tive analysis of GFNR expression, average red fluorescence was subtracted from average green fluorescence to obtain an estimate of specific GFNR fluorescence.

Identification of trapped genes and northern blot. Total RNA was prepared from cell cultures using the Trizol reagent (Gibco BRL), according to the manufacturer's protocol. To recover and identify trapped genes, we did a 5′ RACE on total RNA by using the Smart RACE cDNA amplification kit (Clontech), according to the manufacturer's protocol, using two EGFP primers (5′-CTTGTGGC-CGTTTACGTCGCCG-3′, 5′-CGGTGAACAGCTCCTCGCC-3′) in the first round and nested PCR, respectively. Inverse PCR was performed as described[21], using a SupF primer (5′-GGAGCAGGCCAGTAAAAGCATTACCCGTG-3′) and a NTR primer (5′-AGTAGCGTTTTGATCTGCTCGGCCTGTTCC-3′), followed by PCR with two nested primers (5′-CTTCCCCCACCACCATCACTTT-3′, 5′-TAGTGGAATGACGCTTTAAGGC-3′). PCR products were cloned using the Invitrogen (Carlsbad, CA) TOPO TA cloning kit and sequenced using fluorescent dye terminators on a Perkin-Elmer (Foster City, CA) 310 sequence analyzer, according to the manufacturer's protocols. For quantitative northern blot analysis, bound radioactivity was detected and quantified using a STORM 840 phosphorimager apparatus (Molecular Dynamics, Sunnyvale, CA).

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Efficient FLPe recombinase enables scalable production of helper-dependent adenoviral vectors with negligible helper-virus contamination

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Helper-dependent (HD), high-capacity adenoviruses are one of the most efficient and safe gene therapy vectors, capable of mediating long-term expression[1–12]. Currently, the most widely used system for HD vector production avoids significant contamination with helper virus by using producer cells stably expressing a nuclear-targeted Cre recombinase and an engineered first-generation helper virus with parallel *loxP* sites flanking its packaging signal[1,3–12]. The system requires a final, density-based separation of HD and residual helper viruses by ultracentrifugation to reduce contaminating helper virus to low levels. This separation step hinders large-scale production of clinical-grade HD virus[13]. By using a very efficient recombinase, *in vitro*–evolved FLPe (ref. 14), to excise the helper virus packaging signal in the producer cells, we have developed a scalable HD vector production method. FLP has previously been shown to mediate maximum levels of excision close to 100% compared to 80% for Cre (ref. 15). Utilizing a common HD plasmid backbone[1,7,8,10–12], the FLPe-based system reproducibly yielded HD virus with the same low levels of helper virus contamination before any density-based separation by ultracentrifugation. This should allow large-scale production of HD vectors using column chromatography–based virus purification[13].

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A helper virus, a first-generation adenovirus with a packaging signal flanked by FLPe-recombination target (FRT) sites and excisable by FLPe-mediated recombination, was constructed as illustrated in Figure 1A. The genome of this helper virus (designated FL helper) is similar to that of the helper widely used in the Cre-based system, namely the AdLC8luc virus[1,4,6–12]. The packaging signal of the FL helper is flanked by parallel FRT sites, instead of the *loxP* sites of AdLC8luc. Both viruses encode a luciferase expression cassette driven by the human cytomegalovirus (hCMV) promoter (in the same orientation in both genomes) and a stuffer of similar size, inserted into the E3-deleted region. FL helper virus has the essential core packaging-signal (domains A1–A5), shown not to have deleterious effects on replication and packaging of adenoviral genomes[16,17].

Human embryonic kidney (HEK) 293 cells were transfected with a vector for strong, constitutive expression of a nuclear-targeted FLPe recombinase gene operatively linked by an internal ribosome entry site (IRES) to a puromycin resistance gene. Cells with stable expression of this cassette were selected for growth in the presence of puromycin, and characterized as described in the Experimental Protocol section. Clone 293-FLPe6 was chosen for further work. Efficient excision of the packaging signal was shown functionally (data not shown), and confirmed at the DNA level (Fig. 1B).

To rescue a β-galactosidase–expressing HD virus from HD plasmid (pGS46), 293-FLPe6 cells were transfected with linearized pGS46 (refs 10,12) using a high-efficiency variant of the calcium phosphate transfection method[18] (Fig. 2A). Plasmid pGS46 consists of the adenoviral inverted terminal repeats (ITRs), the wild-type packaging signal, noncoding human stuffer DNA sequences, and a β-galactosidase expression cassette[10,12] (Fig. 2B). The transfected cells were subsequently infected with FL helper virus using a multiplicity of infection (MOI) of 5, and virus was harvested after full cytopathic effect (CPE). Titers of GS46-FLPe HD virus generated after the initial rescue step were 5×10^4 to 2×10^5 blue-forming units (BFU)/ml (i.e., 1.3 to 5.3×10^4 BFU/μg of transfected DNA) (Fig. 2A).

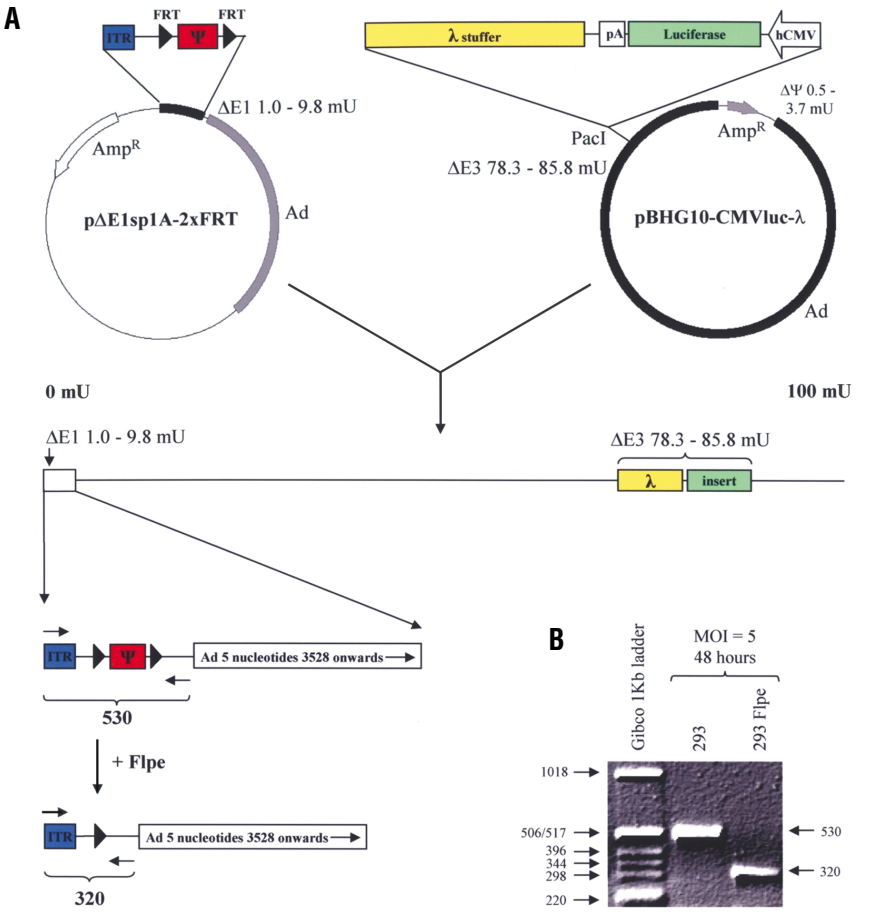

Figure 1. An E1-, E3-deleted helper adenovirus (FL helper) with a packaging signal sensitive to FLPe-mediated excision. (A) FL helper was generated by homologous recombination in 293 cells after co-transfection with plasmids pDE1sp1A-2xFRT and pBHG10-CMVluc-l. The bottom half of the figure indicates the the left-end region of the helper virus genome, before and after FLPe-mediated recombination; the figure also indicates the location of the PCR primers used to determine excision of the packaging signal (black arrows). (B) The result of PCR amplification of the the left-end region of the helper genome 48 h after infection of either 293 cells, or 293-FLPe6 cells with an MOI of 5. Notice that growth in 293-FLPe6 cells leads to the excision of the packaging signal, visible as a band of ~320 bp. No higher molecular weight band is detected in this lane. Helper viral genomes were extracted from 293 or from 293-FLPe6 cells using a modified Hirt procedure[19]. mμ, Map units.

GS46-FLPe HD virus was scaled up through three to four amplification steps, by co-infecting 293-FLPe cells with FL helper virus plus lysate from the previous step. The titers of GS46 HD and FL helper vectors for each step are shown in Figure 2A. In two independent vector preparations, the final HD vector yield in the crude lysate was 5×10^{10} BFU from 9×10^7 cells, and 1.8×10^{11} BFU from 3.6×10^8 cells, respectively (i.e., ~500 BFU/cell in the final step for both preparations), similar to that reported for HD vectors produced with the same HD plasmid backbone using the AdLC8luc helper/293-Cre system[1,7,10,12].

The level of helper virus in the final steps of both preparations was 0.05% and 0.04% (IU/BFU), before any density-based separation by ultracentrifugation. This is an improvement over previous results obtained for HD vector production using the same HD plasmid backbone and the 293-Cre system[1,7,8,10–12], which at best reached these low levels of contamination only after one or two ultracentrifugation steps. The use of GS46-FLPe HD virus *in vivo* indicated a qualitative level of transduction comparable with a first-generation virus containing a similar transgenic cassette (Fig. 3) and to a HD vector tested by us previously[10,12].

If the level of helper vector contamination is assessed by a quantitative TaqMan real-time PCR method, contamination levels are at most 1.4% before, and 0.22% after CsCl gradient centrifugations (Table 1). Compared with data reported in a previous study using quantitative PCR (ref. 11), and the same plasmid vector backbone, our levels of helper virus contamination before CsCl gradient centrifugation are as good as those obtained with a Cre-based system after gradient centrifugation. For both recombination systems, helper virus contamination calculated by quantitative TaqMan real-time PCR differs by a factor of 1–1.5 logs to that calculated by biological titration (Table 1). The reasons for these differences remain to be determined. The level of contamination of replication-competent $E1a^+$ genomes in our preparation, determined by quantitative PCR, was below $1:10^9$ copies of HD genomes.

It is well known that CsCl density gradient purification limits large-scale production of HD vectors for clinical use. In contrast, FLPe-based excision of the helper packaging signal should enable purification of HD vectors directly from the final crude lysates or

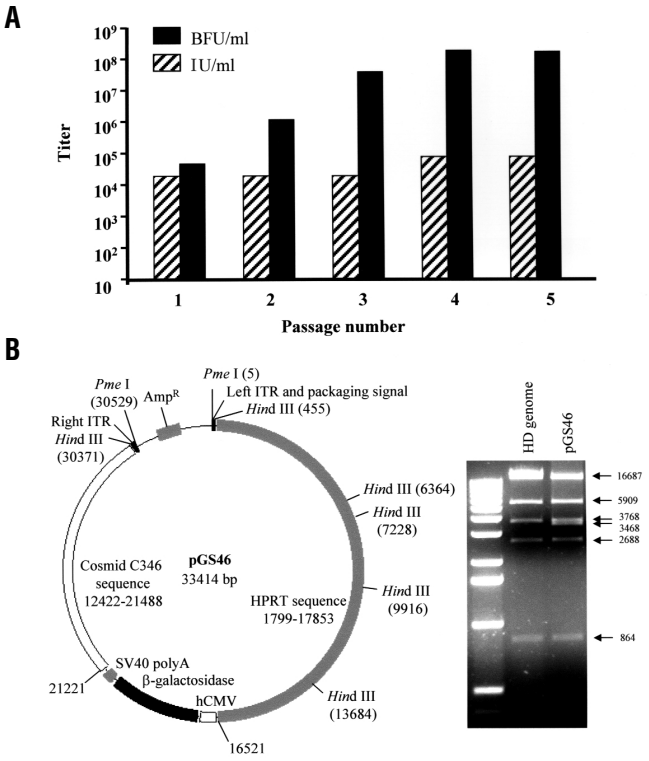

Figure 2. Rescue and amplification of a β-galactosidase–expressing HD virus using 293-FLPe cells and FL helper virus. (A) 293-FLPe cells were transfected with *Pme*I-digested HD plasmid pGS46, and HD virus was rescued by infection with FL helper virus (passage number 1). HD virus was amplified in a series of four further passages by co-infecting 293-FLPe6 cells with lysate from the previous passage plus FL helper virus. GS46-FLP HD virus titer was measured as blue-forming-units (BFU)/ml, whereas contaminating FL helper virus titer was measured as infectious-units (IU)/ml. (B) Map of plasmid GS46, which was rescued as a HD vector. Viral genome rescued from CsCl gradient-purified HD vector, or pGS46, were digested with *Hind*III and separated on a 1% agarose gel. The identical banding pattern indicates the absence of major rearrangements of the HD vector produced after five passages in 293-FLPe6 cells; the extra band visible in *Hind*III-digested pGS46 (i.e., 3,468 bp), but absent from the HD genome, represents the fragment containing the ampicillin resistance gene removed upon *Pme*I digestion.

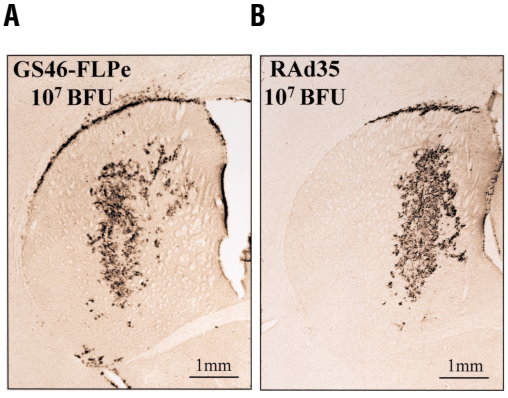

Figure 3. *In vivo* β-galactosidase expression in rat brain infected with either GS46-FLPe HD vector or first-generation adenovirus (RAd35). (A) GS46-FLPe HD virus (7×10^7 BFU) or (B) 7×10^7 BFU of RAd35, a first-generation, replication-deficient adenovirus also expressing β-galactosidase from the hCMV promoter[20], were injected into male Sprague–Dawley rat brains[20]. Six days later animals were perfusion-fixed and 50-μm-thick sections were cut using a vibratome, and β-galactosidase, under the control of the hCMV promoter in both viruses, was detected using immunohistochemistry[21].

Table 1. Helper virus contamination (for pSTK120-derived vectors).

Recombination system	Production stage	Infectious units ratio (biological assay, %)[a]	Genome copies ratio (%)[b]
FLPe	Crude	0.04–0.05 (this study)	1.4 (this study)
	CsCl gradient	0.004–0.01 (this study)	0.22 (this study)
Cre	Crude	Not available	Not available
	CsCl gradient	0.08–0.15 (refs 1,7,8,10,12)	1.0 (ref. 11)

[a]Infectious units ratio, calculated as the ratio between the titer of helper virus (determined as infectious units) to the HD vector titer (e.g., determined as blue-forming units in the case of vectors expressing β-galactosidase); for a double CsCl gradient–purified material, 1×10^6 IU/ 2.4×10^{10} BFU = 0.004.
[b]Genome copies ratio, determined by quantitative Taq Man real-time PCR.

extracts using a column chromatography step, a process that easily lends itself to large-scale production methods[13]. Important modifications introduced recently by others to the HD plasmid backbones and helper virus for this system[11] may be combined with the FLPe-mediated recombination described here and lead to widespread use of HD adenoviral vectors for gene therapy.

Experimental protocol

Introduction of FLP-recombinase target (FRT) sites flanking the adenoviral packaging signal. Minimal (34 bp) FRT sites (5′-gaagttcctattctctagaaagtataggaacttc) were introduced in parallel orientation flanking the adenoviral packaging signal by PCR and reassembling the left end adenoviral sequences in plasmid pΔE1sp1A (Microbix, Toronto, ON, Canada). The resulting plasmid was designated pΔE1sp1A-2xFRT. Further details of the cloning are available from the authors upon request.

Subcloning of a luciferase expression cassette and stuffer DNA in the adenoviral ΔE3 region. A luciferase expression cassette under the control of the hCMV promoter and including a simian virus 40 (SV40) polyadenylation sequence was subcloned into pABS.4 (Microbix), a plasmid designed to facilitate the introduction of DNA into the adenoviral ΔE3 region[17]. A 2.9 kbp, *Pst*I restriction fragment of bacteriophage λ-DNA was subsequently introduced as a stuffer into the modified pABS.4. The luciferase mammalian expression cassette, the stuffer DNA fragment, and a bacterial kanamycin resistance cassette were then subcloned into the *Pac*I site of adenoviral-backbone plasmid pBHG10 (Microbix). The resulting plasmid was designated pBHG10-CMVluc-λ.

Generation of a helper virus with a packaging signal sensitive to FLPe-mediated excision. pΔE1sp1A-2xFRT and pBHG10-CMVluc-λ DNA were co-transfected by a calcium phosphate method[18] into low-passage 293 cells (Microbix). A first-generation, replication-defective, E1- and E3-deleted helper adenovirus, with its packaging signal flanked by FRT sites and with a luciferase expression cassette plus stuffer DNA subcloned into the ΔE3 region (total DNA size of 35.7 kbp), was generated by homologous recombination in co-transfected 293 cells as described[19,20]. The resulting virus (FL helper virus) was isolated and amplified by standard methods and titrated by an end-point dilution, CPE assay (see below)[19,20]. To determine the excision of the packaging signal, PCR primers were designed that corresponded to Ad 5 base pairs 1–35 (5′-catcatcaataatataccttattttggattgaagc-3′) and the reverse complement of pΔE1sp1a base pairs 418–450 (5′-ccccaccttatatattctttcccaccctaacc-3′). Two nanograms of viral DNA extracted from either 293 or 293-FLPe6 cells were subjected to 20 cycles of PCR with an annealing temperature of 63°C.

Calcium phosphate transfections. 293 cells were transfected using a version of the calcium phosphate transfection method[18] using 10% fetal calf serum.

Establishment of a 293-FLPe cell line. Low-passage 293 cells were transfected with linearized plasmid pCAGGSFLPeIRESpuro (a gift of Francis Stewart, EMBL, Heidelberg, Germany)[18]. pCAGGSFLPe-IRESpuro carries a strong, constitutive expression cassette for an *in vitro*–evolved FLP recombinase (FLPe)[14] under the control of the chicken β-actin promoter and a hCMV immediate early

enhancer. The FLPe recombinase carries a minimal, nuclear localization signal from the SV40 large T antigen and is operatively linked by an IRES to a puromycin resistance gene. Additional elements of the expression cassette are an intron with splice donor and acceptor sites upstream of the FLPe gene and a bovine growth hormone polyadenylation signal downstream of the puromycin resistance gene. Transfected cells were selected for stable expression of transgenes by culturing in the presence of 1.5 μg/ml of puromycin. Individual cell clones were isolated, amplified, and compared by a functional assay.

HD virus rescue and amplification. To rescue HD virus from HD plasmid, 293-FLPe6 cells were first transfected with *Pme*1-linearized, β-galactosidase–expressing HD plasmid pGS46 (ref. 16) (a gift of S. Kochanek and G. Schiedner)[18]. Following 16 h of incubation, cells were infected with FL helper virus using an MOI of 5. Upon full CPE, virus was harvested lysing the cells by three freeze–thaw cycles. The lysate from this initial rescue was used to amplify the HD virus in three to four serial steps, co-infecting 293-FLPe cells (at ~80% confluency) with FL helper virus plus lysate from the previous step. First, three wells of a six-well plate with 293-FLPe cells were co-infected with all of the lysate from the rescue step plus FL helper virus at an MOI of 5 (for passages 1 and 2, and MOI of 3.5 for subsequent passages). Plates were centrifuged at 1000 *g* for 90 min at room temperature and incubated at 37°C for 3 h. The medium was replaced by fresh culture medium and cells were incubated until complete CPE. The same procedure was repeated for the third step, but infecting six instead of three wells of the six-well plate, and using an MOI of FL helper of 3.5. All of the lysate from the third step was used to infect nine large T182 culture flasks (182 cm^2 of culture area each) adding FL helper at an MOI of 3.5. A volume of 0.5 ml of culture medium per cm^2 of culture area was used in all steps. Alternatively, for a larger preparation, all the lysate from the third step was used to infect 3 T75 flasks adding FL helper at an MOI of 3.5. All of the lysate from this step was used to infect 30 T182 culture flasks, again adding FL helper at an MOI of 3.5.

Titration of HD and helper virus. HD virus was titrated by quantifying the BFU (ref. 4). Cell lysates or purified viruses were serially diluted (10^{-2}–10^{-10}), and 0.5 ml of each dilution was used to infect in triplicate 293 cells cultured at ~90% confluency in 24-well plates. The plates were centrifuged as described above and incubated at 37°C for 20 h. Cells were fixed in 0.5% glutaraldehyde in PBS and stained using the X-gal method[4]; BFUs were calculated by counting blue cells per well under an inverted microscope, taking the average of blue cell counts within at least two different dilutions within the linear range of the assay, and dividing the values obtained by the volume tested (0.5 ml) and by the dilution factor. FL helper virus was titrated by quantifying the infectious-units (IU) in 96-well plates by end-point CPE as described[19], using 5×10^3 293 cells per well, assaying each dilution in quadruplicate, incubating the plates for 14 days, and inspecting the plates visually for CPE every 24 h. Titers expressed as IU/ml were calculated by multiplying the number of wells with CPE at the highest dilution and dividing by the total volume tested and the dilution factor.

Molecular quantification of relative levels of vector and helper virus genomes. The amount of helper genomes was measured relative to HD vector genomes by quantitative TaqMan real-time PCR (Perkin-Elmer, Boston, MA). DNA was extracted from either crude freeze–thaw lysates (subjected to DNase digestion, followed by pelleting by centrifugation through a 20% sucrose cushion, and heat inactivation) or CsCl gradient–purified vectors, using the QIAamp DNA Kit (Qiagen, Basel, Switzerland). Specific sets of primers and probes labeled with 6-carboxyfluorescein and 6-carboxyltetramethylrhodamine were designed for both helper and HD vector genomes using the program Primer Express (Perkin-Elmer). A plasmid containing the target sequences for both the vector and helper virus was constructed and used as a standard. pJM17 (Microbix) was used as a standard when quantifying the relative concentration of replication-competent virus and helper virus genomes. DNA copy numbers were calculated by using the 7700 software (Perkin-Elmer). Primers were as follows: HD vector forward: 5′-ATGCCAGGACCACCAGGAA-3′ (bases 2,554–2,572 from the C346 stuffer in pGS46); HD vector reverse: 5′-TGCAGGGCTGTCATAGGGA-3′ (bases 2,602–2,620); HD TaqMan probe: 5′-AAGCCTCAGTCCTCTTCTCAATGTCCT-3′ (bases 2,574–2,600); helper forward: 5′-GTTGGCACCCCTATTCGACA-3′ (bases 14,322–14,351 in the adenovirus type 5 genome); helper reverse: 5′-GGATGCCACATCCGTTGACT-3′ (bases 14,379–14,398); helper TaqMan probe: 5′-ACCCGTGTGTACCTGGTGGACAAC-3′ (bases 14,354–14,377); E1a forward: 5′-CCAGTGACGACGAGGATGAA-3′ (bases 951–970 in the adenovirus type 5 genome); E1a reverse: 5′-CCGTATTCCTCCGGTGATAATG-3′ (bases 1,031–1,052); E1a TaqMan probe: 5′-CAAGACCTGCAACCGTGCCC-3′ (bases 1,010–1,029).

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