected with combinations of RAd-CAG-tTA(nls):RAd-TRE- β -galactosidase or RAd-hPRL-tTA(nls):RAd-TRE- β -galactosidase at a tTA(nls):TRE ratio of 10:1 in the absence or presence of doxycycline, after which specific β -galactosidase activity was quantitated. The ratios used relate to the number of infectious units and not to the number of tTA(nls) molecules to TRE elements, as the level of tTA(nls) is dependent on both the cell type(s) infected and the

promoter (CAG or hPRL) used. When cultures were infected with RAD-CAG-tTA(nls):RAD-TRE- β -galactosidase in the absence of doxycycline, specific β -galactosidase activity was induced to 21.65 ± 2.5 U/ μ g protein (Fig. 4). When cultures

TABLE 1. Cell-type specific and inducible expression of β -galactosidase driven by the CAG and human PRL promoter via the tTA(nls) and TRE from the tetracycline regulatable system in primary AP cell cultures

Hormone	Promoter	% of total endocrine cell population positive for single hormone	% of hormone-positive cells expressing β -galactosidase in the absence of doxycycline
PRL	hPRL	35 ± 2.4	64 ± 2.3
	CAG	34 ± 2.7	63 ± 2.0
GH	hPRL	26 ± 1.7	13 ± 1.1
	CAG	23 ± 1.5	65 ± 1.8
LH	hPRL	5 ± 1.4	0
	CAG	7 ± 1.0	60 ± 0.8

AP cells in primary culture were grown in 48-well plates and 7 days after plating were infected with RAD-CAG-tTA(nls):RAD-TRE- β -galactosidase or RAD-hPrl-tTA(nls):RAD-TRE- β -galactosidase at a ratio of 10:1 (total of 550 IU/cell). Hormone-producing AP cells were stained for the various hormones and analyzed for the coexpression of β -galactosidase whose expression was driven by the TRE. Expression from the TRE was, in turn, controlled by binding of the tTA(nls) whose expression was driven by either the hPRL or CAG promoters. Note that when the hPRL promoter is used, approximately 87% of the total β -galactosidase-immunoreactive cell population are also PRL positive and about 13% are also GH positive. No expression of β -galactosidase was observed in the absence of doxycycline in AP cells expressing FSH, TSH, or ACTH. When the CAG promoter is used, approximately 39% of the total β -galactosidase-immunoreactive cell population are also PRL positive, about 27% are also GH positive, and about 7.6% are LH positive. Ten fields were counted under $\times 40$ magnification. Results are expressed as the mean \pm SD. Note that percentages of each cell population are not representative of the AP gland, as values were taken 9 days after the plating of AP cells in culture.

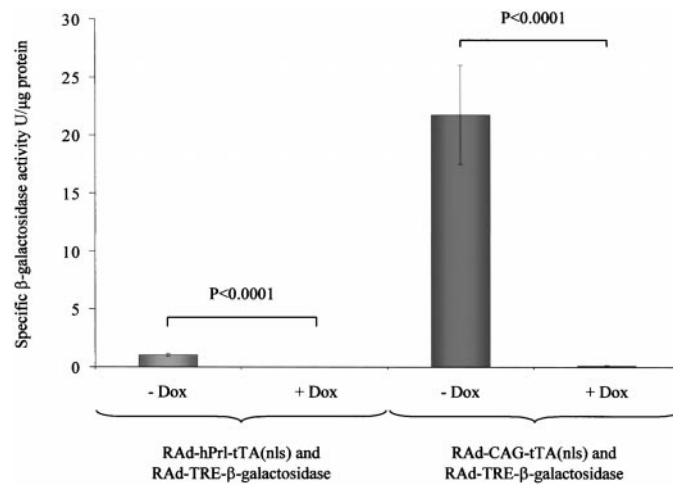
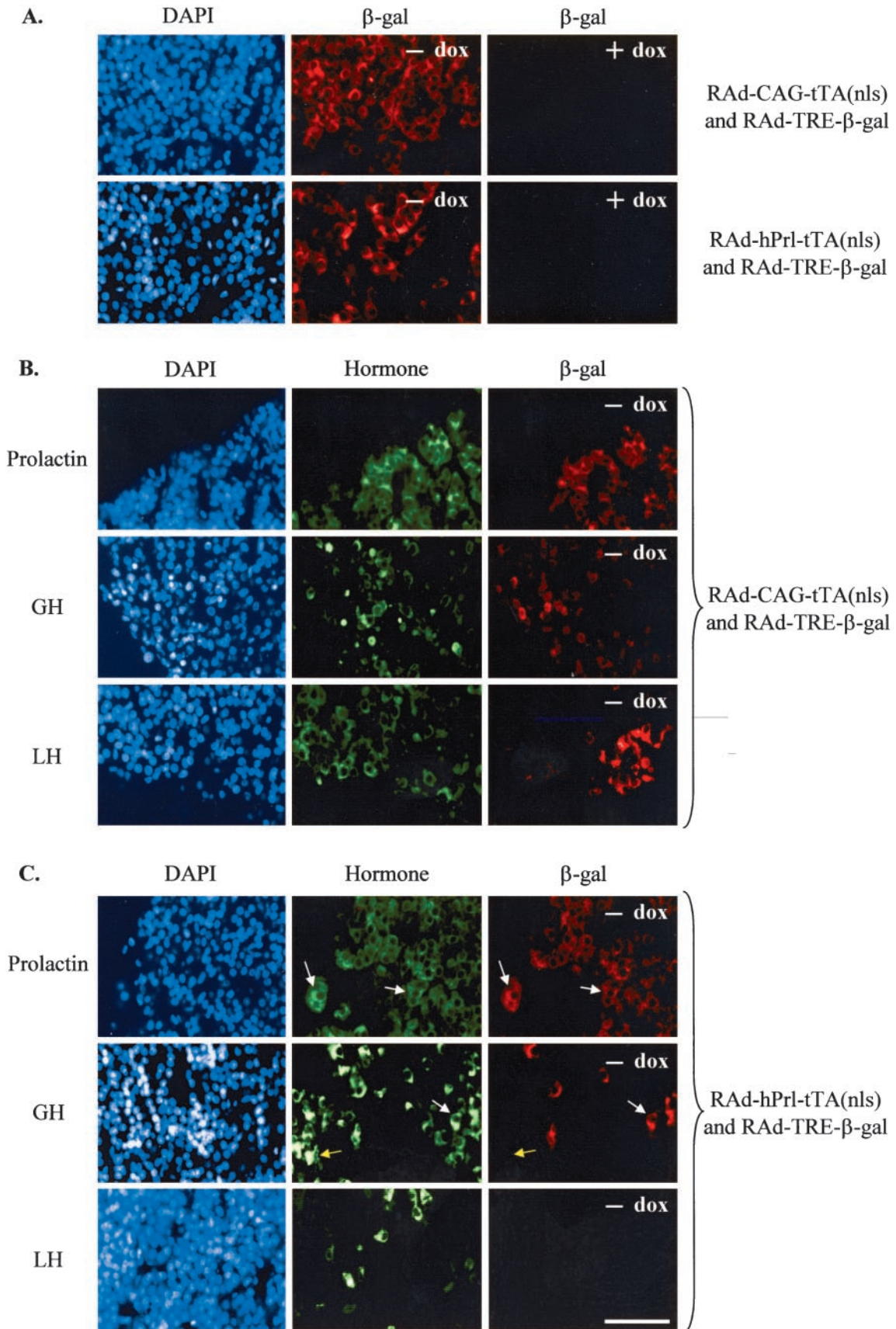


FIG. 4. Quantitative analysis of β -galactosidase expression in primary AP cultures after infection with RAD-CAG-tTA(nls):RAD-TRE- β -galactosidase (right bar) or RAD-hPrl-tTA(nls):RAD-TRE- β -galactosidase (left bar) at a tTA(nls):TRE ratio of 10:1 in the absence and presence of doxycycline. Error bars represent the SEM ($n = 4$). The fold difference between β -galactosidase levels obtained with hPRL and CAG RADs in the absence of doxycycline is indicated.

were infected with RAD-hPRL-tTA(nls):RAD-TRE- β -galactosidase in the absence of doxycycline, specific β -galactosidase activity was induced to 1.03 ± 0.08 U/ μ g protein (Fig. 4). Negligible basal levels of specific β -galactosidase activity were seen in all groups in the presence of doxycycline. The RAD-CAG-tTA(nls):RAD-TRE- β -galactosidase-infected cultures showed a 21-fold increase in induction of β -galactosidase activity compared with RAD-hPRL-tTA(nls):RAD-TRE- β -galactosidase-infected cultures (Fig. 4). Higher levels of β -galactosidase expression obtained in the on state with RAD-CAG-tTA(nls):RAD-TRE- β -galactosidase compared with RAD-hPRL-tTA(nls):RAD-TRE- β -galactosidase were expected, because expression from the hPRL promoter is only seen in lactotrophic cells, which represent about 35% of the total endocrine cell population within primary AP cultures (Table 1), although the lower percentage of cells in which the transgene is expressed cannot explain the 21-fold difference observed.

Lactotroph-specific and regulatable transgene expression in the AP gland *in vivo*

To assess whether lactotroph-specific and regulatable expression could be achieved using this dual adenoviral vector system *in vivo*, the cell types in which β -galactosidase expression was seen were analyzed in the AP gland *in situ* after transcranial stereotaxic delivery. AP glands of male Buffalo rats that had been given water with or without doxycycline were injected with a total of 1×10^8 infectious units of RAD-CAG-tTA(nls):RAD-TRE- β -galactosidase or RAD-hPRL-tTA(nls):RAD-TRE- β -galactosidase virus combinations at a tTA(nls):TRE ratio of 10:1. We then analyzed the cell types in which β -galactosidase expression was seen and compared it with the expression of specific hormones, *i.e.* ACTH, PRL, LH, FSH, TSH, and GH, using double immunofluorescence techniques. Infection with RAD-CAG-tTA(nls):RAD-TRE- β -galactosidase or RAD-hPRL-tTA(nls):RAD-TRE- β -galactosidase resulted in expression of β -galactosidase within the AP gland in the absence, but not the presence, of doxycycline (Fig. 5A). Infection with RAD-CAG-tTA(nls):RAD-TRE- β -galactosidase resulted in expression of β -galactosidase in cells expressing PRL, GH, or LH (Fig. 5B) or FSH, TSH, and ACTH (data not shown) in the absence, but not the presence, of doxycycline. Infection with RAD-hPRL-tTA(nls):RAD-TRE- β -galactosidase resulted in expression of β -galactosidase in fewer cells in the absence, but not the presence, of doxycycline. Expression was restricted mainly to lactotrophic cells and a subpopulation of cells expressing GH that are likely to be mammosomatotrophs and also express PRL (Fig. 5C and Table 2). The cell type specificity displayed by the RAD-hPRL-tTA(nls):RAD-TRE- β -galactosidase dual virus system explains the lower number of cells expressing β -galactosidase compared with RAD-CAG-tTA(nls):RAD-TRE- β -galactosidase and is expected, as PRL-producing cells comprise approximately 32% of all endocrine AP cell types around the injection sites of these animals (Table 2). As β -galactosidase expression was seen in $25 \pm 2.6\%$ of PRL-positive cells (Table 2), then approximately 6% of the total cell population were positive for both PRL and β -galactosidase. No β -galactosidase expression was seen in cells expressing the other AP hormones, *i.e.* LH (Fig. 5C and Table 2) or FSH, TSH,



or ACTH (data not shown) in either the presence or absence of doxycycline.

Switching transgene expression on and off in the AP gland in vivo

To assess whether transgene expression could be switched from on to off and from off to on using this dual adenoviral vector system *in vivo*, rats were given water in the presence or absence of doxycycline. Four days after injection of 1×10^8 iu RAD-CAG-tTA(nls):RAD-TRE- β -galactosidase at a tTA(nls):TRE ratio of 10:1, doxycycline was either withdrawn or administered (Fig. 6). The expression of β -galactosidase was then assessed 11 and 18 days after surgery. In the animals that received doxycycline 4 days after surgery, β -galactosidase expression was found in a very small number of cells sparsely scattered around the AP gland at 11 days, although almost all of the β -galactosidase expression was switched off (Fig. 6). Eighteen days after surgery, no β -galactosidase-positive cells were found in the presence of doxycycline (Fig. 6). In the animals that had doxycycline withdrawn at 4 days, expression of β -galactosidase was seen at both 11 and 18 days (Fig. 6). The levels of β -galactosidase expression seen in the absence of doxycycline on days 11 and 18 showed a gradual decline over time compared with the levels on day 3 (Fig. 5A), in accordance with our previous observations (7).

Discussion

In this study we have investigated the cell type-specific and regulatable expression of the reporter gene β -galactosidase *in vitro* and *in vivo* from a dual adenoviral vector system. Either of two viruses driving the expression of a nuclear localized tTA from the lactotroph-specific hPRL promoter or the ubiquitous CAG promoter were used in combination with a virus driving expression of β -galactosidase from the TRE promoter. In contrast to the ubiquitous CAG promoter, hPRL promoter-regulated β -galactosidase expression was restricted to AP tumor cell lines that synthesize and secrete PRL, *i.e.* GH₃ cells. Also within primary AP cultures and the AP gland *in situ*, regulated β -galactosidase expression was restricted to lactotrophic cells with the hPRL, but not CAG, promoter. When doxycycline was withdrawn or administered 4 days after surgery, β -galactosidase expression was switched on or off, respectively.

Although both cell type-specific promoters and the tetracycline-responsive system have been successfully used to express transgenes within RAdS (6–8, 17, 18, 29), this is the first report on using a combined cell type-specific and regulated system within the AP gland *in vitro* and *in vivo*. We inserted the hPRL-tTA(nls) and TRE- β -galactosidase cassettes in two separate RAdS so that we could address the

issues of optimal tTA(nls) to TRE ratio and promoter interference.

There are conflicting data indicating the ratio of *trans*-activator to TRE at which the best regulation of the tet system is seen. Previously, it had been shown that an excess of TRE is needed to achieve efficient regulation from RAdS when using either the tTA or rTA *trans*-activator (17). Corti *et al.* (18), however, showed that a 1:1 ratio of tTA to TRE is able to mediate tight control of gene expression, whereas previous results from our laboratory have consistently shown that an excess of tTA to TRE in a two plasmid or adenoviral system improves regulated gene expression compared with other ratios (30) (data not shown). Unlike the studies by Harding *et al.* (17, 31) and Corti *et al.* (18), the tTA used in our system contains a nuclear localization signal, and as the compartmental concentrations of tTA or TRE within the cell are likely to influence the control and levels of tet-regulated expression, this may play an important role in both the regulation and the leakiness obtained with this system. Previously, the addition of a nuclear localization signal to the rTA resulted in reduced induction and increased basal gene expression from RAdS (31); however, the addition of an nls to the tTA caused increased induction and decreased basal gene expression from RAdS at a variety of tTA to TRE ratios (15). It is possible that an excess of nuclear rTA may result in a reduced level of TRE binding, whereas excess nuclear tTA may result in increased TRE binding. The exact reasons behind this anomaly are unclear, so with these factors in mind we decided to determine the tTA(nls) to TRE ratio that would provide the highest levels of transgene expression without causing leaky gene expression. When tTA(nls) to TRE ratios of 10:1 and 1:1 were used in pituitary tumor cell lines we saw tight regulation with both ratios, but higher induction levels with a ratio of 10:1, and therefore decided to use this ratio for the subsequent experiments. It is possible that the increased induction levels of gene expression seen at a ratio of 10:1 are caused by increased intracellular levels of tTA(nls) up until a point at which TRE induction plateaus and/or becomes inhibited. Such a scenario might explain the lack of increase in induction between the 1:1 and 10:1 ratios when using the stronger CAG promoter in GH₃ cells (Fig. 2), as this ratio in GH₃ cells, and not BHK, AtT20, or CNS-1 cells, may produce inhibitory levels of tTA(nls). Alternatively, the levels of tTA(nls) reached within GH₃ cells using the CAG promoter at a 10:1 ratio may be toxic.

In addition to its use in assessing the optimal tTA to TRE ratio, our system was designed as a dual adenovirus system to circumvent problems of interference with the transcriptional or regulatory elements. It has been suggested that by placing the promoter driving expression of the tTA in tan-

FIG. 5. A, Expression of β -galactosidase in the AP gland *in vivo* after injection through the transcranial route with RAD-CAG-tTA(nls):RAD-TRE- β -galactosidase or RAD-hPRL-tTA(nls):RAD-TRE- β -galactosidase virus combinations at a tTA(nls):TRE ratio of 10:1 (10^8 iu/pituitary). Note widespread and efficient β -galactosidase expression in the absence of doxycycline, whereas in the presence of doxycycline, β -galactosidase expression is completely switched off. B, Expression of β -galactosidase in the AP gland *in vivo* within immunohistochemically identified hormone-producing cells after infection with RAD-CAG-tTA(nls):RAD-TRE- β -galactosidase in the absence of doxycycline. C, Expression of β -galactosidase in the AP gland *in vivo* within immunohistochemically identified hormone producing cells after infection with RAD-hPRL-tTA(nls):RAD-TRE- β -galactosidase in the absence of doxycycline. White arrows indicate cells double labeled for hormones and β -galactosidase, and yellow arrows indicate cells labeled for hormone alone. The presence or absence of doxycycline is indicated by + dox or – dox in the *top righthand corner*. Scale bar (*bottom right image*), 100 μ m.

dem with or in the same backbone as the TRE promoter, nonspecific induction of gene expression can occur (32), and this may be coupled to reduced inducibility. Although leaky expression is not observed when an alternative regulatory system to the tet system is used in tandem within a RAd (33), this may not be the case with the tet system, as tail to tail insertion of the tTA/TRE cassettes within a RAd (34) resulted in leaky gene expression. The insertion of an insulator sequence between the two cassettes (18) or the division of the two cassettes between two RAds (15, 17, 31) appears to avoid this interference of the regulatory elements within a single RAd, and our results confirm the validity of the later approach. It has also been suggested that the backbone of all RAd vectors may be deleterious to either transcriptional or regulatory systems due to the presence of the E1A enhancer within the lefthand ITR. As β -galactosidase expression from

TABLE 2. Cell-type specific and inducible expression of β -galactosidase driven by the human PRL promoter via the tTA(nls) and TRE from the tetracycline regulatable system in the AP gland *in vivo*

Hormone	Promoter	% of total endocrine cell population positive for single hormone	% of hormone positive cells expressing β -galactosidase in the absence of doxycycline
Prolactin	hPRL	32 \pm 3.6	25 \pm 2.6
GH	hPRL	22 \pm 2.2	5 \pm 5
LH	hPRL	3 \pm 0.8	0

AP glands were injected transcranially with RAd-hPrl-tTA(nls):RAd-TRE- β -galactosidase at a ratio of 10:1 (total of 1×10^8 IU). Hormone-producing AP cells were stained for the various hormones and analyzed for the coexpression of β -galactosidase whose expression was driven by the TRE. Expression from the TRE was, in turn, controlled by binding of the tTA(nls) whose expression was driven by the hPRL promoters. No expression of β -galactosidase was observed in the absence of doxycycline in AP cells expressing FSH, TSH, or ACTH. Five fields were counted under $\times 100$ magnification. Values are the mean \pm SD. Note that percentages of each cell population are not representative of the entire AP gland, as counts were taken from sections around the injection sites only.

our dual RAd system was both cell type specific and tightly regulated, we concluded that the transcriptional and regulatory functions of our dual virus system were not affected by the RAd genetic backbone. It is likely that by placing the tTA(nls) and TRE elements on two different RAds we have avoided the basal promoter leakage and weak repression of expression previously seen with the tet system within a single herpes virus amplicon vector (35).

Although β -galactosidase expression was seen in GH-producing AP cells using our system, this is probably due to the presence of mammosomatotrophic cells within the AP gland that secrete both GH and PRL (36) and not to interference of the hPRL promoter elements. Previously, transgenic mice expressing hGH from a full-length rat PRL promoter restricted expression of hGH to lactotrophs and mammosomatotrophs (24). Although it cannot be excluded that this expression may be due to hPRL expression in somatotrophs, it would seem unlikely, as the number of β -galactosidase/GH-positive cells is considerably lower than the number of β -galactosidase/Pro cells, and male rats generally have higher numbers of somatotrophs than lactotrophs. Also somatotrophs are readily infected by adenoviruses (5–8, 12, 20) and when the ubiquitous CAG promoter was used similar levels of transduction (60–65%) were seen in all cell types, so a lack of somatotroph infectivity cannot be the reason for the reduced levels of β -galactosidase expression seen in somatotrophs compared with lactotrophs when using the hPRL promoter, if this were the case. Furthermore, it has been shown previously that mammosomatotrophs in mice and rats make up between 1–30% of the total GH-positive population in the AP gland (22, 37, 38). Both *in vitro* (13%) and *in vivo* (5%) using the hPRL promoter, we observed β -galactosidase expression in a proportion of the total GH-positive cells that correlates with this observation.

For such a cell type-specific system to be of use in a therapeutic setting, the levels of expression would have to be

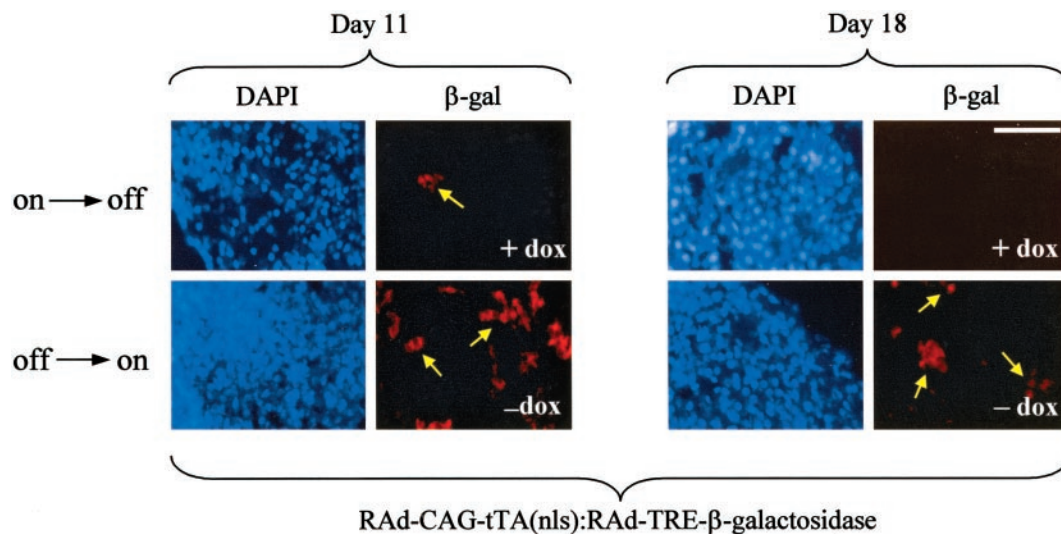


FIG. 6. Switching of β -galactosidase expression in the AP gland *in vivo* 7 and 14 days after the administration or withdrawal of doxycycline. Animals with or without doxycycline in their drinking water were injected through the transcranial route with RAd-CAG-tTA(nls):RAd-TRE- β -galactosidase virus at a tTA(nls):TRE ratio of 10:1 (10^8 iu/pituitary), and 4 days later doxycycline was switched. The presence or absence of doxycycline is indicated by + dox or - dox in the bottom righthand corner. Scale bar (top right image), 100 μ m.

high enough to achieve a beneficial therapeutic outcome, so the efficiency of the hPRL promoter was assessed quantitatively. Although transgene expression from the hPRL promoter within this study appears to be restricted to PRL-secreting cells, it is apparent that the relative levels of inducible expression seen *in vitro* are lower than those obtained with the ubiquitous CAG promoter. Although a lower number of β -galactosidase-transduced cells would be expected using the hPRL promoter in mixed cell populations due to the cell type-specific nature of the promoter, this does not explain the lower levels of specific β -galactosidase activity, compared with the CAG promoter, seen at both ratios in PRL-secreting GH₃ cells in the absence of doxycycline. Additionally the 21-fold increase in specific β -galactosidase activity between hPRL and CAG in primary AP cultures in the absence of doxycycline does not correlate with the 2.4-fold increase (data not shown) in total endocrine cells in primary AP cultures expressing β -galactosidase when using the CAG promoter. Even if β -galactosidase expression from the CAG promoter is seen in 100% of the fibroblasts that account for the remaining 65% of cells within primary AP cultures at 7 days (data not shown), this would only result in an 8.6-fold increase (data not shown) in total cells expressing β -galactosidase. It appears that *in vitro* the hPRL promoter is a weaker promoter than the CAG promoter, and this correlates with previous *in vitro* and *in vivo* observations (6, 7).

As our system is reliant upon a cell being infected by two different viruses, one of its main limitations is the level of cell type-specific transduction achievable (64% of PRL-positive cells *in vitro* and 25% *in vivo*). By increasing the amount of virus used it would be possible to transduce a higher number of cells, but there is a level, both *in vitro* and *in vivo*, at which adenovirus becomes toxic, and the levels of virus used in our experiments were deliberately chosen to be below those thresholds (as determined by us previously, data not shown) (7). Even so it would be difficult to achieve an overall transduction efficiency *in vivo* much greater than 25%, because the cells closer to the injection site are more accessible to infection and *vice versa*. Even so, levels of 25% expression would be sufficient for many therapies, such as the production of hormones, which are secreted molecules. In this case the tet system could be used to regulate hormone release to therapeutic levels independently of the number of cells in which the hormone is expressed. Also, certain tumor treatments, such as the suicide gene therapy approaches (*i.e.* herpes simplex virus 1-thymidine kinase), do not rely upon high transduction efficiencies, because they have efficient bystander-killing effects.

It has previously been shown using dual adenovirus tet off or on regulated systems that expression can be switched on and off in the brain. Harding *et al.* (17) showed that using the tet off system enhanced green fluorescent protein expression was reduced 5 days after doxycycline administration and abolished by 10 days, whereas using the tet on system, enhanced green fluorescent protein expression was reduced 7 days after doxycycline withdrawal and abolished by 10 days. With our system we also saw a slow complete switching off of transgene expression in the presence of doxycycline that took more than 7 days. Although this could be caused by the

slow switching off of transcription, due to poor accessibility of doxycycline to the AP gland, it is more likely to be due to the half-life of β -galactosidase, as expression of β -galactosidase in the AP gland is prevented 4 days after a single doxycycline administration. Unlike when it is switched off, the switching on of transgene expression can be seen at 7 days, although the levels are reduced by 14 days. This quicker switching from one state to the other may be due to the shorter half-life of doxycycline within the body, and the reduced levels seen from 7–14 days are expected because we have previously observed a drop in RAD-mediated AP-directed transgene expression over time (7).

To aid the development of our understanding of AP function and to advance current gene therapy strategies for therapeutic treatment of pituitary diseases, advances in gene delivery technology need to be made. These ought to address putative side-effects (39–41), efficiency, and longevity of transgene expression. With this in mind, we have developed a combined cell type-specific and regulatable expression system that is able to efficiently express a transgene in a cell type-specific and regulatable fashion within the AP gland *in situ*. Although the combined use of cell type-specific and regulatable systems has been shown previously in transgenic animals in the brain (42, 43) and liver (44) or from RADs in the liver (33), tumor cells (45), or brain (30, 46), to our knowledge this is the first reported use of a combined lactotroph-specific and regulatable expression system. The data presented in this paper indicate that transcriptional and reversible inducible transgenesis can be achieved using adenoviral vectors that allow spatial and temporal restriction of transgene expression within the adult AP gland *in vivo*.

Acknowledgments

We are grateful to Mrs. R. Poulton and Ms. T. Maleniak for expert secretarial and technical assistance, respectively. We also thank Dr. A. F. Parlow, National Hormone and Pituitary Program, Harbor-University of California-Los Angeles Medical Center, for the supply of hormone immunocytochemistry antibodies specific for the pituitary hormones, and Prof. J. Davis, Endocrine Sciences Research Group, University of Manchester School of Medicine and Biological Sciences, for the provision of the hPRL promoter complementary DNA for the construction of RAD-hPRL-tTA(nls). We thank Prof. A. M. Heagerty for his continuous support.

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