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Regulated Expression of Adenoviral Vectors-Based Gene Therapies:

Therapeutic Expression of Toxins and Immune-Modulators

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Summary

Regulatable promoter systems allow gene expression to be tightly controlled in vivo. This is highly desirable for the development of safe, efficacious adenoviral vectors that can be used to treat human diseases in the clinic. Ideally, regulatable cassettes should have minimal gene expression in the "OFF" state, and expression should quickly reach therapeutic levels in the "ON" state. In addition, the components of regulatable cassettes should be non-toxic at physiological concentrations and should not be immunogenic, especially when treating chronic illness that requires long-lasting gene expression. In this chapter, we will describe in detail protocols to develop and validate first generation (Ad) and high-capacity adenoviral (HC-Ad) vectors that express therapeutic genes under the control of the TetON regulatable system. Our laboratory has successfully used these protocols to regulate the expression of marker genes, immune stimulatory genes, and toxins for cancer gene therapeutics, i.e., glioma that is a deadly form of brain cancer. We have shown that this third generation TetON regulatable system, incorporating a doxycycline (DOX)-sensitive rtTA2S-M2 inducer and tTSKid silencer, is non-toxic, relatively non-immunogenic, and can tightly regulate reporter transgene expression downstream of a TRE promoter from adenoviral vectors in vitro and also in vivo.

Keywords

Adenoviral vectors; tetracycline; TetON; inducible; regulatable; gene therapy; doxycycline; glioma; Pseudomonas exotoxin A (PE)

1. Introduction

Gene expression can be regulated using a promoter that is sensitive to environmental (e.g., heat), physiological (e.g., steroids), or chemical (e.g., tetracycline) changes in the body (1–3). In general, chemical switches are preferable to either physiological or environmental switches because pharmacokinetic/pharmacodynamic properties can be determined, reducing the fluctuations that may otherwise interfere with environmental or physiological regulators. An ideal regulatable system for gene therapy requires that the following conditions are met: (1) regulation of gene expression in vivo should be achievable using a compound that is nontoxic; (2) the compound must be able to penetrate into the desired target tissue or organ at effective concentrations; (3) it should have a half-life of a few hours (as opposed to minutes or days), so that when withdrawn or added (depending on the regulatable system used), gene expression can be turned on or off quickly and effectively; (4) the genetic switches employed should ideally be non-immunogenic in the host; (5) expression in the "OFF" state should be minimal, and expression in the "ON" stale should be sufficiently high for therapeutic efficacy of the transgene (4). We have developed a regulatable cassette based on the TetON system (see Note 1). In this cassette, therapeutic or reporter transgenes are under the control of the TRE promoter (5). The

Tet-sensitive transactivator, rtTA2S-M2 (see Note 2) (6,7), and the repressor element tTSkid (8) are both constitutively expressed by a murine CMV promoter. In the “OFF” state, tTSkid suppresses unwanted activation of TRE and lowers basal therapeutic gene expression (9). In the “ON” state doxycycline (DOX) (a water soluble Tet analog that can be administered in food and water) binds with the rtTA2S-M2 transactivator, inducing a conformational change in rtTA2S-M2 that confers binding affinity for TetO repeat elements present in the TRE promoter, thus inducing expression of genes downstream from the TRE promoter (see Fig. 1). Tet regulatable vectors have been used in our laboratory for inducible expression of therapeutic transgenes in vitro (10) and in vivo (11). These Tet-dependent regulatable expression systems constitute an ideal platform to develop vectors that will allow regulated transgene expression for both basic and translational research applications and also to generate vectors expressing genes that could be toxic to the producer cell line, i.e., FasL, TNFα, Pseudomona exotoxin A, Diphteria toxin. In this chapter, we will describe in detail the protocols our laboratory has successfully used for (1) cloning plasmids containing transgenes under the control of the TetON regulatable cassette; (2) cloning mammalian expression plasmids where transgene expression is tightly regulated by the TetON promoter; (3) validation of cloning by Southern blot; (4) scale-up of HC-Ad vectors; (5) confirming DOX-inducible gene expression in vitro; and (6) in vivo.

2. Materials

2.1. Cloning of Tet-ON Regulated Plasmids

1. Plasmids (see Table 1 for description and sources).
    a. pΔE1sp1A.
    b. pAL120.
    c. pIRES-tTSkid-pA.
    d. pUHS 6-I.
    e. pIRES.
    f. pUHrT 62-I.
    g. prtTA2S M2-IRES-tTSkid -pA.
    h. pSP72Bgl2.
    i. pSP72-mCMV.
    j. pSP72[mCMV-rtTA2S M2-IRES-tTSkid -pA].
    k. pBS2 SK(+) [Tre-MCS-pA]-Kana.
2. Restriction endonucleases (REs) and REs buffer (NEB, Ipswich, MA, USA).
3. 1% Agarose gel: 1% w/v agarose in 0.5× TBE buffer, 300 ng/ml ethidium bromide.
4. Gel Purification Kit (available from Qiagen, Valencia, CA, USA).
5. T4 DNA ligase and ligase buffer.
6. Maxi Prep kit (Qiagen).

2.2. Generation of pmCMV-TetON Plasmid Driving Inducible Expression of Gene of Interest

1. Plasmids (see Table 1 for description and sources).
    a. pmCMV-TetON.

b. pSP72Bgl2-MCS.

2. REs and buffer; Swa1, Bcl1, Afl2 or Age1.
3. Calf intestinal phosphatase (CIP) (NEB)
4. 1% Agarose gel: 1% w/v agarose in 0.5× TBE buffer, 300 ng/ml ethidium bromide.
5. Gel documentation system.
6. UV light box.
7. Gel Purification Kit (available from Qiagen).
8. Quick Ligase and Ligase buffer (NEB).
9. Maxi Prep kit (Qiagen).
10. Luria–Bertani media (LB): LB + 50 μg/ml Ampicillin (LB-AMP), LB-AMP agar plates (12).

2.3. Confirming Insertion of Transgene by Southern Blot

1. 0.25 M HCl (500 ml).
2. 0.4 M NaOH(1 l).
3. Tris–HCl pH 9.00 (500 ml).
4. Tris–HCl pH 8.00 (500 ml).
5. 20× SSC: 3 M NaCl, 0.3 M Na-Citrate, pH 7.0.
6. Pre-hybridization solution (5× SSC): 1% blocking reagent, 0.1% N-lauroylsarcosine, 0.02% sodium dodecyl sulfate (SDS), heat to 60°C for 30 min or until fully dissolved. Store at −20°C.
7. Buffer 1: 100 mM Tris–HCl, 150 mM NaCl, pH 7.5.
8. Buffer 2: 100 mM Tris–HCl, 150 mM NaCl, 1% Blocking reagent (Roche, Indianapolis, IN, USA), pH 7.5.
9. Buffer 3: 100 mM Tris–HCl, 150 mM NaCl, 50 mM $MgCl_2$, pH 9.5.
10. Buffer 4: 10 mM Tris–HCl, 1 mM EDTA, pH 8.0.
11. Color substrate: 200 μl NBT/BCIP in 10 ml Buffer 3 (see step 9).

2.4. Scale-up of HC-Ad Vector Expressing Transgene Regulated by Tet-ON

1. 293 FLPe cells (293 cells stably transfected with Flippase expressing (FLPe) recombinase were generated by our laboratory (13)).
2. 293 FLPe medium: 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA), 1% non-essential amino acids (Gibco), 1% L-Gln (Gibco), 10 IU/ml Penicillin–Streptomycin (Gibco), 1.5 μg/ml Puromycin (Sigma, St. Louis, MO, USA).
3. First-generation FLPe-sensitive helper adenovirus (FL helper) (generated by our laboratory (13)).
4. pSTK120 plasmid with TetON regulatable cassette driving transgene expression (see Table 1 for description and sources).
5. Ultracentrifuge (Beckman Coulter, Fullerton, CA, USA).
6. 5% sodium deoxycholate in H_2O.

7. 2 M $MgCl_2$.
8. 3-ml sterile syringe (Becton Dickenson, Franklin lakes, NJ, USA).
9. 5-ml sterile syringe (Becton Dickenson).
10. 18-G, 3.5-inch spinal needle (Becton Dickenson).
11. 21-G, 1′-long needle (Becton Dickenson).
12. 1.33 g/ml = 8.349 g CsCl dissolved in 16 ml CsCl buffer.
13. 1.45 g/ml = 8.349 g CsCl dissolved in 11.4 ml CsCl buffer.
14. CsCl buffer: 5 mM Tris–HCl, pH 7.5, 1 mM EDTA, pH to 7.5 with 1 M sodium hydroxide, and then filter sterilize using a 0.45-μm filter.
15. TE buffer: 10 mM Tris–HCl, 1 mM EDTA, pH to 7.8 with 1 M sodium hydroxide, and then sterilize by autoclaving.
16. Mineral oil.
17. Buffer A: 7.092 g Tris–HCl, 0.4284 g $MgCl_2$, 35.5 g NaCl, in 4.5 l total volume with H_2O and autoclaved.
18. Buffer B: Buffer A with 10% glycerol and sterilized by autoclaving.

2.5. Confirming Inducible Gene Expression in Vitro

1. Cell lines.
    a. Cos7 (ATCC) African Green Monkey SV40-transfected kidney fibroblast cell line.
    b. GL26 (ATCC) C57BL/6 mouse glioma cell line.
    c. CNS1 (ATCC) Lewis rat glioma cell line.
    d. U87 MG (ATCC) human, caucasian female 44 years old; glioblastoma-astrocytoma.
    e. U251 (ATCC) human glioma cell line.
    f. IN859 (human biopsy primary culture obtained by our laboratory (14)).
    g. IN2045 (human biopsy primary culture obtained by our laboratory (14)).
2. DOX (Sigma).
3. Culture media; MEM media (Gibco) containing 10% FBS (Gibco), 1 mM L-Gln (Gibco), 1mM non essential amino acids (Gibco), 10 IU/ml Penicillin/Streptomycin (Gibco).
4. ELISA specific for transgene (Flt3L ELISA from R&D Systems, Minneapolis, MN, USA).
5. Immunoglobulins specific for Flt3L (generated in our laboratory (15)), PE toxin (generated in our laboratory), IL4 (R&D). IL13 (R&D), and IL13-alphaR2 (R&D).
6. Immunoglobulins specific for β-galactosidase (generated in our laboratory (15)).
7. Secondary anti-Ig-specific immunoglobulins-HRP conjugated (Dako Cytomation, Glostrup, Denmark).
8. SDS–PAGE equipment (BioRAD).

9. RIPA Lysis buffer: 50 mM Tris–HCl, pH 7.4 (50 μl of 1 M Stock), 150 mM NaCl (30 μl of 5 M Stock), 1 mM NaF (20 μl of 50 mM Stock), 1 mM $NaVO_4$ (8 μl of 125 mM Stock), 1 mM EGTA, pH 8.0 (20 μl of 50 mM Stock), 1% NP40 (10 μl), 0.25% sodium deoxycholate (25 μl), 1× PI (1 μl of 1000× Stock), 1× PMSF (1 μl of 1000× Stock), made up to 1 ml with 831 μl dH_2O.

10. Mini Trans-blot cell for Western Blotting (BioRad, Hercules, CA, USA).

11. PowerPac 300 Power supply (BioRad).

12. Film and automated film developing machine (Kodak, Rochester, NY, USA).

2.6. Confirming Inducible Gene Expression in Vivo

1. Male Fisher rats, 200–250 g (Harlan Sprague Dawley).

2. DOX (Sigma).

3. Stereotactic apparatus (Stoelting).

4. Anesthetics and analgesics.

    a. 100 mg/ml Ketamine HCl (Phoenix Pharmaceuticals Inc., St. Joseph. MO, USA).

    b. 1 mg/ml Medatomidine HCl (Pfizer, Exton, PA, USA).

    c. 0.3 mg/ml Buprinorphine (Reckitt Benckiser, Richmond, VA, USA).

    d. 5 mg/ml Atipamazole HCl (Pfizer).

5. First generation Ad and HC-Ad vectors.

6. Surgical equipment.

    a. Scalpel and sterile blades.

    b. Surgical Scissors, 14-cm long, straight, Sharp (World Precision Instruments, Sarasota, FL, USA).

    c. Iris Forceps, 10-cm long, serrated straight 0.8-mm tips (World Precision Instruments).

    d. Wire Retractors, 5-cm long, 15-mm wire blades, maximum spread 25 mm (World Precision Instruments).

    e. Nylon Ethicon Sutures 3-0 (Cardinal Health, Dublin, OH, USA).

    f. Dremel Stylus cordless drill and drill bits (Dremel, Racine, WI, USA).

7. 26-G, 10-μl Hamilton needle and syringe.

8. Tyrode's 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 $NaHCO_3$.

9. 4% Paraformaldehyde (w/v) in PBS.

10. PBS + 0.1% sodium azide.

11. Electronic VT1000S vibrating blade vibratome (Leica, Wetzler, Germany).

12. Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA, USA).

3. Methods

3.1. Cloning of Tet-ON Regulated Plasmids

1. We have generated both Ad (TetOFF or TetON) and HC-Ad (TetON) vectors expressing inducible transgenes using Tet-regulatable system (5,16,17). Because of genomic size constraints (up to 7 kb can be inserted into first generation, E1a/E4 deleted viruses), first-generation Ad vectors require the Tet-regulatable cassette to be expressed by one adenoviral vector, and the transgenes under the control of the TRE promoter to be expressed by another vector. Thus, inducible gene expression in cells requires co-infection with Ad vectors expressing Tet-regulatable elements and Ad vectors expressing TRE-driven transgene elements. High multiplicity of infection (MOI) can be used in vitro, and first-generation Ad vectors are relatively easy to scale up, thus Ad vectors are useful for in vitro or in vivo studies of transgene biological activity or preclinical efficacy. The latest generation, high capacity, gutless Ad vectors (HC-Ad), with a theoretical capacity of ~35 kb, can encode for Large transcriptional units, and transgene expression form of these vectors is stable even in the presence of a peripheral anti-Ad immune response as could be encountered in patients undergoing clinical trials. They are therefore the preferred choice for pre-clinical in vivo studies (13). Here we will describe in detail the generation of HC-Ad vectors; first-generation Ad vectors can also be generated by excising either TetON or Tre-transgene-pA elements and inserting them into first-generation Ad shuttle plasmids (e.g., pΔE1sp1A or pAL120).

2. To generate the TetON cassette, the transcriptional silencer carrying plasmid p[IRES-tTSKid-pA] was generated by digesting pUHS 6-1 with Xba1 and Hind3 to liberate tTS$_{kid}$, ligating tTS$_{kid}$ into the shuttle plasmid pΔE1sp1A and digesting with Xba1 and Sal1 before ligation into pIRES digested with Xba1 and Sal1. This generated a plasmid containing IRES followed by tTSkid and a polyAdenylation (pA) signal.

3. The rtTA$_{2S}$M2 transactivator was excised using EcoRI and BamHI from plasmid pUHrT 62-1; the BamHI site was adapted using short oligonucleotide dimers to add an Mlu1 restriction site. The adapted rtTA$_{2S}$M2 insert was directionally cloned into a previously generated plasmid p[IRES-tTSKid-pA], digested with EcoR1 and Mlu1, resulting in intermediate plasmid, p[rtTA2SM2-pIRES-tTSKid-pA]. This generated a plasmid where the rtTA$_{2S}$M2 and tTS$_{kid}$ coding sequences were separated by an IRES (5).

4. Next, we placed a constitutive mCMV promoter upstream or rtTA$_{2S}$M2 to drive expression of rtTA$_{2S}$M2 and tTS$_{kid}$. We excised the mCMV promoter from pAL120 with EcoRI and HindIII and ligated into a pSP72-Bgl2 shuttle vector (Clontech, Mountain View, CA, USA), producing intermediary plasmid pSP72[mCMV]. Plasmid p[rtTA2SM2-pIREStTSKid-pA] was excised with XhoI and SalI to release cassette [rtTA2SM2-pIRES-tTSKid-pA] and subsequently cloned into its corresponding sites into plasmid pSP72[mCMV], generating the intermediate plasmid pSP72[mCMV-rtTA2SM2-pIRES-tTSKid-PA].

5. The [mCMV-rtTA2SM2-pIRES-tTSKid-pA] regulatable Tet-ON cassette was then excised with BgIII and cloned into the BgIII site of intermediate plasmid pBlueScript II SK(+)[TRE-MCS-polyA]-Kanamycin, thus generating the final intermediate plasmid, pBlueScript II SK(+)[TRE-MCS-polyA]-[mCMV-rtTA2SM2-IRES-tTSKid-pA]-Kanamycin.

6. The MCS contains REsites for Bcl1, Afl2, Age1, and Swa1 and can be used to directly insert transgenes downstream of TRE. A shuttle vector, pSP72-Bgl2-MCS, also exists that facilitates the cloning of transgenes into pmCMV-TetON.

3.2. Generation of pmCMV-TetON Plasmid Driving Inducible Expression of the Gene of Interest

1. Digest 1 μg pmCMV-TetON (see Note 3) and 1 μg pSP72-Bgl2-MCS containing the transgene with compatible restriction enzyme (Swa1, Bcl1, Afl2, or Age1). Thy reaction mixture should contain 10 μl 10× RE buffer (see Note 4), 1 μg DNA, 5 μl RE, and H_2O bringing entire volume to 100 μl and needs to be digested at 37°C (55° C for Bcl1) for at least 2 h. This will linearize the pmCMV-TetON plasmid and drop out the transgene with compatible ends, allowing the ligation of the therapeutic gene downstream of the TRE promoter and upstream of pA sequence.

2. Digestion must be to completion, confirm that no undigested DNA remains by running a 1% agarose gel with 5 μl reaction mixture (and undigested DNA as a control in another gel lane) before continuing.

3. Run the entire sample on a 1% agarose gel and use a new razor blade to cut out the band of interest from the gel, visualizing the DNA using a UV illumination source (see Note 5).

4. Purify the DNA from Agarose gel using DNA purification columns from Qiagen.

5. Elute the DNA in 30 μl TE buffer, and run 2 μl on an agarose gel to quantify DNA concentration.

6. Dephosphorylate the ends of the vector using CIP. This prevents religation of vector.

7. Set up the ligation reaction with (10 min, room temperature) Quick Ligase (NEB). We generally ligate 200 ng vector with a threefold molar excess of insert.

8. Transform competent DH5α cells with ligated product (use 5 μl ligation reaction) and culture overnight at 37°C or Agar plates with LB + Ampicillin (50 μg/ml).

9. Pick colonies and grow for 8 h at 37°C in 2 ml LB + Ampicillin (50 μg/ml).

10. Transfer 1–200 ml LB + Ampicillin (50 μg/ml) and grow overnight at 37°C.

11. MAXI prep DNA from bacteria and use REs to confirm that the transgene has been inserted in the correct orientation. Expression of the transgene can be verified here by transient transfection before proceeding to insert the cassette into pSTK120 vector.

3.3. Confirming Insertion of Transgene by Southern Blot

1. Probe preparation: Digest 5–10 μg of plasmid DNA containing the sequence of preference (e.g., IL13) to liberate the probe. (10 μl DNA, 10 μl Buffer, 5 μl RE, 75 μl H_2O). Digest for 2 h at 37°C and run 1 μl to check completion of the digestion, together with undigested DNA and DNA ladder (Bioline Hyperladder I). Gel extract the desired fragment using Qiagen Gel Extraction kit in 30 μl total of milliQ water.

2. Sample preparation: Nucleic acids absorb strongly at 260 nm. The optical density at 260 nm (OD_{260}) of DNA samples (RNA free) can be measured using spectrophotometry to determine the concentration of DNA in a sample. Other major components of cells, i.e., proteins, lipids, etc., do not absorb strongly at 260 nm. Dilute 1 μl plasmid DNA with 100 μl H_2O, transfer to a quartz cuvette, and determine the OD_{260} after first blanking the machine with H_2O only. The concentration of DNA can be calculated by multiplying the OD_{260} of each sample by 5000 (Concentration will be μg/ml DNA). Digest 1 μg plasmid DNA with a restriction enzyme that drops out a band containing the DNA sequence (1 μg plasmid DNA; 3 μl HindIII; 2 μl Buffer 2; H_2O up to 20 μl). Incubate at 37°C for 2 h and run 1 μl to check that the digestion has proceeded to completion.

3. Gel Electrophoresis: Run DNA ladder, and Plasmid DNA in a 0.8% agarose gel at 95 V (Biorad, Minisub-cell GT; Biorad, PowerPac 300), for 1 h. Take a photograph of the gel using a gel documentation system (Alpha Innotech Corporation).

4. Transfer: Use a positively charged membrane to bind negatively charged DNA (Roche). Rinse the membrane with MilliQ water and soak in 0.25 M HCl, 15–39 min. Rinse the membrane twice with MilliQ water then soak in 0.4 M NaOH. Rinse with MilliQ water and set up the transfer apparatus. Trim off the edges of the gel that do not contain DNA, including the wells. Cut the top right corner of the gel with a blade (this will allow the orientation of the gel to be determined later). Cut thick Whatman 3 M filter paper with the exact measures of the gel. Cut the membrane to the same size as the gel and filter paper. Soak filters and membrane in 0.4 M NaOH and cut a piece of filter paper (Whatman, 3 M) long enough to form the “bridge” over the tray (Life Technologies, Blot transfer system 20–25). Place the Whatman filter paper bridge on the Blot transfer system with the ends immersed in 0.4 M NaOH. Place the gel on this bridge and place the membrane above it taking care not to leave any bubbles between the gel and the membrane (see Note 6). Put the thick filter paper and paper towels on top of the membrane and place the weight on top of the paper towels. Transfer overnight.

5. Labeling the probe: [Note; 10 ng to 3 μg of DNA can be labeled (Roche, DNA labeling and detection kit).] Add 1 μg the DNA and MilliQ water to a final volume of 16 μl. Take OD260 of the purified probe and calculate the volume needed for 1 μg of DNA. Denature the diluted probe DNA in a boiling water bath for 10 min and quickly transfer to ice. Add the rest of the reagent needed For the labeling reaction (2 μl Hexanucleotide Mix 10×; 2 μl dNTPs labeling mix; 1 μl Klenow enzyme). Vortex, spin down and incubate overnight at 37°C a longer incubation will increase the yield of the labeled DNA. Stop the reaction by adding 2 μl of 0.2 M EDTA; pH 8.00 and precipitate labeled DNA with 2.5 μl of 4 M LiCl and 75 μl 100%EtOH. Mix well and leave 2 h at −20°C, centrifuge 15 min 15,000 g rinse the pellet with 50 μl of 70% ice cold EtOH, dry and resuspend the labeled pellet in 50 μl of MilliQ water. Store at −20°C until use.

6. Prehybridization: Briefly soak the membrane in 5× SSC buffer and transfer the membrane to a hybridization tube (TECHNE, FHB11). Prepare the prehybridization solution (10 ml/membrane) (15 ml 5× SSC; 150 mg blocking reagent (Roche DNA labeling and detection kit); 15 mg 0.1% N-lauril sarcosine and 3 μl 10% SDS). Incubate for 30 min at 65°C, until dissolved, add 10 ml of prehybridization solution taking care of avoiding air bubbles, again with the help of a pipette and incubate for 2 h, at least, at 68°C.

7. Hybridization: Denature the probe by heating for 10 min at 95°C and transfer to ice immediately. Remove the membrane from the incubator and add the 50 μl of labeled probe to the prehybridization solution. Incubate at 68°C overnight. Remove the membrane from the cylinder and wash in 2× SSC/0.1% SDS at room temp. 15 min on a shaker. Change washing solution to 0.2× SSC/0.1% SDS, incubate at room temperature, 15 min on a shaker and wash once with 0.1× SSC/0.1% SDS at 65°C, 30 min.

8. Immunodetection: [Note: all the washes are done while shaking.] Wash the membrane in buffer 1, for 1 min and incubate the membrane for 30 min in buffer 2. Wash the membrane with buffer 1 for 5 min, then dilute 4 μl of anti-digoxigenin/Alkaline Phosphatase conjugate (vial 8, provided in the kit) in 10 ml of buffer 1 add to the membrane and incubate at room temperature, 30 min while shaking. Wash with buffer 1, for 30 min and incubate in buffer 3 for 2 min. Transfer the membrane to the color

substrate solution (10 ml buffer 3 + 200 µl NBT/BCIP). Incubate in the dark until the bands are clearly visible and then stop the reaction using buffer 4. This incubation can be prolonged until bands are evident. Archive the image using a scanner or a gel documentation system. The image can be compared with the original agarose gel to determine sizes of the DNA (see Fig. 2 as an example).

3.4. Scale up of HC-Ad Vectors Expressing Transgenes Regulated by TetON

1. The generation of first-generation Ad vectors has been described in great detail before (18). Here we will focus on the production and generation of HC-Ad vectors containing the TetON regulatable cassette.

2. The entire cassette encoding the transgene regulated by TRE promoter and the TetON cassette expressed by mCMV must be transferred into a plasmid that will allow production of adenoviral particles. We use pSTK120 to generate high-capacity Ad vectors. pSTK120 is a large plasmid that does not contain any known genes or regulatory sequences and is relatively stable; recombination events are low compared with other large plasmids. pSTK120 contains Amp resistance and also contains Ad-5 LTR sequences and the packaging sequence (Ψ), allowing production of HC-Ad vectors. pSTK120 is ~28 kDa but can be reduced in size if necessary by digestion with RE (e.g., Nhe1 and Nar1 can each remove 3 Kb from the final plasmid). Inserts can be cut with Eag1 compatible enzymes (e.g., NotI), or Nhe1 compatible enzymes (e.g., Avr2) (see Table 2 for the complete list of pSTK120 plasmids and cloning capacities).

3. The insert [TRE-hsFlt3LpolyA]-[mCMV-rtTA2SM2-pIRES-tTSKid-pA]-Kana was excised from the shuttle plasmid using NotI and cloned into the compatible EagI site of HC-Ad plasmid pSTK120.2, generating pSTK120.2-[TRE-hsFlt3L-polyA]-[mCMV-rtTA2SM2-pIRES-tTSKid-pA]-Kana (HC-Ad-mCMV-TetON-Flt3L). The fragments were ligated overnight, transformed into DH5α cells, and plated on LB plates with 25 µg/ml kanamycin (12). Plasmids were isolated from overnight cultures using miniprep and screened by HindIII digestion. Maxiprep purifications were performed on the correct clones (Qiagen). Restriction enzymes were used to confirm the integrity of the plasmids (see Note 7).

4. The major steps in HC-Ad vector production are rescue, amplification, scale up, and purification of HC-Ad vectors as previously described (19). For a summary of this protocol (see Fig. 3).

5. Rescue: Seed 2 × 10^6 293 FLPe cells (seeNotes 8 and 9) into 60-mm dish the day before transfection. (Note: 293 cells FLPe cells grow in MEM with puromycin 1.5 µg/ml). Transfect the 293 FLPE cells (80% confluent cells) with 10 µg of gutless plasmid DNA linearized with PmeI, using the calcium phosphate precipitation technique (seeNote 10). Incubate the cells at 37°C, 5% CO_2. (Include one flask transfected with pMV12, a plasmid expressing β-galactosidase as a transfection positive control, and another flask transfected with no plasmid DNA as a transfection negative control.) Sixteen hours later, wash the 293 FLPe cells first with PBS and second with 293FLPe culture media. Infect the cells with helper virus at an MOI 5 diluting the virus in 5 ml of medium. Incubate at 37°C, 5% CO_2. Harvest the cells when full CPE appears (usually 2–3 days). Tap the side of the dish to dislodge the cells (or use scraper) and lyse the cells by three freezing and thawing cycles.

6. Amplification: For each amplification passage (P1 to P6), seed 293 FLPe cells into a 60-mm dish the day before infection to obtain approximately 80% confluence of cells the next day. Cells are grown and incubated at 37°C, 5% CO_2. Infect using 10% of the volume collected from the previous passage lysate and coinfect with FL helper

vims at MOI = 1. Harvest the cells when full CPE appears. Tap the side of the dish to dislodge the cells (or use scraper) and lyse the cells by three freezing and thawing cycles.

7. Scale-up: For P7, seed a 150-mm dish with 1 × 10^7 cells and infect using 100% of the lysate from P6 and co-infect with FL helper virus at MOI = 1. Keep 10% of the lysate (usually 50–100μl), for analysis, BFU, IU, molecular titration by duplex RT–PCR (18). Harvest the cells when full CPE appears and pellet down the cells by centrifugation. 10 min at 1500 g, 4°C; and resuspend in 500 μl of PBS. Seed 30 dishes (150 mm) with 1 × 10^7 cells the day before infection. Prepare the medium by adding the viral vector suspension and the FL helper virus at MOI 1, and infect the cells. Harvest the cells when full CPE appears (2–3 days) pellet down the cells by centrifugation 10 min at 1500 g, 4°C; and resuspend in 8 ml of 100 mM Tris–HCL pH 8.0. Store at −80°C until CsCl purification is performed.

8. We have also used a similar method for rescuing and scaling up of HC-Ad vectors developed by Philip Ng and colleagues that utilizes a Cre recombinase sensitive helper adenovirus to package HC-Ad genomes in trans (19,20). We have found that this method, allows larger number of cells to be infected in the final scale-up step; thus, we routinely achieve higher titers (∼one log higher titer) of HC-Ad vectors using the Cre method. This method has been described in detail by Ng et al. (19).

9. Purification: Cesium chloride (CsCl) purification has been described before for the purification of first generation adenoviral vectors (13,19). Briefly, 4 ml of 5% sodium deoxycholate is added to the cell pellets and incubated at room temperature for 30 min to lyse the cells. Add 400 μl of 2 M $MgCl_2$ and 100 μl of DNaseI. Incubate at 37°C for 1 h to remove genomic and non-packaged Ad DNA. Centrifuge at 20,000 × g for 15 min. Pipette 2.5 ml of CsCl at a density of 1.33g/ml in a Beckman 14-ml centrifuge tube (Beckman, 331374) (to fit a SW40 swing bucket rotor). Then, using a 5-ml syringe equipped with a wide bore 18-G needle, place the needle at the bottom of the centrifuge and very slowly inject 1.5 ml of CsCl at a density of 1.45 g/ml, so the less dense layer floats on top of the denser layer. (Densities were calculated by the determination of the refractive index.) CsCl was dissolved in 5 mM Trizma HCl, 1 mM EDTA pH 7.8. Overlay 7 mL of the virus solution on the CsCl gradient. Overlay mineral oil on the viral layer until the meniscus reaches 2 mm from the top of the tube. Place the tubes in the centrifuge buckets SW40Ti (Beckman Coulter) and seal before leaving Class 2 hood. Weigh a set up balance tube to an identical weight (±0.02 g) before placing tubes in rotor and centrifuge in an ultracentrifuge for 2 h at 90,000 × g (22,500 rpm for the SW40 rotor). Remove tubes from the rotor in the Class 2 hood. Puncture side wall approximately 1 cm below the level of the band with a wide bore 21-G syringe needle. This will allow you to get a large enough angle to remove the entire viral band. The viral band is the lowest of the three bands that should be visible. The volume is NOT important at this step. Dilute the virus fraction with half a volume of TE pH 7.8. Layer the virus fraction on top of a second CsCl gradient prepared with 1 ml of 1.45 g/ml CsCl and 1.5 ml of 1.33 g/ml CsCl. Overlay mineral oil until the meniscus is 2 ml from the top of the tube and centrifuge in an ultracentrifuge for 18 h at 100,000 × g (23,800 rpm for the SW40 rotor). Recover the viral band as before (see Note 11). At this stage, the volume recovered is very important. Inject virus into a dialysis cassette and dialyze the banded virus twice against 1.5 l of Buffer A for 2 h and once against 1.5 l of Buffer B for 2 h at room temperature. Aliquot the virus and store at −70°C. Maintain sterility at all times.

10. Titration of HC-Ad vectors is usually performed by measuring the total viral particles using Optical Density (see Note 12).

11. In vitro and in vivo assessment of gene expression must be carried out before assessing the therapeutic efficacy of the vector in preclinical models (see Note 13).

3.5. Confirming Inducible Transgene Expression in Vitro

1. In vitro expression can be confirmed using ELISA, immunocytochemistry, or Western blotting (seeNote 14). It is preferable to use more than one method to confirm expression in vitro before proceeding. In this example, we tested HC-Ad-mCMV-TetON-Flt3L for transgene expression in the presence and absence of Dox by ELISA and by immunocytochemistry. We tested Ad-IL4-TRE-IL-13.PE for expression by immunofluorescence and bioactivity by the production of cytotoxic IL13.PE (see Note 15).

2. ELISA: Flt3L is a secretable protein: we measured Flt3L expression in the media of HC-Ad-infected glioma cells from mouse, rat, and human in the presence and absence of Dox. Cells were plated in 12-well plates at 50,000 cells (GL26: CNS-1; U251; U87 MG; IN859; IN2045) per well and allowed to adhere overnight. The following morning, media was replaced with 0.5 ml of media containing DOX (1 μg/ml) or without DOX and cells were then infected with HC-Ad-TetON-Flt3L (50,000 VP/ CELL). After 72 h, Flt3L (transgene) was determined in the cell culture supernatant using ELISA (R&D Systems), exactly as outlined in the manufacturer's guide (see Note 16). Inducible expression of Flt3L was detected in the media by ELISA after six glioma cell lines were infected with HC-Ad-TetON-Flt3L and incubated with or without Dox (see Fig. 4).

3. Immunocytochemistry: GL26, CNS-1, U251, U87, IN859, and IN2045 cells were seeded in 12-well dishes (50,000 cells/well), on sterile coverslips, and allowed to adhere overnight. The following day, cells were incubated with 1 μg/ml DOX or without and were infected with HC-Ad-mCMV-TetON-Flt3L (50,000 VP/Cell). After 72 h, cells were washed cells two times with PBS and fixed for 10 min in 4% PFA at room temperature. Cells were then blocked (1 h, room temperature) with 10% Horse Serum in TBS + 0.5% Triton-X100. Stain 2 h with primary antibody (against transgene), use the dilution recommended by the manufacturer. Wash the cells and stain with secondary antibody (these can be either fluorescent tagged or Biotin tagged). If using biotinylated secondary Ab, incubate for 1 h, wash with PBS three times and add avidin-HRP (1:1000 in PBS, 1 h, room temperature). Wash in PBS, wash two times in 100 mM sodium acetate, and stain with DAB. Allow precipitate to develop (usually 1–5 min), wash with sodium acetate and then with PBS + 0.1% sodium azide. Mount on slides and visualize gene expression using visible light microscopy (see Fig. 4) (see Note 17).

4. Western blotting: Lyse cells in RIPA buffer and quantify protein concentration of cell lysates. For each sample dilute 30 μg of the ceil lysate in 20 μl total volume in SDS–Tris buffer containing DTT. Boil samples for 10 min to denature proteins. Run 30 μg samples on an SDS–polyacrylamide gel (with protein standard markers) and transfer to nitrocellulose membrane (Amersham Biosciences). Block membranes and incubate with primary and secondary antibodies as recommended by the manufacturer. We use our custom Flt3L antibody at 1:1000 in blocking solution [TBS + 0.05% Tween20 (TBS-T) with 5% dry milk powder] overnight at 4°C on membranes blocked previously for l h with blocking solution. Membranes are then washed three times for 5 min each using TBS-T before addition of secondary antibody for l h at room temperature (goat anti-rabbit-HRP, 1:2000 in blocking solution, DAKO). Membranes are washed three times for 5 min each with TBS-T and developed using ECL Western Blotting Analysis kit (Amersham Biosciences) and visualize on X-OMAT LS film (Kodak).

5. Immunofluorescence (Ads or HC-Ad): Cos7 cells were seeded at 50,000 cells per well in a 12-well dish, on coverslips, and allowed to adhere overnight. Cells were infected with Ad-IL4-TRE-IL-13.PE and Ad-TetON and were incubated with or without 1 μg/ml DOX for 48 h. After fixing as described above, transgenes were detected by immunofluorescence using primary antibodies against IL-4 or IL-13 (R&D Systems) in combination with a primary antibody against PE toxin developed in our laboratory. Wash cells in TBS+0.5% TritonX100 and incubate cells for 1 h with fluorescent labeled secondary antibody, protecting from light. Wash in PBS three times. On the final wash, add DAPI to visualize nuclei, wash once more with PBS and mount on slides with anti-Fade mounting media (ProFade) for analysis using an immunofluorescent microscope. Colocalization of the transgenes was observed by Confocal microscopy (see Fig. 5). Note that transgene expression was only observed when cells were incubated in the presence of Dox.

6. Viability assay (MTS): Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) assay. Cos7, IN859, and U251 cells were seeded at 5000 cells per well in a 96-well dish and infected with Ad-IL4-TRE-IL-13.PE and Ad-TetON. Cells were incubated with or without 1 μg/ml DOX for 48 h. Twenty microliters of reaction solution containing MTS (final concentration 333 μg/ml) and an electron coupling reagent (phenazine ethosulfate, final concentration 25 μM) were added to each well containing 100 μl of culture medium. After 3 h at 37°C. the OD was read in a micropore spectrophotometer at a wavelength of 495 nm. The quantity of formazan product is directly proportional to the number of living cells in culture. Ad-IL4-TRE-IL13.PE was designed to produce PE toxin conjugated to a variant of IL-13 that binds specifically with the IL-13α2R receptor found almost exclusively on glioma cells. Cell viability was reduced 70% when the U251 and IN859 human glioma cells expressing the IL-13α2R (see Fig. 6) were incubated in the presence of Ad-IL4 TRE-IL13.PE in the “ON” state (Dox+) but remained unaffected in the “OFF” state, indicating that the expression of the chimeric toxin can be tightly regulated. Ad-IL4-TRE-IL13-PE did not affect the viability of COS-7 cells (African Green Monkey SV40-transfected kidney fibroblast cell line), which do not express the IL13alpha2 receptor (see Fig. 5). These results suggest that the cytotoxic effect of the chimeric is specific to glioma cells. The control vector Ad-IL4-TRE-IL13 did not affect the viability of human glioma cells or COS-7, neither in the presence nor in the absence of Dox.

3.6. Confirming Inducible Gene Expression in Vivo

Here we describe the detection of transgene expression in vivo using immunohistochemistry after stereotactic injection of inducible adenoviral vectors into the brain striatum. However, these methods can be applied to immunohistochemical analysis of any tissue of interest.

1. Male Fisher 344 rats of 200–250 g of body weight (Harlem Sprague Dawley Inc.) were used for in vivo HC-Ad-mediated gene delivery. Rats were given drinking water containing 2.0 mg/ml Dox (Sigma) and 1% sucrose or 1% sucrose alone 24 h before brain surgery and HC-Ad delivery.

2. On the day of surgery, the animal was anesthetized with ketamine and medatomadine, the head area was shaved, prepared with betadyne, and placed in a stereotactic frame ready for surgery. A total of 1×10^9 vp of HC-Ad-mTetON-Flt3L, 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% w/v 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 before

careful needle retraction. As a negative control, one group of rats received 3-μl saline injections.

3. After postoperative surgery, both untreated and treated animals received drinking water containing Dox and 1% sucrose, 1% sucrose alone, or water alone during the required times, and drinking water was changed daily.

4. After 6 days, animals were then sacrificed and brains were perfused with approximately 200 ml oxygenated Tyrode's solution by means of trans-cardial perfusion. Rats were next perfused with 250 ml of 4% paraformaldehyde, pH 7.4 (4% PFA), fixative, and brains were stored postfixed in 4% PFA for 3 days at 4°C followed by 3 days of washing in PBS containing 0.1% sodium azide.

5. Brains were serial sectioned using a vibratome to obtain 50-μm free-floating sections. Sections were stored in PBS containing 0.1% sodium azide at 4°C until ready for use.

6. Free-floating brain sections were washed with TBS + 0.5% Triton X-100 followed by 0.3% H_2O_2 incubation to inactivate endogenous peroxidase. Non-specific antibody sites and Fc receptors were blocked with 10% normal horse serum for 1 h.

7. Sections were incubated for 48 h at room temperature with rabbit polyclonal anti-Flt3L primary antibody (1:1000) (generated in our laboratory (15)) 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).

8. 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 (see Fig. 7).

4. Notes

1. TRE promoters can be either unidirectional or bidirectional. We have used both successfully to produce Ad vectors. Bidirectional promoters allow the simultaneous expression of two or more genes. In our experience, expression of two genes from a bidirectional promoter is more robust than expression of two genes, separated by an IRES, from a unidirectional promoter. We have also used bidirectional TRE promoter to simultaneously express four genes upon addition of DOX by using an IRES to separate two open reading frames (ORE) on either side of the bidirectional TRE promoter. However, if expression of only one transgene is required, then a unidirectional promoter is sufficient.

2. The transactivator used. rtTA2SM2, is a chimeric protein consisting of the DNA-binding domain of the Tet repressor rtTA fused with the transactivation domain of VP-16. Point mutations changing the amino acid sequence of rtTA2SM2 have improved the induction of gene expression in the presence of DOX (8).

3. We have determined that optimal transgene expression can be achieved by generating an HC-Ad genome where mCMV-TetON elements are in the opposite direction as Tre-transgene elements, and where mCMV and TRE are juxtaposed (see Fig. 4). The tTSkid ensures that basal activity of the TRE promoter remains low in the “OFF” state, whereas the close association of mCMV transcriptional enhancer elements in the mCMV promoter increases the activity of TRE promoter in the “ON” state.

4. Different RE require different buffer conditions for optimal activity. Product literature accompanying the RF will explain what conditions are required for optimal activity

for each enzyme. Usually, two different RE can be used at the same time by selecting a buffer with optimal activity for both. However, sometimes, the RE buffers are not compatible and lead to suboptimal RE enzymatic activity. In this situation, sequential digestion of DNA with the RE may be performed, as long as DNA is precipitated from the first RE buffer before starting the second digestion.

5. Wear protective eye gear (mask or goggles) when cutting DNA bands out of the agarose gel. It is also important to note that UV light can cause point mutations, for example, by oxidizing Guanine to 8-Oxyguanine that binds with Adenine, not Cytosine, resulting in a GC-AT transition. Thus, it is important to limit the time that DNA is exposed to UV light. We use a low power UV box and limit DNA exposure to the UV light to less than 2 min.

6. Bubbles between the gel and the membrane can be removed by first overlaying one layer of filter paper on the membrane. Firmly but carefully a 4-ml tube or a 25-ml pipette can be rolled over the filter paper from one edge to the opposite edge 2–3 times. When doing this, it is important to take care not to break the gel or slide the membrane off the gel.

7. Cloning of HC-Ad vectors requires the manipulation of large plasmids, and recombination is often an issue. It is essential to use a recombinase deficient Escherichia coli bacterium, we use DH5α. Furthermore, we find that culture of bacteria transformed with pSTK120-transgene plasmids is more successful when bacteria are grown at 32°C and 160 g. After cloning, plasmids should be checked rigorously using REs to confirm that recombination events have not occurred. This can identify anomalies that otherwise would not be detected until the HC-Ad vectors are produced. In addition, although it has not been specifically mentioned here, DNA sequencing to confirm that the gene is present is advisable.

8. Considering that during viral replication transgenes are expressed in vector-producing cells, production of vectors encoding toxic transgenes is difficult and requires special strategies. We have produced adenoviral vectors encoding IL-13-PE toxin in 293 cells that are resistant to the toxin. During viral replication, IL-13-PE is expressed in 293 cells and can inhibit protein synthesis by ADP-ribosylation of EF-2, leading to low vector liters. Thus, we used 293 cells stably transfected with pHED-7 (21), which encodes the gene for ADP ribosylation-resistant elongating factor-2 (EF-2) from CHO cells (22). In these cells. ADP-ribosylating toxins are incapable of EF-2 inactivation; thus, they can support the growth of adenoviruses expressing Pseudomonas or Diphtheria chimeric toxins, yielding high viral titers.

9. Another strategy to block the toxicity of these vectors during adenovirus production is to block the intracellular processing of the chimeric toxin. To inhibit protein synthesis, IL-13-PE toxin requires furin-mediated proteolytic cleavage that activates the exotoxin releasing the catalytic Domain III Thus, adenoviral vectors encoding Pseudomonas or Diphtheria chimeric toxins can be produced in wild-type 293 cells in the presence of furin inhibitor Decanoyl-RVKR-CMK (5 μM, Calbiochem).

10. Transfection of pSTK 120-transgene plasmids into 293Flpe cells can be achieved with high efficiency using the calcium phosphate precipitation method. It is necessary to make new transfection reagents and to avoid repeated freezing and then thawing of the reagents before use. Shorter limes where DNA is added to 2× HBS (e.g., 10 s) work much better than longer times (e.g., 5 min). Polypropylene tubes improve transfection efficiency in our hands compared with polystyrene tubes. We have also tested various commercially available transfection reagents, we find that TransIT (Mirus) and HyFect (Denville) achieve similar high levels of transfection and high-quality viral preparations. Although useful for other applications, we did not have any

success using either JetPEI (PolyPlus) or GeneJuice (Novagen) for transfecting HC-Ad vectors. Plasmid DNA must be of high quality to rescue HC-Ad vectors. The concentration of DNA must be at least 1 μg/ml and the OD_{260} : OD_{280} ratio must not be above 1.9.

11. Usually, only one viral band is evident during CsCl purification of HC-Ad viral vectors. This is desirable. Occasionally, we have observed a second viral band present: these preparations are usually of a lower quality when tested in vitro and in vivo.

12. Real-time qPCR can also be used to quantify the number of viral genomes present. We have found that the information obtained by qPCR is very useful in characterizing HC-Ad preparations. In particular, it allows a sensitive, quantitative measure of the presence of helper virus contamination in the sample. This protocol has already been described in detail (see Puntel et al., (23)).

13. In our experience, a good-quality HC-Ad vector preparation should meet the following in vitro criteria:

    a. There should be less than 30-fold greater viral particles (measured with OD_{260nm}) than viral genomes as determined using qPCR.

    b. There should be less than 10 times as many viral genomes (qPCR) than transgene expressing units (TEU, determined by ICC on Flow cytometry).

    c. Contaminating helper viral particles (measured with plaque forming units assay) should be $<10^5$ pfu/ml.

14. Transgene expression in the presence of DOX can be confirmed using a myriad of techniques, the best of which is completely dependent on the transgene that will be detected. We have outlined three such techniques in vitro, ELISA, ICC, and Western blotting that we most commonly use to titrate HC-Ad vectors in vitro. Several other techniques including reverse transcriptase PCR and flow cytometry may also be used and may be preferable depending on the availability of reagents to detect the transgene of interest.

15. Expression of many transgenes can be confirmed using immunoglobulins purchased from specific companies. However, it may be more desirable to “tag” the transgene (FLAG or Myc tags work well) before cloning it into pmCMV-TetON. Specific, high-affinity immunoglobulins have been developed against these tags improving the sensitivity of the assay for detecting the transgene.

16. For secretable proteins, e.g., human soluble Flt3L, much of the protein produced will be secreted. Thus, cell culture media from transfecred cells can be used to determine expression of transgene in vitro.

17. Of the techniques we described to determine whether viral preps induce transgene expression in vitro (i.e., ELISA, ICC, and Western blot), only ICC can give an accurate assessment of the total number of transgene expressing particles in a viral preparation. We have recently attempted to overcome this problem by developing a method of titrating gene expression from Ad vectors using flow cytometry. We have found that this can give a very accurate and comparable assessment of the number of transgene expressing particles in a viral prep compared with ICC.

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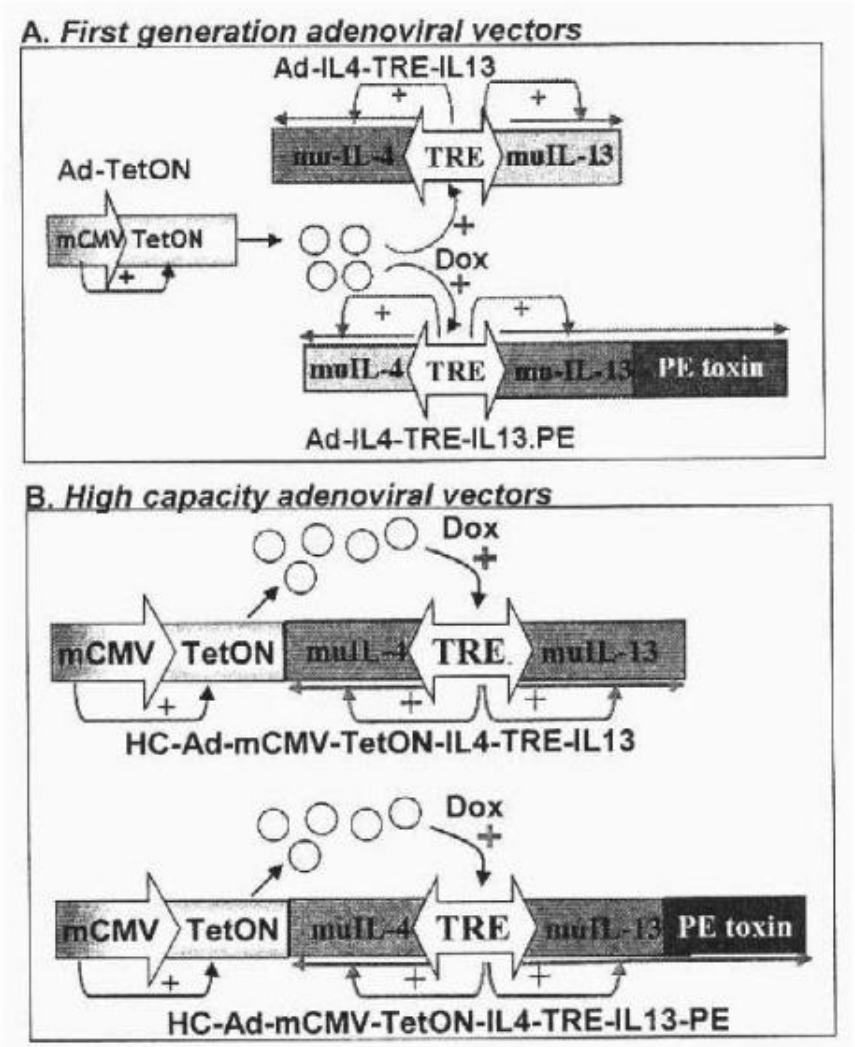

Fig. 1.

Structure of regulatable first generation (A) and high capacity (B) adenoviral vectors. These novel adenoviral vectors express mulL4 and mlL13 or the cytotoxin mulL-13-PE under the control of the bidirectional TRE promoter. The TRE promoter is activated by the transactivator (TetON) in the presence of the inducer Dox. The expression of the TetON is driven by the strong murine CMV promoter from an additional first generation adenovirus that is administered with the therapeutic virus, or is included in the high capacity adenovirus, since it allows the expression of larger transgenes.

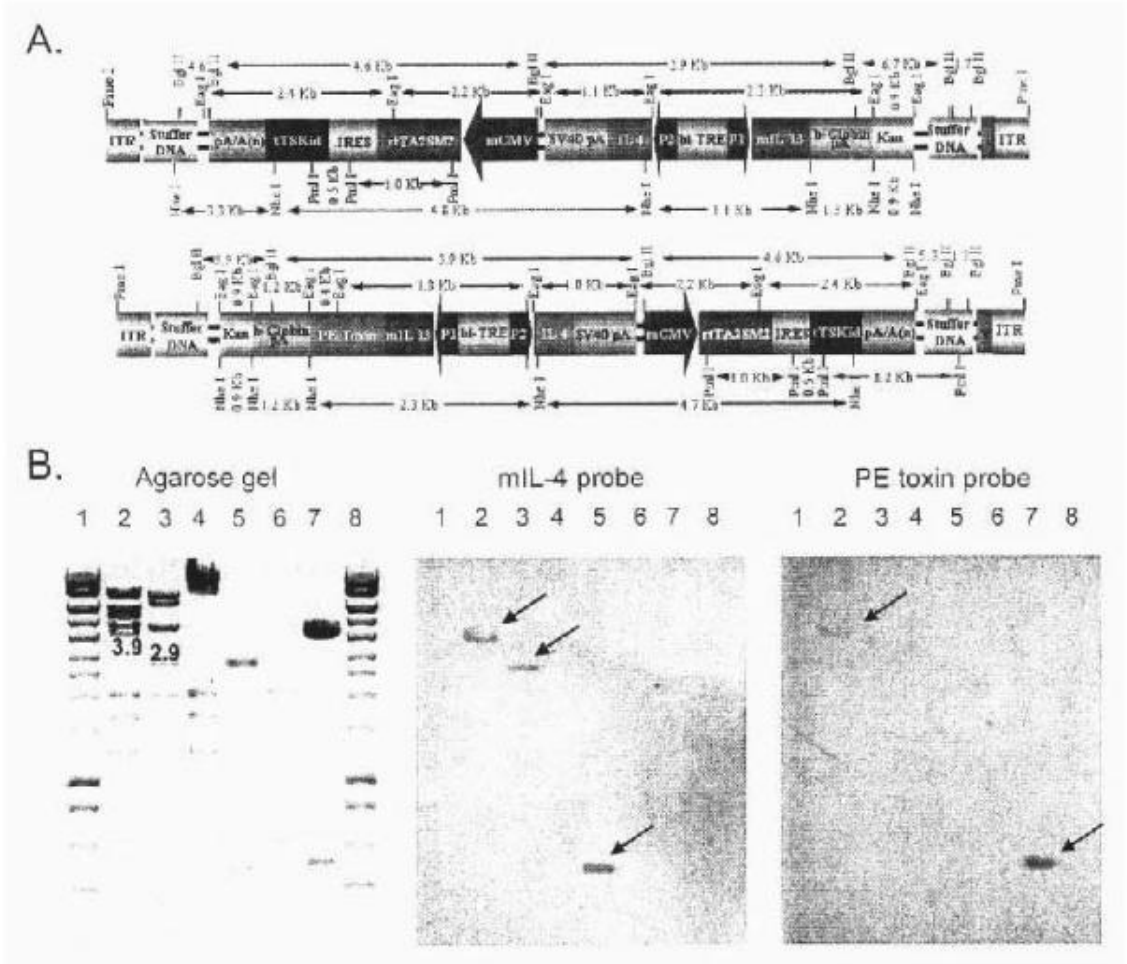

Fig. 2.

A) Linear depiction of the HC-Ad vector encoding the mIL-4 and mIL-13 without or with the PE toxin transgene and mCMV driven regulatable TetON switch cassette. The constructs indicate the individual components and 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 part A. B) Southern blot analysis of IL4 and PE toxin enconded by the therapeutic vector. HC-Ad plasmids were digested and lanes are as follows: lanes 1 and 8 Hypperladder; lane 2, pSTK120mA.mIL-4.mIL-13PE Bgl II digest; lane 3, pSTK120mA.mIL-4.mIL-13 Bgl II digest; lane 4, pSTK120mA Bgl II digest; lane 5, pLS.IL-4 Sal I/EagI digest; lane 6, pLS.IL-13 EcoRV/HindIII digest; lane 7, pRB 39.1 PE toxin EcoRI/ HindIII digest. Southern blot hybridization was performed with specific probes to IL4 and PE toxin. Both of the recombinant HC-Ad vectors, and the control plasmid were positive for mIL4 (lanes 2, 3 and 5); the hybridization for PE toxin was positive only in the therapeutic HC-Ad vector and in the control plasmid (lanes 2 and 7).

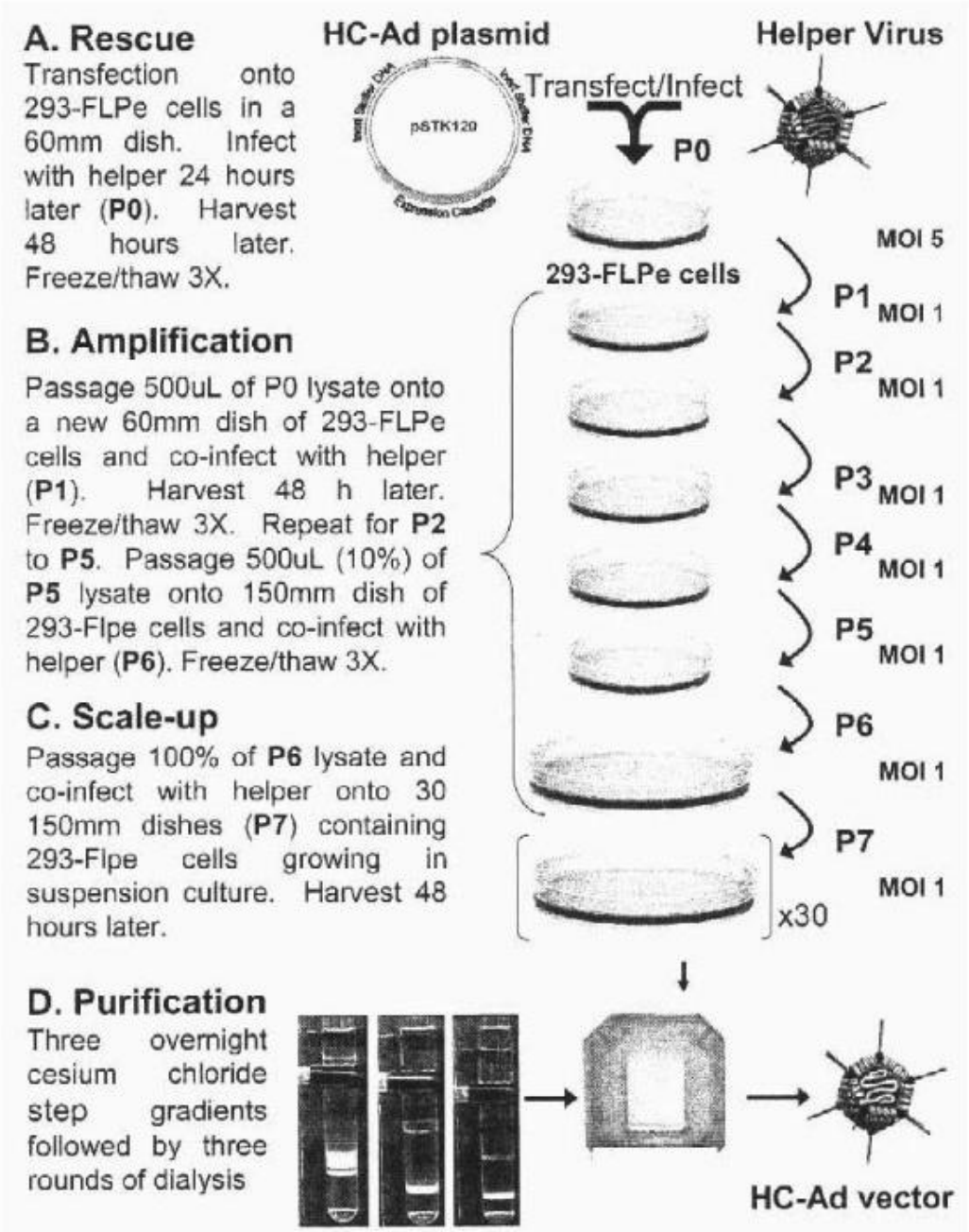

Fig. 3.

Construction of High-Capacity adenoviral vectors (HC-Ad). A. HC-Ad vectors are rescued by transfecting 293 cells expressing FLPe recombinase with linearized pSTK120 plasmids containing two adenoviral ITR's, a packaging signal, an expression cassetle (Tet ON regulated), and inert stuffer DNA. The cells are infected with FL helper virus containing two loxP sites flanking its packaging signal 24 hours later (MOI 5 pfer per cell). The cells are harvested and lysed 48 hours later. B. The HC-Ad vectors are amplified on 293-FLPe cells by 5–7 more serial passages of 10% of the cell lysate from the previous passage, the last of which onto one 150 mm dish with the addition of fresh FL helper virus (MOI 1). The tites of the HC-Ad vectors increases with each serial passage, eventually surpassing the titer of the FL helper which is not

efficiently packaged due to the excision of its packaging signal. C. During scale up, 100% of P6 lysate is co-infected with fresh FL helper virus into 30 150 mm dishes containing 293-FLPe cells. Cells are harvested 48 hours later. D. Cells are lysed with 5% deoxycholate and DNaseI and HC-Ad vectors are purified over three CsCl step gradients. The vectors are dialyzed against three changes of dialysis buffer supplemented with 10% glycerol, aliquoted, and stored at −80° C.

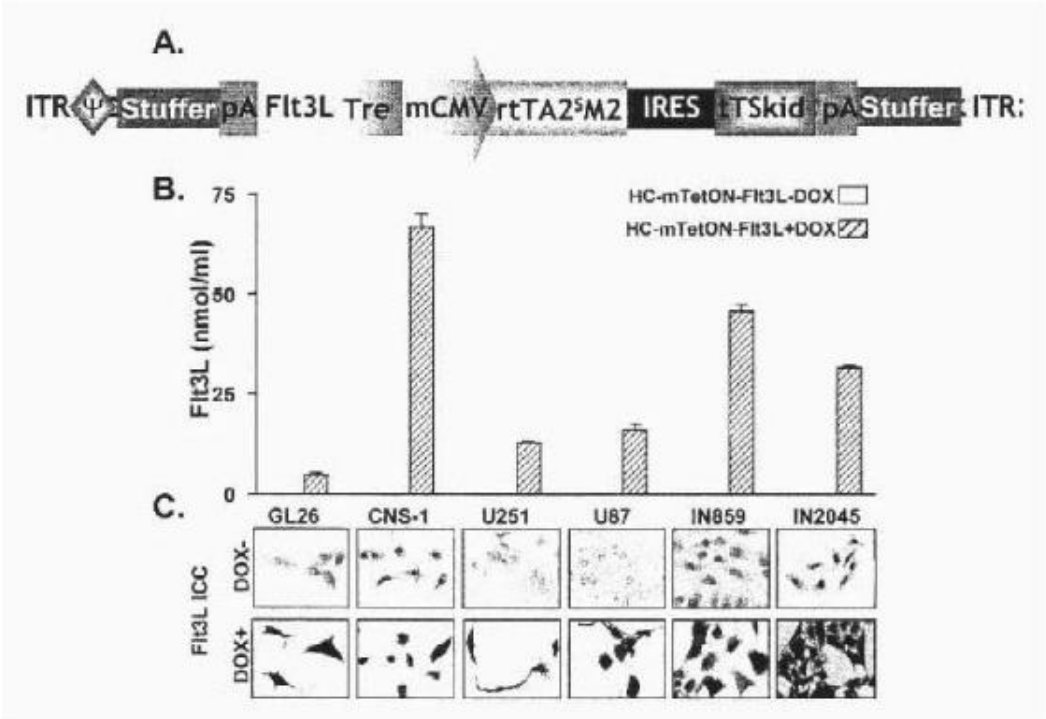

Fig. 4.

Regulated expression of Flt3L from the high capacity adenoviral vetor(HC-Ad-mTetON-Flt3L) in human and murine glioma cells. Established human glioma cell lines (U251, U87), cultures from human glioma biopsies (IN859, IN 2045), rat (CNS-I), and mouse (GL26) glioma cells were infected with HC-Ad vector encoding Flt3L under the control of a TetON system driven by the murine CMV promoter (HC-Ad-mTetON-Flt3L) for 72 h in the presence or absence of the inducer doxycyline (Dox, 1 μg/ml). Transgene expression was determined by ELISA and immunocytochemistry (ICC).

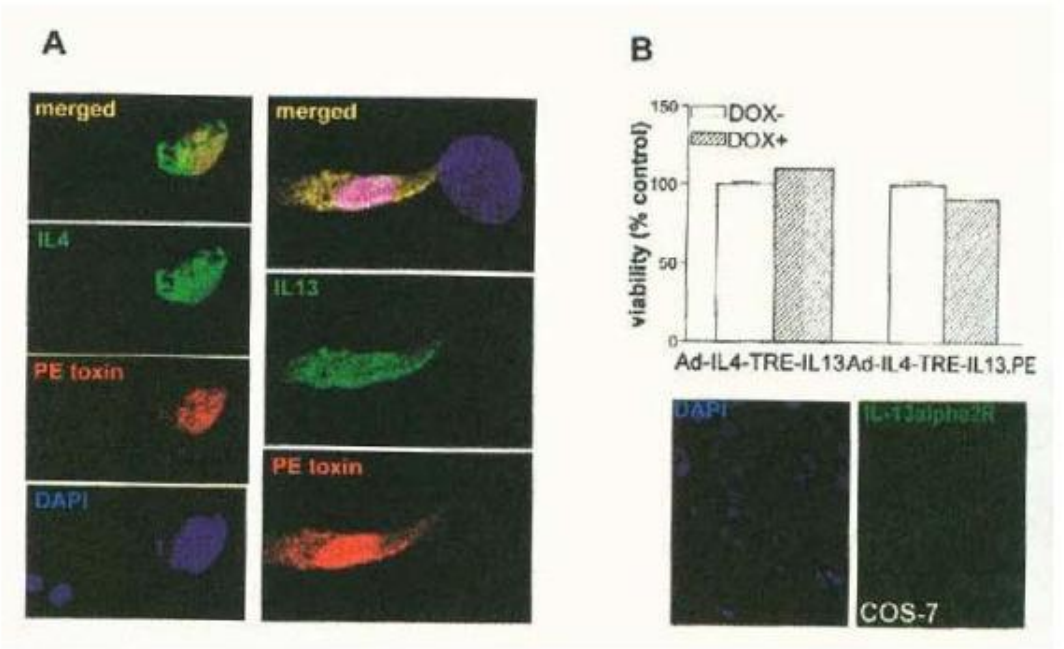

Fig. 5.

mIL-13-PE toxin expression from the therapeutic (Ad-IL4-TRE-IL13.PE) adenoviral vector in COS-7 cells. A. COS-7 cells were infected with the therapeutic adenoviral vector (Ad-IL4-TRE-IL13. PE) that express mulL4 and mIL13 or the cytotoxin mulL-13-PE under the control of the bidirectional TRE promoter, which is activated by the transactivator (TetON) in the presence of the inducer Dox. Microphotographs show the regulated expression of the mIL-13-PE toxin, as determined by immunocytochemistry in the presence (Dox+) or absence (Dox−) of the inducer. B. COS-7 cells (were infected with control (Ad-IL4-TRE-IL13) and therapeutic (Ad-IL4-TRE-IL13.PE) adenoviral vectors. Upper panels: Cell viability was determined by MTS assay. Lower panels: IL-13alpha2R expression was determined by immunocytochemistry. Nuclei were stained with DAPI. Note that the therapeutic Ad-IL4-TRE-IL13.PE exert its cytotoxic effect only in cells expressing the glioma-restricted IL-I 3alpha2R.

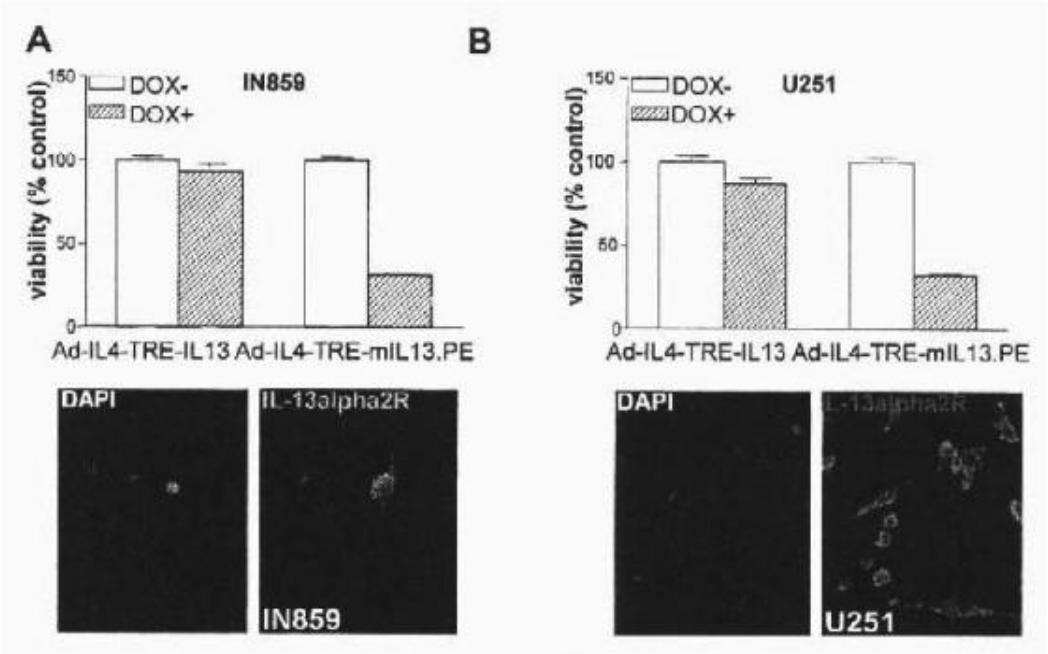

Fig. 6.

mIL-13-PE toxin induces cell death in glioma cells. A. IN859 and B. U251 human glioma cells were infected with the therapeutic adenoviral vector (Ad-IL4-TRE-IL13.PE) or control vector Ad-IL4- TRE-IL13. Upper panels: Cell viability was determined by MTS assay. Lower panels: IL-13alpha2R expression was determined by immunocytochemistry. Nuclei were stained with DAPI. Note that the therapeutic Ad-IL4-TRE-IL13.PE exert its cytotoxic effect only in cells expressing the glioma-restricted IL- 13alpha2R.

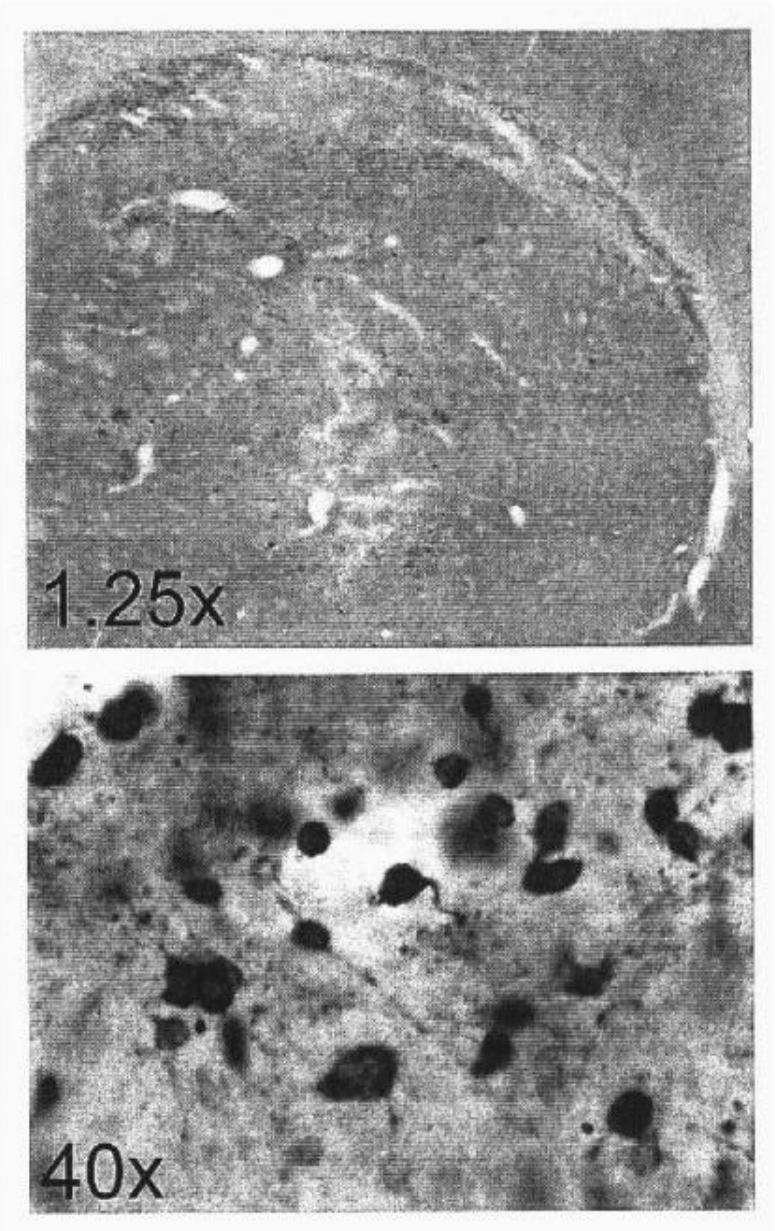

Fig. 7.
Inducible Flt3L expression in vivo. Male Lewis rats fed Dox supplemented chow 24 h before injection of 1×10^9 vp HC-Ad-mCMV-TetON-Flt3L into the brain striatum. After 7 days, rats were euthanized, fixed with PFA and coronal sections of the brain were taken using a vibratome (Leica). Brain sections were stained with rabbit anti-Flt3L as outlined in the materials and methods section and images were visualized on a Zeiss Axioplan 2 microscope under 1.25× n d 40× objectives. Expression of Flt3L was not observed in rats fed normal chow without Dox (not shown).

Table 1

Plasmid Sources and Function

Name	Function	Reference
pΔE1sp1A	A first generation shuttle plasmid containing Xba1, Hind3 and Sal1 (among others) in the multiple cloning site.	Microbix Biosystems
pAL120	A first generation shuttle vector containing the mCMV promoter and a pA signal sequence. Sal 1 is used for cloning transgenes downstream of the mCMV promoter and upstream of the pA.	Generated in our laboratory (24)
pIRES-tTS$_{Kid}$-pA	An intermediate plasmid in the cloning of the TetON cassette, it is a modified pIRES plasmid containing the tTSkid-pA sequence downstream of the IRES.	Generated in our laboratory (2)
pUHS 6-1	This plasmid contains the tTSkid repressor and polyadenylation (pA) signal flanked by Xba1 and Hind3.	H. Bujard at ZMBH, Germany (8)
pIRES	Cloning plasmid containing the Internal Ribosome Entry Site (IRES) from the encephalomyocarditis virus.	Clontech
pUHrT 62-1	This plasmid carries the rtTA$_{2S}$-M2 Tet-sensitive transactivator gene flanked by EcoR1 and BamH1.	H. Bujard at ZMBH, Germany (8)
prtTA$_{2S}$ M2-IRES-tTS$_{kid}$-pA	8200 bp plasmid based on pIRES containing rtTA$_{2S}$ M2 upstream and tTS$_{kid}$ immediately downstream of the IRES sequence (i.e., the TetON cassette) flanked by Xho1 and Sal1.	Generated in our laboratory (2)
pSP72-Bgl2	Modified cloning plasmid (original from Clontech) with a multiple cloning site that contains Bgl2, Hind3 and EcoR1 among others.	Generated in our laboratory (2).
pSP72-mCMV	pSP72-Bgl2 containing the mCMV promoter and pA sequence (from pAL120).	Generated in our laboratory (2).
pSP72[mCMV-rtTA$_{2S}$M2-IRES-tTS$_{kid}$-pA]	Intermediate plasmid containing the TetON cassette regulated by the mCMV promoter. The entire cassette is flanked on both ends by Bgl2.	Generated in our laboratory (2)
pBS2SK+ [Tre-MCS-pA]-Kana	Intermediate vector based on the bluescript 2 SK+ plasmid.	Generated in our laboratory (unpublished)
pmCMV-TetON	pBlueScript based vector containing the TetON cassette and also containing the Tet-sensitive promoter TRE upstream of a multiple cloning site (Age1, Afl2, Swa1, Bel1) and pA.	Generated in our laboratory (unpublished)
pSP72Bgl2-MCS	Adaptor plasmid generated in our laboratory containing two copies each of the Age1, Afl2, Swa1, and Bcl1 restriction endonuclease sites. By cloning transgene through this vector, these rare restriction sites can be used to clone directly into pmCMV-TetON.	Generated in our laboratory (unpublished)
pSTK120	Plasmid used to generate high capacity adenoviral vectors. Plasmid only shares long terminal repeat (LTR) and packaging (Ψ) sequences of wild type Adenovirus. Several variants have been created, e.g., pSTK120.1, pSTK120.2, and pSTK120.3.	Generated by S. Kochanek (25)

Table 2

HC-Ad Plasmids and Predicted Cloning Capacities for Transgene Cassettes

Plasmid	Cloning capacity (Kb)	Restriction sites for cloning in transgene cassette
pSTK120	0–7	Eag1
pSTK120.1	2–10	Nhe1, Eag1
pSTK120.2	4–13	Nhe1, Eag1, Nar1
pSTK120.3	8–18	Nhe1, Eag1, Nar1, Ncol