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Regulatable Gutless Adenovirus Vectors Sustain Inducible Transgene Expression in the Brain in the Presence of an Immune Response against Adenoviruses

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In view of recent serious adverse events and advances in gene therapy technologies, the use of regulatable expression systems is becoming recognized as indispensable adjuncts to successful clinical gene therapy. In the present work we optimized high-capacity adenoviral (HC-Ad) vectors encoding the novel tetracycline-dependent (TetOn)-regulatory elements for efficient and regulatable gene expression in the rat brain in vivo. We constructed two HC-Ad vectors encoding β-galactosidase (β-gal) driven by a TetOn system containing the rtTAS^{s}M2 transactivator and the tTS^{Kid} repressor under the control of the murine cytomegalovirus (mCMV) (HC-Ad-mTetON-β-Gal) or the human CMV (hCMV) promoter (HC-Ad-hTetON-β-Gal). Expression was tightly regulatable by doxycycline (Dox), reaching maximum expression in vivo at 6 days and returning to basal levels at 10 days following the addition or removal of Dox, respectively. Both vectors achieved higher transgene expression levels compared to the expression from vectors encoding the constitutive mCMV or hCMV promoter. HC-Ad-mTetON-β-Gal yielded the highest transgene expression levels and expressed in both neurons and astrocytes. Antivector immune responses continue to limit the clinical use of vectors. We thus tested the inducibility and longevity of HC-Ad-mediated transgene expression in the brain of rats immunized against adenovirus by prior intradermal injections of RAds. Regulated transgene expression from HC-Ad-mTetON-β-Gal remained active even in the presence of a significant systemic immune response. Therefore, these vectors display two coveted characteristics of clinically useful vectors, namely their regulation and effectiveness even in the presence of prior immunization against adenovirus.

The capacity to tightly and effectively turn "on" or switch "off" the expression of a therapeutic gene is critical to achieve successful short- and long-term therapeutic benefits in clinical gene therapy. An inducible system will allow the turning "off" of the therapeutic gene during disease remission or if toxic side effects arise. It will also allow the gene to be turned "on" during exacerbation periods of the disease. Four main regulatory systems are currently available and include the tetracycline-, the progesterone antagonist RU486 (9, 61)-, the insect hormone ecdysone (24)-, and the rapamycin (FK506)-dependent systems (17, 42). We have chosen to use the tetracycline (Tet)-dependent inducible system for transgene expression regulation in the central nervous system (CNS), since the inducers are nontoxic, cross the blood-brain barrier, and provide tight regulation within adenovirus (19, 21, 22, 40, 47, 48, 62).

The original tetracycline (Tet)-regulated system is constitutively active, but in the presence of the tetracycline analog, doxycycline (Dox), gene expression is switched "off" and therefore is known as the "tet off" variant (20, 27). A mutant tetracycline-dependent transactivator (rtTA) was found to become active only in the presence of Dox (15). The rtTA system is thus called "TetOn," since transgene expression is induced in the presence of Dox and inhibited in its absence. Therapeutically, it may be advantageous to control levels of transgene expression. Thus, the TetOn system is a more attractive option to develop regulatable gene expression vectors for preclinical testing, with the aim to prepare this technology for clinical implementation. Another advantage of the TetOn system is that, after addition of the inducer, it has a faster induction period than the TetOff system following depletion of Dox (22).

The generation of rtTA2^{S}M2, an rtTA TetOn transactivator mutant, led to the development of superior TetOn switch systems that exhibit much more stringent regulatable expression (58). Mutated forms of the rtTA transactivator were found to have a lower affinity for the Tet operon DNA in the absence of Dox, therefore giving a much lower basal activity in the "off" state. The rtTA2^{S}M2 mutation also led to strong inducibility with a lower concentration of Dox than rtTA or other mutations (10, 38). The increased sensitivity to Dox and decreased basal activity in the absence of Dox provide clear advantages to the new TetOn system.

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Additionally, the tetracycline-controlled transcriptional silencer, tTS^{Kid}, allows for even tighter regulation of the inducible system (12). The silencer was generated by fusing the DNA binding domain of the Kid-1 protein (*kid-1* is a rat zinc finger gene) to the Tet repressor (63). In vitro assays determined that in the absence of Dox, the silencer inhibits basal levels of gene expression from the tetracycline-inducible promoter even further (43). It has also been determined that the silencer reduces gene expression down to background levels in the absence of Dox in in vivo models (31, 40, 44, 65). Finally, the transcriptional repressor enhances transactivator expression levels by inhibiting its degradation through the ubiquitin-dependent proteasomal degradation pathway (25). Therefore, the addition of the Dox-dependent transcriptional repressor, tTS^{Kid}, to the $rtTA2^{S}M2$ TetOn-inducible system results in a regulatable switch with stringent regulation kinetics and virtually no basal expression in the "off" state (26, 33, 45).

The ideal TetOn gene regulatory system for effective and safe short- and long-term regulation of the therapeutic gene should exhibit (i) no basal activity of the transactivator in the absence of the tetracycline inducer, (ii) fast induction and shutoff kinetics, (iii) specific regulation of transgene expression, (iv) negligible cytotoxic or inflammatory responses associated with the regulatory elements within the TetOn switch system, and (v) no pleiotropic consequences for other cellular transcription units (16).

Regulatable switches have been previously engineered into several vector systems, i.e., lentivirus (13, 23, 38, 59), retrovirus (19), adeno-associated virus (10, 11, 20, 27, 33, 40, 44), first-generation adenovirus (11, 27, 33, 44, 47, 48), herpesvirus (18), and high-capacity adenoviral vectors (5, 6, 45, 57, 60, 64). Wang et al. determined that an inducible system within a high-capacity adenovirus (HC-Ad) led to prolonged periods of regulatable transgene expression in the liver (60). A helper-dependent adenoviral vector encoding alpha interferon under the control of a TetOn system showed regulatable expression after systemic administration in rodents and nonhuman primates (5, 6). The combination of $rtTAS^{s}M2$ with tTS^{Kid} in an HC-Ad vector was previously shown to exert tight control of transgene expression, as measured by serum levels of the reporter gene of soluble alkaline phosphatase, after intramuscular administration in mice (45).

The inducible system drives expression of the transgene encoding β-galactosidase (β-gal) from the inducible Tet promoter. The entire regulatable switch was cloned into a high-capacity, helper-dependent adenoviral vector. We engineered the regulatory TetOn switch ($rtTA2^{S}M2$ transactivator and tTS^{Kid} repressor) under the control of either the strong murine cytomegalovirus (mCMV) or human CMV (hCMV) promoter (14). To avoid inserting a third expression cassette, an internal ribosome entry site (IRES) was added between the $rtTA2^{S}M2$ transactivator and the tTS^{Kid} repressor to allow translation and expression of these proteins from a single promoter. The use of strong promoters will elicit high transactivator gene expression levels with lower doses of viral vector, which in the presence of Dox will bind to the TRE promoter to elicit high levels of gene expression. This will minimize the chances of vector-induced toxicity and inflammation due to high viral vector doses needed to achieve therapeutic efficacy.

It has been previously demonstrated that peripheral immune responses against adenovirus completely inhibit transgene expression from first-generation adenoviral vectors in the brain (53, 54). In the present work we constructed and characterized in detail two novel inducible HC-Ad vectors in the rat brain in vivo and tested the inducibility and longevity of HC-Ad-mTetON-β-Gal-mediated transgene expression in rats preimmunized against adenovirus. To do so, we constructed two high-capacity adenoviral vectors encoding β-galactosidase driven by a TetOn system that contains the $rtTAS^{s}M2$ transactivator and tTS^{Kid} repressor under the control of the murine cytomegalovirus promoter (mCMV; HC-Ad-mTetON-β-Gal) and the human CMV promoter (HC-Ad-hTetON-β-Gal). Both vectors exhibited good inducibility and were capable of being turned "off" by the depletion of Dox, with HC-Ad-mTetON-β-Gal showing higher levels of transgene expression. The HC-Ad vector was able to regulate and exhibit persistent transgene expression even in the presence of a systemic immune response against the vector. Therefore, this vector constitutes an attractive candidate for developing human gene therapy approaches.

MATERIALS AND METHODS

Engineering of transgene and regulatable TetOn switch components. The β-galactosidase (β-gal) transgene cassette [TRE-β-gal-polyA], approximately 4.5 kb in size, was excised using XhoI and HindIII from plasmid pΔE1-TRE-β-gal-polyA (Clontech), and the XhoI site was adapted to a HindIII end. The HindIII-flanked transgene cassette was then ligated into the HindIII site of intermediate plasmid pBlueScript II SK(+) to generate pBlueScript II SK(+)[TRE-β-gal-polyA]. The kanamycin resistance gene was excised using NheI from plasmid pcDNA3.1/Zeo(+)-Kanamycin (Clontech) and cloned into the NheI site of plasmid pBlueScript II SK(+)[TRE-β-gal-polyA] to create an intermediate plasmid, pBlueScript II SK(+)[TRE-β-gal-polyA]-Kanamycin. The kanamycin sequence was used as a selection marker for positive screening of clones for generation of HC-Ad plasmids.

Construction of murine and human CMV-driven regulatable TetOn switches. The $rtTA2^{S}M2$ transactivator was excised using EcoRI and BamHI from plasmid pUHrT 62-1, approximately 4.2 kb in size (provided by H. Bujard, ZMBH, Germany); the BamHI site was adapted with MluI, and the insert was directionally cloned into a previously generated plasmid p[IRES-tTS^{Kid}-pA], resulting in intermediate plasmid, p[$rtTA2^{S}M2$-pIRES-tTS^{Kid}-pA]. The transcriptional silencer carrying plasmid p[IRES-tTS^{Kid}-pA] was generated using IRES and tTS^{Kid} sequences from commercial plasmid pIRES, 6.1 kb (Clontech), and plasmid pUHS 6-1, 4.3 kb (provided by H. Bujard, ZMBH, Germany), respectively. The mCMV promoter was excised from pAL120 (generated in our laboratory [14]) with EcoRI and HindIII and ligated into a pSP72 shuttle vector (Clontech), producing intermediary plasmid pSP72[mCMV]. Plasmid p[$rtTA2^{S}M2$-pIRES-tTS^{Kid}-pA] was excised with XhoI and SalI to release cassette [$rtTA2^{S}M2$-pIRES-tTS^{Kid}-pA] and subsequently cloned into its corresponding sites into plasmid pSP72[mCMV], generating the intermediate plasmid pSP72[*mCMV*-$rtTA2^{S}M2$-pIRES-tTS^{Kid}-pA]. The [*mCMV*-$rtTA2^{S}M2$-pIRES-tTS^{Kid}-pA] regulatable TetOn switch was then excised with BglII and cloned into the BglII site of intermediate plasmid pBlueScript II SK(+)[TRE-β-gal-polyA]-Kanamycin, thus generating the final intermediate plasmid, pBlueScript II SK(+)[TRE-β-gal-polyA]-[*mCMV*-$rtTA2^{S}M2$-IRES-tTS^{Kid}-pA]-Kanamycin. A similar strategy was used to generate the final intermediate plasmid pBlueScript II SK(+)[TRE-β-gal-polyA]-[*hCMV*-$rtTA2^{S}M2$-IRES-tTS^{Kid}-pA]-Kanamycin.

Engineering of HC-Ad plasmids. The insert [TRE-β-gal-polyA]-[*mCMV*-$rtTA2^{S}M2$-IRES-tTS^{Kid}-pA]-Kanamycin or [TRE-β-gal-polyA]-[*hCMV*-$rtTA2^{S}M2$-IRES-tTS^{Kid}-pA]-Kanamycin was excised and cloned into the compatible NheI site of HC-Ad plasmid pSTK120m, generating pSTK120m[TRE-β-gal-polyA]-[*mCMV*-$rtTA2^{S}M2$-IRES-tTS^{Kid}-pA]-Kanamycin (HC-Ad-mTetON-β-Gal) and pSTK120.1 [TRE-β-gal-polyA]-[*hCMV*-$rtTA2^{S}M2$-IRES-tTS^{Kid}-pA]-Kanamycin (HC-Ad-hTetON-β-Gal). The following vectors were used as positive controls in our experiments: first-generation vectors (RAd) RAd35 [hCMV-Lac Z-pA] (46) and RAd36 [mCMV-Lac Z-pA] (14); RAd-hypoxanthine phosphoribosyltransferase (RAd-HPRT) (50, 53, 54) was used for peripheral preimmunizations.

Production, scale up, and purification of HC-Ad vectors. HC-Ad vectors were generated using 5 μg of HC-Ad plasmid DNA that was linearized using PmeI, heat inactivated, and transfected into 293FLPe cells using the calcium phosphate method. Transfected 293 cells were coinfected with helper virus (FL) (57) (previously generated in our laboratory) with a multiplicity of infection (MOI) of 5 (passage 0). All subsequent infections in the amplification were done using an MOI of 1. After full cytopathic effect (CPE) was observed for each passage, 1.5×10^6 of preseeded confluent 293FLPe cells were adsorbed for 1 h with 0.5 ml of the cell lysate from the preceding passage (after three cycles of freeze/thaw for membrane lysis and viral release), followed by infection with FL helper virus with an MOI of 1. Cells were incubated at 37°C for 2 to 3 days until full CPE was observed. Subsequent passages were performed in the same manner with identical corresponding MOIs until passage 7 was reached. Subsequent scale up, purification, and end point titrations of HC-Ad vectors were done as previously described (28, 29, 57). High titers of HC-Ad vectors, which were used to assess regulated and persistent gene expression in preimmunized animals, were scaled up and purified using the 293Cre cell system as previously described (35, 37).

In vitro HC-Ad infection and β-galactosidase activity. Regulatable expression of β-galactosidase from HC-Ad vectors was tested in COS7 and CNS-1 cell lines. Twenty-four hours prior to HC-Ad virus infection, cells were plated in 6-well plates at a density of 8×10^5 cells/well either in the presence or absence of 1 μg/ml doxycycline in Dulbecco's modified Eagle medium (DMEM) containing 10 μl/ml nonessential amino acids, 10 μl/ml L-glutamine, 10 μl/ml penicillin-streptomycin, and 10% fetal calf serum (FCS; Invitrogen). Cells were infected with 30 blue forming units (BFU) of HC-Ad-mTetON-β-Gal, HC-Ad-hTetON-β-Gal, and the first-generation vectors RAd-hCMV-β-Gal and RAd-mCMV-β-Gal and incubated for 72 h either with or without 1 μg/ml doxycycline in DMEM. After incubation, cells were subjected to a single gentle wash with phosphate-buffered saline (PBS) and then gently scraped, centrifuged, and resuspended in a volume of 100 μl of PBS. The cell suspensions were flash frozen and thawed three times and cellular debris were removed by centrifugation. The supernatants containing protein extracts were transferred into fresh tubes, and 1 μl of Halt Protease inhibitor cocktail EDTA-Free (Pierce) was added to the samples and stored at −70°C until use. The β-galactosidase assays were performed to measure the enzymatic activity of the transgene by means of color detection. β-Galactosidase activity was measured by conversion of *o*-nitrophenyl-β-D-galactopyranoside in 10 mM $MgCl_2$–0.45 M 2-mercaptoethanol. All samples were incubated at 37°C, and the enzymatic reaction was stopped with 510 μl 1 M Na_2CO_3 (2). β-Galactosidase activity measurements were recorded at 420 nm absorbance from a nitrophenol standard curve using *o*-nitrophenol substrate. Protein sample measurements were determined at 562 nm absorbance from a standard protein curve produced using bicinchoninic acid protein assay reagent (Promega). The β-galactosidase activity was determined using the following mathematical equation: enzymatic activity = *o*-nitrophenol/(time × protein).

Stereotactic neurosurgery. Male Fisher 344 rats of 200 to 250 g of body weight (Harlem Sprague Dawley Inc.) were used for in vivo HC-Ad-mediated gene delivery. Animals were housed at constant temperature and humidity with a 12-h light/dark cycle and had free access to food and water containing Dox and 1% sucrose, 1% sucrose only, or water alone. Rats were given drinking water containing 2.0 mg/ml Dox (Sigma) and 1% sucrose or 1% sucrose alone 24 h prior to brain surgery and HC-Ad delivery. On the day of surgery, the animal's head area was shaved, prepared with betadyne, and anesthetized with ketamine and xylazine and placed in a stereotactic frame ready for surgery. A total of 1×10^7 BFU of the HC-Ad vector, either HC-Ad-mTetON-β-Gal or HC-Ad-hTetON-β-Gal, was injected in the rat striatum (coordinates from bregma [the contact between sagittal and coronal sutures] were the following: anterior, 1.0 mm; lateral, 3.0 mm; ventral, 4.0 mm) using a 10-μl Hamilton syringe (47). A total volume of 3 μl of HC-Ad vector diluted in 0.9% (wt/vol) saline per animal was injected in the striatum over a 5-min period. Subsequent to vector injection, the needle was left in place for a further 2 min prior to careful needle retraction. Control rats received 3-μl saline injections. After postoperative surgery, both untreated and treated animals were housed in the above-described conditions for the time indicated in each experimental condition. Animals operated on received drinking water containing Dox and 1% sucrose, 1% sucrose alone, or water alone during the required times, and drinking water was changed daily. Animals were then sacrificed and brains were perfused with approximately 200 ml oxygenated tyrodes solution (0.14 M NaCl, 1.8 mM $CaCl_2$, 2.7 mM KCl, 0.32 mM NaH_2PO_4, 5.6 mM glucose, and 11.6 mM $NaHCO_3$) by means of *trans*-cardial perfusion. For immunohistochemistry, brains were perfused with 250 ml of 4% paraformaldehyde, pH 7.4 (4% PFA), fixative and stored postfixed in 4% PFA for 3 days at 4°C followed by 3 days of washing in PBS containing 0.1% sodium azide. Brains were serial sectioned using an electronic VT1000S vibrating blade vibratome (Leica) to obtain 50-μm free-floating sections. Sections were stored in PBS containing 0.1% sodium azide at 4°C until ready for use.

Preimmunization studies. The surgical procedures and stereotactic coordinates described in the above section were employed in the preimmunization experiment involving intrastriatal injection of HC-Ad-mTetON-β-Gal. Animals were injected intradermally in the lower posterior area with 1×10^9 infectious units of first-generation vector RAd-HPRT in a volume of 100 μl 3 weeks before intrastriatal injections. Animals were housed under previously described normal conditions for 3 weeks to generate an immune response to the recombinant adenoviral vector. The nonimmunized group (control group) received intradermal injections of saline in a 100-μl volume also in the lower posterior area and were housed under previously described normal conditions for 3 weeks. Three weeks after preimmunization, all saline-treated and virus-immunized animals ($n = 3$ per group) were anesthetized with ketamine and medatomidine and placed in a stereotactic frame ready for surgery. Prior to stereotactic surgery, anesthetized animals received a second immunization with either RAd-HPRT or saline in a volume of 100 μl in the lower posterior area to boost the immune response. A total of 1×10^7 blue forming units (BFU) of the HC-Ad vector HC-Ad-mTetON-β-Gal were injected in the rat striatum. After postoperative surgery, each saline-treated and preimmunized animal group received drinking water containing Dox and 1% sucrose (transgene expression) or 1% sucrose with water alone (transgene expression "off"). For each group, animals were sacrificed at the end of each time point for determination of β-galactosidase activity assay.

Anti-adenovirus neutralizing antibody assay. Blood samples were collected by retro-orbital bleeding 10 days after intradermal injection of RAd-HPRT or saline to measure anti-adenovirus-neutralizing antibodies from both nonimmunized (saline) and preimmunized animal groups. Serum samples were heat-inactivated at 56°C for 30 min and serially diluted twofold in minimal essential medium (Invitrogen) containing 2% FCS. The range of dilutions was 1:2 to 1:4,096. Each 50-μl serum dilution was incubated with 1×10^7 BFU of HC-Ad-mTetON-β-Gal (in a 10-μl volume) for 90 min at 37°C. The 50 μl of sample containing sera and virus was then added to the wells of a 96-well plate containing preseeded (1.5×10^4) HEK 293 cells per well and were incubated at 37°C for 1 h. A further 50 μl of medium containing 10% FCS was added to each well, and cells were incubated at 37°C for 20 h before fixing with 4% paraformaldehyde in PBS (pH 7.4) and staining with 5-bromo-4-chloro-indolyl-β-D-galactoside (X-gal) (Sigma). The neutralizing antibody titer for each animal is given as the reciprocal of the highest dilution of serum at which 50% of HC-Ad-mTetON-β-Gal-mediated transduction was inhibited (50, 53).

Western blotting. Sera were collected from each rat by retro-orbital bleeds 10 days after intradermal injection of RAd-HPRT or saline and used for the Western blots. Sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis was performed with adenoviral lysates made by infecting COS-7 cells with 100 virus particles/cell, resuspending them in a volume of 100 μl, adding sodium dodecyl sulfate-Tris buffer to a final concentration of 2%, and boiling for 10 min. Other proteins used were purified recombinant penton and fiber in bacterial and insect cells, respectively. These proteins were made as previously described (32, 41). The gels were then transferred to nitrocellulose and probed with either anti-Ad5 antibody (from AbCam; 1/4,000) or rat serum (1/100). Antibody binding was determined by using a secondary sheep anti-rabbit or rabbit-anti-rat, both of which were horseradish peroxidase (HRP) conjugated, and then developed with the ECL Western Blotting Analysis kit (Amersham Biosciences).

Tissue harvesting and β-galactosidase activity. At the end of the experiments, animals were perfused with approximately 200 ml oxygenated tyrodes solution followed by brain tissue resection around the injected site for tissue harvesting. The dissected tissue was homogenized using a tissue grinder pestle and tube in 500 μl of Halt Protease inhibitor cocktail EDTA-Free (Pierce), and the lysate was flash frozen and thawed three times for cell membrane lysis and protein release. Cellular debris was removed by centrifugation, and the supernatant protein extract was stored on ice until use. After the β-galactosidase enzymatic reactions were set up, all samples were incubated at 37°C for 1 h. The enzymatic reaction was stopped with 510 μl of 1 M Na_2CO_3 (2). β-Galactosidase assays, protein assays, and enzymatic activity rate were performed and measured as described earlier (49, 56).

Immunohistochemistry. Free-floating brain sections were washed with Tris-buffered saline (TBS) and 0.5% Triton X-100 followed by 0.3% H_2O_2 incubation to inactivate endogenous peroxidase. Nonspecific antibody sites and Fc receptors were blocked with 10% normal horse serum for 1 h. Sections were incubated for 48 h at room temperature with rabbit polyclonal anti-β-galactosidase primary antibody (1:1,000) (generated in our laboratory [3, 48, 51, 53]) diluted in TBS-0.5% Triton-1% horse serum-0.1% sodium azide. Sections were washed three times with TBS plus 0.5% Triton and then incubated with goat anti-rabbit biotinylated secondary antibody (1:800) (Dako) for 4 h. The avidin-biotinylated

HRP complex was prepared and used for detection using a Vectastain ABC Elite kit (Vector Laboratories). Following staining with diaminobenzidone (DAB) and glucose oxidase, sections were mounted on gelatin-coated glass slides, dehydrated through graded ethanol solutions, and carefully covered for microscopy. For double fluorescence labeling, sections were incubated with rabbit polyclonal anti-β-gal (1:1,000) combined with chicken polyclonal anti-microtubule associated protein-2 (MAP-2) (1:1,000) and with guinea pig polyclonal anti-glial fibrillary acidic protein (GFAP) (1:500) at room temperature for 48 h, followed by flourescein (Texas Red)-conjugated goat anti-rabbit antibody (1:800) combined with an Alexa-488-conjugated goat anti-chicken antibody (1:1,000) and Alexa-633-conjugated goat anti-guinea pig antibody (1:1,000). Sections were then incubated for 30 min with 4≪,6≪-diamidino-2-phenylindole (1:1,000), washed, and mounted for light and confocal microscopy.

Quantitative stereological analysis. Quantitative analysis to determine the anatomical area occupied by cells immunoreactive with antibodies against β-galactosidase and immune markers in 50-μm brain sections was performed using a Zeiss AxioPlan 2 Imaging microscope (Carl Zeiss Microsystems, Inc., Thornwood, NY) controlled by an electronic MAC 5000 XY stage control (Ludl Electronics Products Ltd., Hawthorne, NY). Brain sections containing the needle track (area of highest levels of immunoreactivity) were used for quantitative analysis. Student's *t* test was used to determine the degree of statistical significance between values from each time point for naïve and preimmunized animal groups (52).

Confocal microscopy. Low-field-magnification images of striatal brain sections were captured using an AxioPlan 2 Imaging microscope (Carl Zeiss Microsystems, Inc., Thornwood, NY) controlled by an electronic MAC 5000 XY stage control (Ludl Electronics Products Ltd., Hawthorne, NY). High-field imagery of β-galactosidase expression in striatal neurons and astrocytes was captured using a confocal microscope (Leica). Brain sections, each 50 μm in thickness, were subjected to sequential laser scanning at 0.5-μm intervals and sequential overlay of layers at the site of interest in the *xyz* axes (52).

Statistical analysis. β-Galactosidase activity data were normalized by protein content and incubation time. They were expressed as means ± standard errors and evaluated by Student's *t* test, two-way analysis of variance (ANOVA) followed by polynomial contrasts or Tukey's test, or one-way ANOVA followed by Dunnet's test. When data failed a normality test, they were evaluated by the nonparametric Kruskal-Wallis test. Results were expressed as the means ± standard errors of the means (SEM). Real-time PCR data were normalized by sample DNA content and analyzed by Student's *t* test. A *P* value of <0.05 was considered significant. All experiments were performed twice.

RESULTS

Generation and characterization of TetOn-regulatable, high-capacity adenoviral vectors (HC-Ad). We have previously demonstrated that a strong promoter is necessary to achieve high levels of transgene expression, allowing the reduction of the vector dose and, thus, eliminating inflammatory side effects (14). We therefore compared the murine major immediate-early cytomegalovirus (mCMV) and the hCMV promoters driving the expression of the rtTA2^{s}M2 transactivator, IRES, and tTS^{kid} repressor cassettes. We constructed two HC-Ad vectors, one encoding the hCMV promoter driving expression of the transactivator cassette (HC-Ad-hTetON-β-Gal) and a second one (HC-Ad-mTetON-β-Gal) encoding the mCMV promoter driving expression of the regulatory proteins (Fig. 1A). In both vectors, the rtTA2^{s}M2 transactivator is responsible for initiating expression of the β-galactosidase transgene by binding to the TRE promoter in the presence of Dox. Following cloning to develop the HC-Ad vector plasmids, restriction digests were performed to map the plasmid DNA constructs (Fig. 1B, C, and D). After confirming the presence and the required orientation of the TetOn-IRES-tTS^{Kid} and the β-gal expression cassettes, we proceeded to construct, scale up, and purify both HC-Ads as previously described elsewhere (29, 36, 37, 57).

Regulated transgene expression in vitro. We first determined the efficacy and inducibility of the novel vectors in vitro. Transgene expression was determined by measuring β-galactosidase activity in HC-Ad-mTetON-β-Gal- and HC-Ad-hTetON-β-Gal-infected cell lines. HC-Ads encoding β-galactosidase under the control of the TetOn system achieved high and regulatable expression in both CNS-1 rat glioma cells (Fig. 2 A) and COS-7 monkey kidney cells (Fig. 2 B). Compared to CNS-1 and COS-7 cells infected with Rad-hCMV-β-Gal or Rad-mCMV-β-Gal (used as positive controls), the activity was significantly higher when these cells were infected with the HC-Ad-mTetON-β-Gal and HC-hTetON-β-Gal vectors (Fig. 2A and B). HC-Ad-TetOn vectors induced strong transgene expression in the presence of Dox, while, in the absence of the inducer, β-galactosidase activity was negligible. Infection with HC-Ad vectors encoding β-galactosidase under the control of the TetOn system driven by the murine or human CMV promoter in the presence of Dox provided 3.24 ± 0.028 times higher β-gal activity in CNS-1 cells ($P < 0.001$) and 1.33 ± 0.07 times higher β-gal activity in COS-7 cells ($P < 0.05$).

In vivo transgene expression in the rat brain: dose response to doxycycline. To evaluate whether these vectors are able to induce successful transgene expression in vivo, we administered them by intracranial injection into the striatum of rats. We first performed a dose curve of doxycycline to determine the optimal dose of the inducer necessary to attain maximal transgene expression in vivo. The inducibility and leakiness of the regulatable TetOn switch from HC-Ad-TetOn-mCMV was determined in intracranially injected rats that received increasing doses of doxycycline (0 to 2.5 mg/ml) in their drinking water. After 7 days, levels of transgene expression were determined in the striatum by the β-gal enzymatic activity assay (Fig. 3). Statistical analysis showed that there was not a significant difference between 0 mg/ml of Dox and either the 0.5 mg/ml or 1 mg/ml concentration. A significant increase in β-galactosidase levels was observed at 2 mg/ml and 2.5 mg/ml compared to the control levels of 0 mg/ml of Dox. In view of these results, we administered 2 mg/ml doxycycline in the drinking water of the rats for all in vivo experiments described below.

We compared the Dox-dependent induction of transgene expression driven by HC-Ad-mCMV-β-Gal and HC-Ad-hTetON-β-Gal using immunocytochemical techniques to determine distribution and cell type-specific expression within the striatum (Fig. 4). Both vectors were injected intracranially in rats, followed by the administration of 2 mg/ml doxycycline in their drinking water for 7 days. Both vectors harboring either the hCMV or the mCMV promoter driving the transactivator showed strong β-galactosidase expression in the presence of Dox and minimal expression in the absence of Dox (Fig. 4A). In order to detect which cell types were transduced after the intrastriatal injection of these vectors, we colocalized β-galactosidase with MAP-2 to identify neurons (Fig. 4B) and GFAP to identify astrocytes (Fig. 4C). Both HC-Ad vectors successfully transduced neurons and astrocytes. Quantitative assessment of β-galactosidase enzymatic activity showed that both HC-Ad vectors displayed stringent Dox-dependent transgene expression in the "on" state, with negligible basal expression in the "off" state (Fig. 5). However, compared to the HC-Ad-hTetON-β-Gal vector, the HC-Ad-mTetON-β-Gal

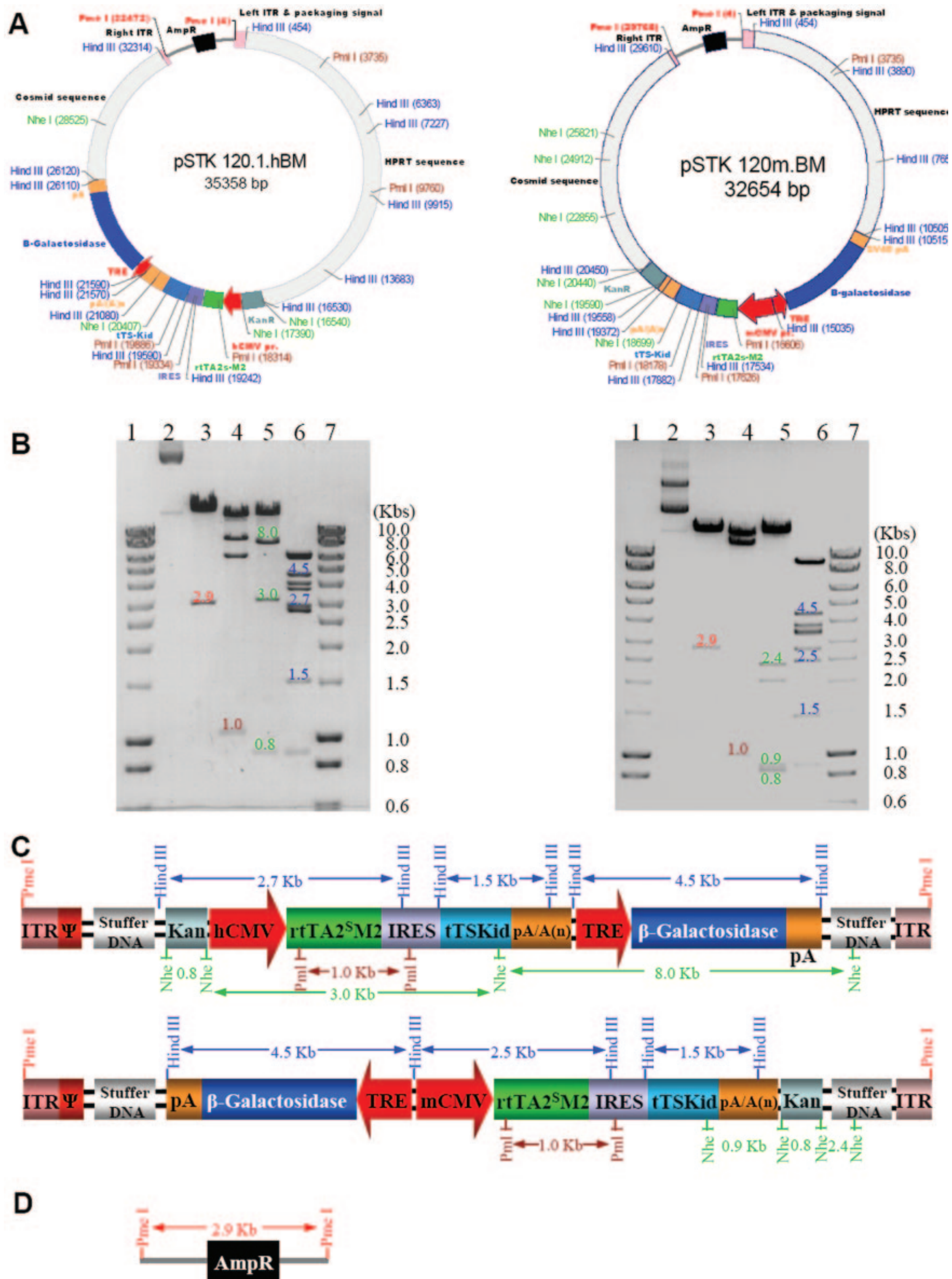

FIG. 1. Schematic representation of pSTK120.1[TRE-β-Gal-pA]-[hCMV-rtTA2^{S}M2-IRES-tTS^{Kid}-pA], approximately 34.857 kb (HC-Ad-hTetON-β-Gal, left panels), and pSTK120m[TRE-β-Gal-pA]-[mCMV-rtTA2^{S}M2-IRES-tTS^{Kid}-pA], approximately 32.464 kb (HC-Ad-mTetON-β-Gal, right panels). (A) HC-Ad plasmid maps indicate the constituents and orientation of the TRE-encoded β-gal cassette and hCMV- or mCMV-driven regulatable TetOn switch cassettes within a pSTK gutless plasmid. (B) Gel electrophoresis and restriction map analysis of HC-Ad plasmid DNA to check for expected band sizes. For both gels, the lanes are as follows: lane 1, hyperladder I (corresponding sizes are labeled to the right of each gel); lane 2, undigested plasmid; lane 3, PmeI digest; lane 4, PmlI digest; lane 5, NheI digest; lane 6, HindIII digest; lane 7, hyperladder I. The colored fragment sizes correspond to the indicated fragments shown in part C. (C) Linear depiction of the HC-Ad vector encoding the β-gal transgene and hCMV- or mCMV-driven regulatable TetOn switch cassette. The constructs indicate the individual components and the orientation of the cassettes and their promoters. Some restriction enzyme sites are shown with the appropriate size fragments which correspond to the sizes indicated in panel B. ITR, inverted terminal repeat. (D) Linear depiction of the ampicillin resistance (AmpR) gene with the restriction enzyme (PmeI) site and fragment size indicated which corresponds to the gels in panel B.

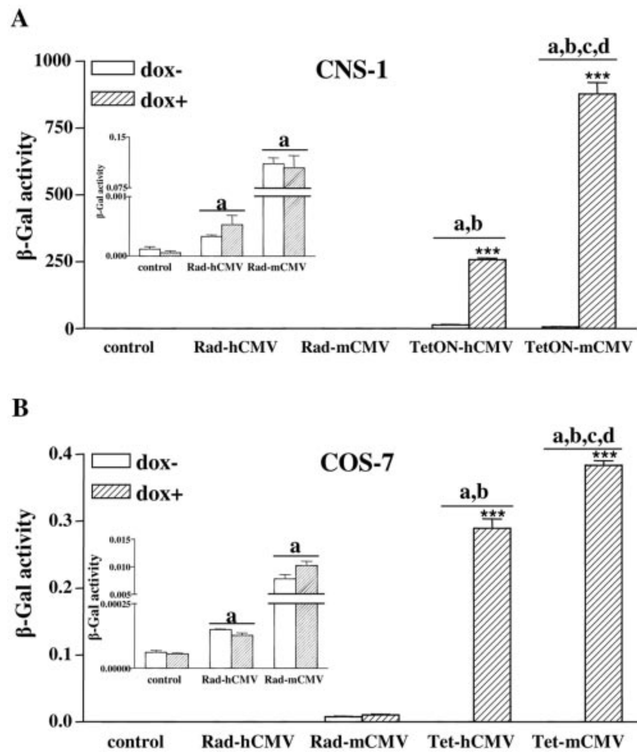

FIG. 2. In vitro β-gal activity in CNS-1 (A) and COS-7 (B) cells infected with high-capacity adenoviral vectors containing a regulatable TetOn switch or first-generation adenoviral vectors (inset). Cells were infected with RAd-hCMV-β-Gal, RAd-mCMV-β-Gal, HC-Ad-hTetON-β-Gal, and HC-Ad-hTetON-β-Gal (30 BFU/cell) in the presence or absence of 1 μg/ml doxycycline for 72 h. After cell harvesting, proteins were purified in the presence of protease inhibitors, and β-gal enzyme activity levels were measured using 2-nitrophenol-β-D-galactopyranoside as substrate. Columns represent the means ± SEM of β-gal activity, calculated as *o*-nitrophenol produced (mg/ml)/sample protein content (mg/ml)/incubation time (min). $n = 3$ wells/group. ***, $P < 0.001$ versus control groups without Dox; a, $P < 0.001$ versus a mock-infected group (control); b, $P < 0.001$ versus a RAd-hCMV-infected group; c, $P < 0.001$ versus a RAd-mCMV-infected group; d, $P < 0.001$ versus a TetON-hCMV-infected group. Statistical analysis was performed using two-way ANOVA followed by polynomial contrasts.

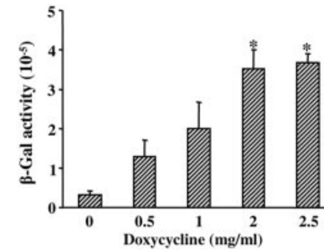

FIG. 3. Doxycycline dose-response curve in the brain of rats intracranially injected with HC-Ad-mTetON-β-Gal. A total of 10^7 blue forming units (BFU) of HC-Ad-mTetON-β-Gal were intrastriatally injected into rats that received a range of doxycycline concentrations (0 mg/ml to 2.5 mg/ml) in their drinking water. After 6 days, transgene expression was determined in the brain by the β-galactosidase activity assay. Columns represent the means ± SEM of β-gal activity, calculated as *o*-nitrophenol produced (mg/ml)/sample protein content (mg/ml)/incubation time (min). Each group was statistically compared with the control group (0 mg/ml Dox). $n = 3$ to 9 animals/group. *, $P < 0.05$ versus control. Statistical analysis was performed using the Kruskal-Wallis test.

vector displayed higher levels of Dox-dependent β-galactosidase activity in the presence of inducer. Considering that the highest in vitro and in vivo transgene expression levels were achieved using HC-Ad-mTetON-β-Gal, we decided to pursue this vector for the rest of the in vivo experiments described below.

In vivo kinetics of induction and inhibition of transgene expression within the CNS from HC-Ad-mTetON-β-Gal-infected rats. The induction kinetics from the HC-Ad-mTetON-β-Gal vector were studied in vivo in intracranially injected rats that received 2 mg/ml doxycycline in their drinking water over a 2-week time course. β-Galactosidase activity was measured in the brain of these animals at different time points over this time period. Figure 6A indicates that maximal β-galactosidase activity can be achieved 6 days after HC-Ad vector injection in the presence of Dox. After reaching peak levels at day 6, β-galactosidase enzymatic activity levels remained stable through the 8- and 14-day time points.

We next examined the kinetics to inhibit or turn "off" HC-Ad-mTetON-β-Gal-driven transgene expression after the withdrawal of Dox. After the intracranial injection with HC-Ad-mCMV, Dox-containing water was administered for 7 days to allow the maximum transgene expression to be reached in the brain. After day 7, Dox-containing water was removed and β-gal activity was determined in the brain at different time points. By the fourth day after Dox removal, β-galactosidase activity was significantly decreased and returned to close to basal levels around 14 days after doxycycline withdrawal. At days 10 and 14 after withdrawal of Dox-containing water, we did not observe significant levels of enzyme activity compared to levels for the saline-injected brains (data not shown). As a control, we show that a nonregulatable HC-Ad vector exerts stable transgene expression at least until day 14 (Fig. 6B, inset). We have previously demonstrated that expression from both RAds and HC-Ad vectors remains stable in the brains of naïve, nonimmunized animals (53, 54).

In vivo HC-mTetON-β-Gal-driven transgene expression in the rat brain after peripheral immunization with a first-generation recombinant adenoviral vector. Since exposure to adenovirus in infancy is common, human patients usually develop anti-adenovirus immune responses. Thus, it is crucial to determine if a preexisting immune response to adenovirus could be detrimental to the regulation of transgene expression by the TetOn switch encoded within HC-Ad vectors. The preimmunization protocol is shown in Fig. 7A. Rats were intradermally injected with saline or 1×10^9 infectious units of first-generation vector RAd-HPRT (50, 53, 54). After 3 weeks, rats received a second intradermal dose of RAd-HPRT or saline. At the same time, HC-Ad-mTetON-β-Gal was injected into the CNS and rats started receiving Dox-containing water for 7 days to allow for optimal transgene expression. Dox was then removed for 21 days to completely turn "off" transgene expression. Following this 21-day Dox depletion period, the TetOn-regulatable virus was turned back "on" by adding Dox to the drinking water for a further 7 days.

In order to confirm the success of RAd-HPRT preimmunization, we checked for the presence of neutralizing antibodies

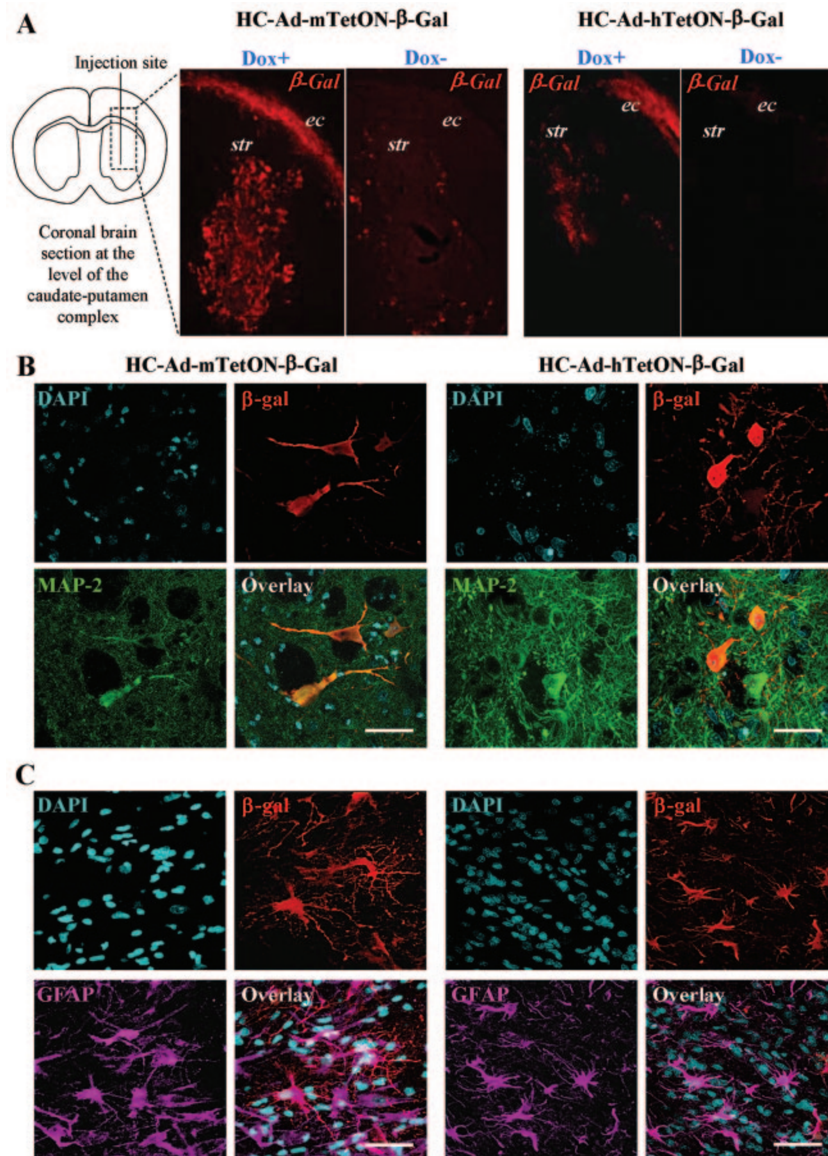

FIG. 4. In vivo transgene expression from HC-Ad-mTetON-β-Gal (left panels) and HC-Ad-hTetON-β-Gal (right panels) within the rat brain. Rats were injected with 1×10^7 blue forming units (BFU) of HC-Ad-mTetON-β-Gal (left panels) or HC-Ad-hTetON-β-Gal (right panels) and received drinking water with 1% sucrose plus 2.0 mg/ml Dox (DOX+) or drinking water with 1% sucrose alone (DOX−) for 6 days. Transgene expression was determined by β-galactosidase immunocytochemistry. (A) Images show β-gal immunopositive cells in striatal sections. (B) Confocal images of striatal sections showing cells positive for β-gal and/or microtubule-associated protein-2 (MAP-2), specific for neurons, and an overlay of the two together. (C) Confocal images of striatal sections showing cells positive for β-gal and/or glial fibrillary acidic protein (GFAP), specific for astrocytes, and an overlay of the two together. Note the colocalization of β-gal and MAP-2 (neurons) and β-gal and GFAP (astroglia). Scale bar, 50 mm. DAPI, 4′,6′-diamidino-2-phenylindole; str, striatum; ec, external cortex.

and antibodies against adenoviral proteins in the sera of animals. Blood samples were collected by retro-orbital bleeding 10 days after intradermal injection of saline or RAd-HPRT, and the presence of anti-adenoviral antibodies was determined by Western blot and the neutralizing antibody assay as described in Materials and Methods. Figure 7B shows the presence of neutralizing anti-adenoviral antibodies in the sera of adenovirus-immunized animals but not in the sera of saline-injected animals. Western blots show that the adenovirus-immunized animals contained antibodies to a variety of adenoviral proteins, in particular the penton protein (Fig. 7C), while no antibodies were found in the sera of animals injected with saline. As expected, the positive control antibody (anti-Ad5 antibody) used detected penton and fiber within the RAd lysate, confirming the specificity of the serum antibodies. These data show that the preimmunization protocol was successful in initiating an effective anti-adenovirus immune response.

Regardless of preimmunization, the TetOn-regulatable HC-Ad was able to regulate β-galactosidase expression over time, even though the adenovirus-immunized animals had a slightly lower enzyme level compared to that of the saline-treated animals. The initial Dox administration for 7 days was successful at

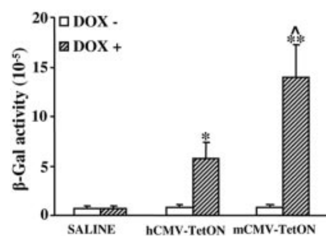

FIG. 5. In vivo doxycycline-dependent transgene expression from HC-Ad-mTetON-β-Gal and HC-Ad-hTetON-β-Gal. Rats were injected with 1×10^7 blue forming units (BFU) of HC-Ad-mTetON-β-Gal (left panels) or HC-Ad-hTetON-β-Gal (right panels) and received drinking water with 1% sucrose plus 2.0 mg/ml Dox (DOX+) or drinking water with 1% sucrose alone (DOX−) for 6 days. Transgene expression was determined by β-galactosidase activity assay. Columns represent the means ± SEM of β-gal activity, calculated as *o*-nitrophenol produced (mg/ml)/sample protein content (mg/ml)/incubation time (min). $n = 4$ animals/group. *, $P < 0.05$; **, $P < 0.01$ versus a control group (Dox−); ^, $P < 0.05$ versus HC-Ad-hTetON-β-Gal. Statistical analysis was performed using two-way ANOVA followed by Tukey's test.

turning β-galactosidase enzyme activity "on" in saline-treated animals, as expected, and also in preimmunized animals (Fig. 7D). Dox depletion for 21 days gave a marked decrease in enzyme activity levels in the brain of both saline-treated and preimmunized rats. The next 7 days of dosing with Dox allowed for a significant increase in enzyme activity, although it was not as high as the initial activity in both groups. Though transgene expression reinduction was weaker in the brain of preimmunized than in saline-treated animals, HC-Ad-mTetON-β-Gal could be rapidly turned on after 3 weeks of transgene inhibition in the presence of an immune response against Ads.

DISCUSSION

Some diseases amenable to treatment with gene therapy approaches will require constant therapeutic gene expression, while others may have periods of remission and exacerbation and therefore will only require treatment during "active" disease progression. A regulatable system that can turn "on" and "off" therapeutic gene expression would prove very beneficial. This will help to avoid unnecessary transgene expression, which could result in adverse immune reactions, and will also allow the turning "off" of therapeutic gene expression if harmful side effects develop. In the present work we have shown that novel regulatable vectors (HC-Ad-mTetON-β-Gal and HC-Ad-hTetON-β-Gal) can be successfully switched "on" and turned "off" both in vitro and in vivo even in the presence of a systemic immune response against the vector, as would be encountered in human patients undergoing clinical trails.

HC-Ad encoding β-galactosidase under the control of the TetOn system achieved higher transgene expression than first-generation adenoviral vectors in CNS-1 and COS-7 cells. Although expression of transgenes from both tetracycline-regulated HC-Ad vectors were shown to be tightly regulatable, HC-Ad-mTetON-β-Gal yielded higher transgene expression levels than HC-Ad-hTetON-β-Gal. We cannot conclusively state that the human CMV is stronger than the murine CMV in the context of HC-Ad-hTetON-β-Gal and HC-Ad-mTetON-β-Gal, respectively, because the orientation of the promoter constructs is

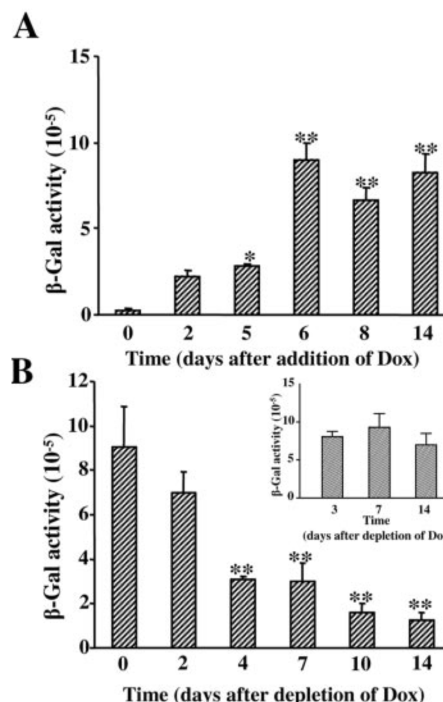

FIG. 6. In vivo induction and deinduction kinetics of HC-Ad-mTetON-β-Gal in the brain. (A) The induction time course for turning on transgene expression was done in the presence of drinking water containing 2.0 mg/ml Dox with 1% sucrose daily. Transgene expression was performed by measuring β-galactosidase enzymatic activity at 0, 2, 5, 6, 8, and 14 days postintracranial administration of 1×10^7 blue forming units (BFU) of HC-Ad-mTetON-β-Gal. Columns represent the means ± SEM of β-gal activity, calculated as *o*-nitrophenol produced (mg/ml)/sample protein content (mg/ml)/incubation time (min). $n = 2$ to 3 animals/group. *, $P < 0.05$; **, $P < 0.01$ versus control (day 0) with one-way ANOVA followed by Dunnet's test. (B) Deinduction time course for turning off transgene expression was done after 6 days of administration of Dox-containing water to HC-Ad-mTetON-β-Gal intracranially injected rats by removing the doxycycline-containing drinking water. After 0, 2, 4, 7, 10, and 14 days, transgene expression in the brain was evaluated by measuring β-galactosidase enzymatic activity. Columns represent the means ± SEM of β-gal activity, calculated as *o*-nitrophenol produced (mg/ml)/sample protein content (mg/ml)/incubation time (min). $n = 3$ to 5 animals/group. **, $P < 0.01$ versus a control group (day 0 after Dox depletion). One-way ANOVA followed by Dunnet's test was used. (Inset) As a control, 1×10^7 blue forming units (BFU) of a nonregulatable HC-Ad encoding β-galactosidase under the control of the mCMV promoter were injected into the striatum, and rats received Dox-containing water for 6 days followed by Dox depletion. Transgene expression was evaluated by β-galactosidase activity at 3, 7, and 14 days after Dox was depleted from the drinking water. Columns represent the means ± SEM of β-gal activity, calculated as *o*-nitrophenol produced (mg/ml)/sample protein content (mg/ml)/incubation time (min). $n = 3$ to 4 animals/group. Statistical analysis was performed using one-way ANOVA.

different within each gutless vector genome. However, previous publications from our group and others have shown that the murine CMV promoter is stronger than the human promoter in first-generation adenoviruses (1, 4, 14, 50). In vivo studies within the CNS showed that although both vectors exerted strong and regulatable transgene expression in the striatum, transgene expression levels from the HC-mTetON-β-Gal vector were 2.5 times higher than those from HC-hTetON-β-Gal. Our results suggest the HC-Ad-mTetON-β-

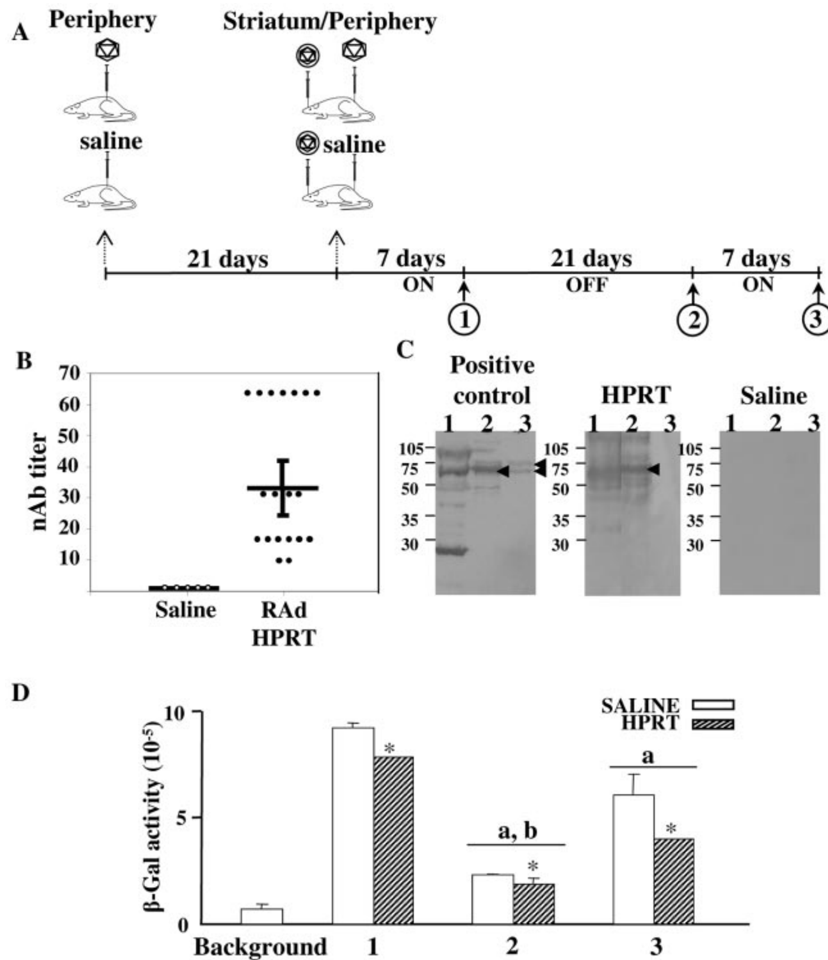

FIG. 7. HC-Ad transgene expression in the presence of a peripheral immune response to adenovirus. Three weeks before intrastriatal injection of 1×10^7 BFU of the HC-Ad-mTetON-β-Gal, rats were injected intradermally with saline or 1×10^9 infectious units of first-generation vector RAd-HPRT in a volume of 100 μl to generate an immune response to the recombinant adenoviral vector. After 10 days, retro-orbital blood was collected and processed for serum extraction to measure serum anti-adenoviral antibodies. The animals received a second immunization with either RAd-HPRT or saline in a volume of 100 μl to boost the immune response immediately prior to stereotactic surgery. Each group was given either Dox (ON) or regular drinking water (OFF) supplemented with 1% sucrose after intrastriatal injection as indicated. (A) schematic representation of the experimental protocol. Numbers represent the end of each on-off period. (B) Serum neutralizing antibody (nAb) titer determination was performed as described in Materials and Methods. Dots represent the titer of individual rats in each group. (C) Western blot analysis of anti-adenoviral antibodies in nonimmunized (saline) and immunized (HPRT) rat sera. Molecular weight markers are shown on the left side of each blot. The arrowhead indicates the protein of interest in the appropriate lanes. Lane 1, RAd lysate. Lane 2, purified penton protein (~68 kDa). Lane 3, purified fiber protein (doublet of ~68 to 70 kDa). The positive control was probed with anti-adenovirus antibody. (D) Transgene expression was determined after each on-off period (indicated by the same numbers as in panel A) by β-galactosidase activity assay. Columns represent the means ± SEM of β-gal activity, calculated as *o*-nitrophenol produced (mg/ml)/sample protein content (mg/ml)/incubation time (min). Background β-gal activity measured in the brain of animals intracranially injected with saline is also shown. $n = 3$ to 4 animals/group. *, $P < 0.05$ versus saline-injected animals; a, $P < 0.001$ versus 1; b, $P < 0.001$ versus 3. Two-way ANOVA followed by polynomial contrasts was used.

Gal vector is more suitable to achieve inducible therapeutic transgene expression due to the higher potency of the mCMV promoter; this would enable us to decrease the overall dose of HC-Ad vector dose used to achieve a desirable therapeutic effect without compromising the safety of the vector or its immune/inflammatory adverse side effects (14, 55).

For therapeutic applications involving the use of regulatable gene expression systems, it is essential to determine how long after the addition of the inducer it will take to achieve maximum levels of gene expression. Likewise, it is crucial to determine how long it will take after the withdrawal of the inducer for gene expression to return to background levels. We elucidated the induction and inhibition kinetics displayed by the HC-Ad-mTetON-β-Gal-driven transgene expression within the CNS. It took 6 days to fully turn "on" transgene expression after the addition of the inducer, Dox, and 10 days to lose transgene expression after Dox withdrawal. This decline was not due to nonspecific loss of transgene expression over time, since expression of transgenes encoded within high-capacity gutless adenovirus vectors is very stable within the brain (53, 54) and also the periphery (7, 8, 30, 34). The leakiness of a regulatable system strongly depends on the half-life of the transgene expressed by the vector. Systemic administration of regulatable vectors indicated that those having short half-life

transgenes, such as alpha interferon (5), showed faster inhibition kinetics than those having transgenes with slower metabolism, i.e., secreted alkaline phosphatase (45). Also, the leakiness of inducible systems relies on the effectiveness of the transactivator, which is constitutively expressed but only activates the minimal TRE promoter element in the presence of the inducer. In our gutless vector constructs, the binding of the transactivator to the TRE promoter element is strongly dependent on the presence of the inducer, doxycycline, since transgene expression levels are negligible in its absence.

Preexisting immunity against gene therapy vectors, as could be present in human patients undergoing gene therapy trials, can have damaging effects on the persistence of therapeutic transgene expression encoded by the vectors and can also elicit very severe systemic adverse immune side effects (30, 39, 54). Thus, although HC-Ad lacks all antigenic adenoviral proteins (29, 54), it is still essential to determine if preexisting immunity could be detrimental to the regulation of transgene expression using the TetOn switch within HC-Ad. Our data determined that preexisting adenoviral immunity will not hinder the ability of a TetOn-regulated HC-Ad to switch transgene expression "on" and "off," and therefore these vectors show promise for long-term regulation of therapeutic gene expression for clinical gene therapy applications. Although there was a slight decrease in transgene expression in the brain of preimmunized rats, we were able to switch "on" the transgene expression after 21 days of being in the "off" state, even in the presence of an anti-adenoviral immune response. In summary, our results indicate that regulated HC-Ad vectors are capable of driving transgene expression from the TetOn-regulatable switch driven by the strong mCMV promoter and efficiently regulate transgene expression even in the presence of a systemic immune response against adenoviruses, as could be encountered in human patients. Our data further support the use of these vectors for gene therapy applications that require turning transgene expression "on" or "off" for either therapeutic or safety reasons. Our data demonstrate that inducible HC-Ads will be useful tools to deliver therapeutic transgenes for the treatment of neurological diseases even in the absence of knowledge regarding the immune status of patients.

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