High-level production of replication-defective human immunodeficiency type 1 virus vector particles using helper-dependent adenovirus vectors

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INTRODUCTION

The genetic modification of somatic cells, for both bench research and clinical purposes, is a fundamental goal of modern molecular and cellular biology. Retroviral vectors, which irreversibly integrate into the genome, remain a popular tool to permanently modify the DNA of the host. Clinical success has been achieved using murine leukemia virus-based vectors to transduce hematopoietic stem cells for X-linked severe combined immunodeficiency1–3 and adenosine deaminase deficiency, 4–6 not to mention topoietic stem cells for X-linked severe combined immunodeficiency type 1 virus vector particles using helper-dependent adenovirus vectors.7–10 and acquired11,12 diseases of the immune system. Because murine leukemia virus vectors favor integration at or near transcriptional start sites,13,14 cannot transduce nondividing cells,15,16 and have been associated with the development of malignancy,17,18 vectors upon human immunodeficiency virus type 1 or HIV have advanced to phase 1 clinical trials, with some recent successes in treating genetic diseases, including forms of adrenal leukodystrophy19,20 and Wiskott-Aldrich syndrome.21 Only in a single patient with β-thalassaemia has there been evidence of clonal predominance after receipt of an HIV-based vector22 and unlike the experience with murine leukemia virus there has been no occurrence of malignant transformation.

The vast majority of the time these HIV-based vectors are prepared by multiplasmid DNA transient cotransfection of 293T cells and harvest of the culture supernatant a few days later. These VSV G-pseudotyped lentiviral vectors (LVs) are subject to standard purity checks, including tests for replication-competent lentivirus.23–26 Unconcentrated vector titers are typically approximately of $10^6$ IU/ml, depending upon the exact vector configuration and reporter gene, which may then be concentrated up to 100-fold by ultracentrifugation. Unfortunately, there is variability in plasmid transfection of 293T cells, and the process scales poorly, especially for clinical trials. VSV G-inducible packaging cell lines have also been developed of similar overall titer,27–33 but these have not been widely adapted for use and of course require rederivation and revalidation, depending upon the encoded gene of interest.

Helper-dependent adenoviral vectors (HDAds), also known as high-capacity adenoviral vectors, are dependent upon helper adenovirus (HV) for replication.34,35 They are essentially “gutted”, with only the inverted terminal repeats and packaging sequence ($\psi$) of adenovirus remaining. Their minimal nature allows 30 kb or more of exogenous DNA to be inserted between the inverted terminal repeats. The HV provides all the trans-functions required for replication of the vector, and $\psi$ of the HV is floxed. During replication in Cre-expressing 293 cells, very little of the HV (typically 0.02% or less) is packaged because $\psi$ is excised by Cre; >99.98% of the adenovirus packaged is the HDAd. After purification, HDAd vector titers approach $10^{12}$ vp/ml, similar to that of first-generation adenoviral vectors.34,35

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Although HDAd systems have been used extensively in preclinical models for gene delivery, we wished to determine whether they could be used to produce VSV G-pseudotyped LV supernatants in a two-step protocol. All necessary cis and trans components of HIV-based vectors would be inserted into an HDAd, which is then amplified in vitro. The HDAd is then used to transduce adenoviral-permissive cells to produce replication-defective, VSV G-pseudotyped HIV vector of high titer. Although similar adenoviral-HIV vector systems were developed more than a decade ago, both the HDAd- and HIV-based vectors were not optimized, with relatively high levels of HIV contamination and low achievable titers. We now report on improved HIV-based vector production from an optimized HDAd system, with more than an order-of-magnitude increase in titer compared to standard plasmid transfection. This simple method of production should advance the use of HIV-based LVs for a myriad of clinical applications now under consideration.

**RESULTS**

In an initial series of experiments, we constructed a single HDAd that encoded SRα promoter driving VSV G and a second-generation HIV-based self-inactivating vector encoding gag, pol, tat, rev, and a CMV IE promoter driving IRES-eGFP reporter cassette. Plasmid transfection of 293T cells using the individual vector components resulted in an end-point titer of ~1.0 × 10^6 IU/ml, with human osteosarcoma (HOS) cells as targets, as assessed by flow cytometry. Shown are results of two separate experiments. HDAd titers, as assessed by lacZ staining, were orders-of-magnitude lower (not shown).

**Figure 1** Schematic of HDAd constructs used here. (a) Starting plasmid pD28E4lacZ (31 kb); (b) HDAd-HIV-1 (33 kb); (c) HDAd-VSVG1/2 (31 kb); (d) HDAd-VSVG3/4 (34 kb); (e) HDAd-PV (39 kb); (f) HDAd-FG12 (37 kb). In each, ψ denotes packaging signal, gray are ITRs, red: SRα driving VSV G, green: FG12 TV, blue: mCMV driving lacZ, yellow: second generation HIV-based vector encoding eGFP, purple: HIV-PV. For HDAd-VSVG1/2 and 3/4 the 2.7 kb SRα-VSV G cassettes are in opposite orientations. E. coli plasmid origin of replication and Knr cassette both lie within 3 kb segment between ITRs. Not shown are the two Pme1 sites that abut the ITRs and allow linearization of the HDAd. Gene lengths and plasmid sizes not precisely to scale.

**Figure 2** Viral titers after transduction of 293T cells with HDAd-HIV-1. Cells were transduced in six-well format at increasing multiplicity of infections and vector supernatant was harvested 48 hours later and titered by limiting dilution on HOS targets, as assessed by flow cytometry (for HIV) and lacZ staining (for leftover, contaminating HDAd). Filled bars are HIV (eGFP) titers; gray bars are HDAd titers. NT, not tested; down arrows indicates titer was <1 IU/ml.

**Figure 3** Viral titers after transduction of various cells with HDAd-HIV-1. Cell lines were transduced in six-well format at increasing multiplicity of infections and vector supernatant was harvested 48 hours later and titered by limiting dilution on HOS targets, as assessed by flow cytometry. Open bars: 293, filled bars: Cos7, striped: HeLa cells. HDAd titers, as assessed by lacZ staining, were orders-of-magnitude lower (not shown).

**Figure 4** Production of HIV over time after transduction using HDAd-HIV-1. 293T cells were transduced in six-well format at multiplicity of infection=3,000 and culture supernatant harvested on consecutive days and titered by limiting dilution on HOS targets, as assessed by flow cytometry. Shown are results of two separate experiments. HDAd titers, as assessed by lacZ staining, were orders-of-magnitude lower (not shown).
washing of the cells the next day, supernatant was harvested at 72 hours post-transduction, and titered on HOS targets. As shown in Figure 2, eGFP+ titers approached $10^7$ IU/ml at the highest MOI (higher MOIs caused 293T cell death and reduced LV production). This eGFP titer reflected VSV G-pseudotyped LV transduction and not from leftover, contaminating HDAd since lacZ titers, which can only originate from the HDAd vector, were orders-of-magnitude lower (Figure 2 and see below).

We then tested the ability of several different cell lines to produce VSV G-pseudotyped LV after HDAd-HIV-1 transduction. COS7, HeLa, and 293 cells survived exposure to very high HDAd MOIs and resultant LV eGFP+ titers ranged from $10^6$ to $5 \times 10^7$ IU/ml from these three cell types (Figure 3). We then examined production from 293T cells using an MOI of 3,000 and harvesting supernatant every 24 hours. Recoverable eGFP+ titer was quite high from d2 to d4, exceeding $10^8$ IU/ml, and was quite reproducible (Figure 4). When scaled up to a 1 l cell factory of 293T cells, ~$10^{11}$ total IU of VSV G-pseudotyped LV were produced.

On the basis of these results with the second-generation HIV vector, we then proceeded to construct an HDAd encoding a third-generation HIV-based LV that may be suitable for clinical use. We initially focused upon a three part HIV vector system, with separate VSV G, HIV-TV, and HIV-PV components (refer to Figure 1 for schematics). The HIV-TV was pFG12, which encodes enhanced green fluorescence protein (eGFP), driven by the UbC promoter, and the HIV-PV encoded HIV gag, pol, tat, rev, driven by the CMV IE promoter. We initially made several HDAds with all three components in various orientations and configurations, and VSV G-pseudotyped LV end-point titers after plasmid transfection of 293T cells was ~$5 \times 10^5$ IU/ml. Unfortunately, in each case, the HDAd either failed to amplify in 116 cells or rearranged. We then made several HDAds with one and two components in various configurations, but the two component

![Figure 5](image-url) Characteristics of HDAd-dependent HIV production and transduction. (a) Production of HIV vector supernatants using all three HDAds (VSVG1, HIV-TV, and HIV-PV) using increasing MOIs of 625, 1,250, and 2,500. Vector supernatant was harvested 48 hours later and titered by limiting dilution on HOS targets, as assessed by flow cytometry. (b) As in (a), but total IU from a single six-well is shown. (C) Stable eGFP+ gene expression for several weeks after transduction of 5 x $10^5$ HOS cells using two different amounts of HIV vector supernatant, with solid line being 1.0 ml and dashed line 0.1 ml, in a total volume of 1 ml. (d) HIV vector supernatant was pre-incubated with anti-Ad5 sera at 1:100 dilution then added to HOS targets, with transduction efficiency assessed by flow cytometry at 48 hours. Wedge indicates decreasing amounts of HIV vector used of 1.0, 0.5, 0.25 µl. Filled bars: nonimmune sera, gray bars immune sera. (e) Ad5 vector encoding LacZ was preincubated with (+) or without (−) anti-Ad5 sera at 1:100 dilution and then titered on HOS cells, staining for lacZ at 48 hours. Relative Ad5 titer is shown. (f) HIV vector transduction of HOS targets was inhibited by increasing concentrations of the NNRTI efavirenz of 1.0, 0.5, 0.25 µl. Filled bars: nonimmune sera, gray bars immune sera. (e) Ad5 vector encoding LacZ was preincubated with (+) or without (−) anti-Ad5 sera at 1:100 dilution and then titered on HOS cells, staining for lacZ at 48 hours. Relative Ad5 titer is shown. (f) HIV vector transduction of HOS targets was inhibited by increasing concentrations of the NNRTI efavirenz of 1.0, 0.5, 0.25 µl. Filled bars: nonimmune sera, gray bars immune sera. (e) Ad5 vector encoding LacZ was preincubated with (+) or without (−) anti-Ad5 sera at 1:100 dilution and then titered on HOS cells, staining for lacZ at 48 hours. Relative Ad5 titer is shown. (f) HIV vector transduction of HOS targets was inhibited by increasing concentrations of the NNRTI efavirenz of 1.0, 0.5, 0.25 µl. Filled bars: nonimmune sera, gray bars immune sera. (g) Ad5 vector encoding LacZ was preincubated with (+) or without (−) anti-Ad5 sera at 1:100 dilution and then titered on HOS cells, staining for lacZ at 48 hours. Relative Ad5 titer is shown.
Production of high-titer HIV-based vectors using HDAd
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ones also failed to amplify or rearranged. During this process, we also attempted to remove all repeated sequences from the HDAd with the exception of the inverted terminal repeats and part of the HIV LTR, but these too could not be amplified.

One issue was that the Rev-response element (RRE), required for nuclear export of intron-containing viral mRNAs, was present in both the HIV-PV and HIV-TV and thus represented an unavoidable repeated sequence of a few hundred bp. Repeated sequences (other than the necessary inverted terminal repeats) may cause rearrangements during HDAd amplification. To circumvent this, we tested alternative Rev/RRE systems in the PV, from FIV, EIAV, and HIV-2. The PVs using the FIV and EIAV Rev/RREs gave relatively poor LV titers after plasmid transfection whereas the LV titer using the HIV-2 Rev/RRE system was similar to that of HIV-1; unfortunately that HDAd could not be amplified. We also replaced the PV RRE with a ×4 multimerized version of the constitutive transport element of the type D retroviruses, which is recognized by the cellular factor TAP (Mex67p).50–53 Although that HIV-PV also produced very good VSV G-pseudotyped LV titers after plasmid transfection of 293Ts, the corresponding HDAd rearranged during amplification, perhaps due to the directly repeated constitutive transport element.

Because of these difficulties and also to maximize flexibility, we decided to construct three separate HDAds, each with a single HIV vector component (see Figure 1 for schematics). Each HDAd easily amplified in 116 cells, with final stock concentrations of >5 × 10^{12} vp/ml, and was used in combination to transduce adherent 293T cells at increasing MOIs. After extensively washing the cells the next day, at 48 hours supernatant was collected and end-point LV titers using HOS targets ranged from 0.3 to 1.2 × 10^{7} IU/ml (Figure 5a), with total IU of 1.5 to 6.0 × 10^{7} IU (Figure 5b). As a quick test to determine whether the eGFP originated from contaminating HDAd versus HIV vector, we measured lacZ titers, since lacZ is encoded within the HDAd backbone, not within any HIV component. LacZ titers were 250 IU/ml (not shown), suggesting that the observed eGFP titers were true HIV titers. Resultant LV titers were absolutely dependent upon using all three components—omission of HDAd-VSV G or HDAd-HIV-PV resulted in LV titer of <100 IU in 0.1 ml, and it is likely that those observed titers were not LV titers but contaminating, left-over HDAd-FG12 titers. Omission of HDAd-FG12 resulted

Figure 6  Expression of HIV vector components after HDAd transduction. (a) Expression of eGFP in 293T producers, as measured by fold-increase in mean fluorescence intensity (MFI) of eGFP+ cells assessed by flow cytometry, 48 hours post-transduction or transfection. Wedge indicates increasing multiplicity of infection (MOI) of 312, 625, 1,250, and 2,500 of HDAd-HIV-TV used to transduce 293T cells; the three gray bars on the right are plasmid transfections, with “all three,” indicating use of pMEVSV G, pHIV-PV, and pFG12. (b) Immunoblot for eGFP after either plasmid transfection (left six lanes) or HIV-HDAd vector transduction (right eight lanes, as bracketed) of 293T cells. 48 hours post-transfection/transduction, cell lysates were subjected to immunoblotting using anti-eGFP antisera. Wedges indicate increasing MOI used, as in (a). eGFP was expressed only when TV was present. (c) Immunoblot for HIV proteins after either plasmid transfection (left six lanes) or HIV-HDAd vector transduction (right eight lanes, as bracketed) of 293T cells. Forty-eight hours post-transfection/transduction, cell lysates were subjected to immunoblotting using anti-HIV antisera. Wedges indicate increasing MOI used, as in (a). HIV proteins were expressed only when PV was included. (d) HIV Capsid (CA) release from cells after either plasmid transfection or HIV-HDAd vector transduction of 293T cells. Forty-eight hours post-transfection/transduction, cell culture supernatants were harvested and CA measured by ELISA.
cotransfection of 293T cells using FG12, HIV-PV, and VSV G resulted in no detectable LV titer (<1 eGFP+ cell in 0.1 ml) and 1,250. No plasmids were transfected in (b), with the different HDAds used indicated at top; MOI of HDAds used were 312, 625, and 1,250. No plasmids were transfected in (b).

Figure 7 Expression of VSV G correlates with HDAd-HIV titers. (a) VSV G expression after either plasmid transfection (left six lanes, bracketed) or HDAd transduction using indicated HDAds (right eight lanes, bracketed) of 293T cells. Cell lysates were electrophoresed and then immunoblotted for VSV G (arrow); wedges represent increasing multiplicity of infection (MOI) used of 312, 625, 1,250, and 2,500. VSV G was expressed only when plasmid or HDAd encoding VSV G was used. (b) Similar to (a), with the different HDAds used indicated at top; MOI of HDAds used were 312, 625, and 1,250. No plasmids were transfected in (b).

To confirm that the high titers with all three components were LV and not HDAd titers, after cellular transduction eGFP expression in HOS cells was stable for many weeks (Figure 5c). HDAd-HIV LV titers were also insensitive to neutralizing anti-AdS sera (Figure 5d), whereas Ad-eGFP titers were reduced to 90% (Figure 5e). Additionally, HDAd-HIV LV titers were reduced by >99% when targets were pretreated with increasing amounts of the HIV NNRTI efavirenz, as were LV titers after plasmid transient cotransfection, whereas Ad-eGFP titers were unaffected (Figure 5f). Not surprisingly, neither HDAd-HIV LV transduction, transduction of LV after plasmid transient cotransfection, nor AdS-LacZ transduction was inhibited by cell cycle arrest caused by mitomycin C (Figure 5g).

We next examined levels of expression of the various protein components of the third-generation HDAd-HIV vectors, in comparison to plasmid transfection. Mean fluorescence intensities of eGFP expression in the 293T producers after HDAd transduction of 293T cells using HDAd-VSVG3, HDAd-VSVG4, or HDAd-HIV-1. Cell lysates were electrophoresed and then immunoblotted for VSV G; wedges represent increasing multiplicity of infection (MOI) used of 312, 625, 1,250, and 2,500. Middle panel is shorter exposure of top panel. Bottom panel is immunoblot of β-tubulin as loading control. (a) Forty-eight hours after transduction of 293T cells in six-well format using HDAd-PV and HDAd-TV, along with either HDAd-VSVG1 (open bars), HDAd-VSVG3 (striped bars), or HDAd-VSVG4 (gray bars), or HDAd-HIV-1 alone (filled bars); resultant vector supernatant was used to infect HOS targets, with titer assessed by flow cytometry 48 hours postinfection; MOI used is indicated.

Figure 8 New HDAd-VSVG vectors lead to higher HIV titers. (a) VSV G expression after HDAd transduction of 293T cells using HDAd-VSVG3, HDAd-VSVG4, or HDAd-HIV-1. Cell lysates were electrophoresed and then immunoblotted for VSV G; wedges represent increasing multiplicity of infection (MOI) used of 312, 625, 1,250, and 2,500. MOI: 2,500, 1,250, 625. (b) Lentivector titer (IU/ml) × 107 IU/ml. Thus, total IU were

in no detectable LV titer (<1 eGFP+ cell in 0.1 ml). Of note, plasmid cotransfection of 293T cells using FG12, HIV-PV, and VSV G resulted in VSV G-pseudotyped LV titers of ~1 × 106 IU/ml, or 3 × 106 IU total in a single six-well.

To confirm that the high titers with all three components were LV and not HDAd titers, after cellular transduction eGFP expression in HOS cells was stable for many weeks (Figure 5c). HDAd-HIV LV titers were also insensitive to neutralizing anti-AdS sera (Figure 5d), whereas Ad-eGFP titers were reduced to 90% (Figure 5e). Additionally, HDAd-HIV LV titers were reduced by >99% when targets were pretreated with increasing amounts of the HIV NNRTI efavirenz, as were LV titers after plasmid transient cotransfection, whereas Ad-eGFP titers were unaffected (Figure 5f). Not surprisingly, neither HDAd-HIV LV transduction, transduction of LV after plasmid transient cotransfection, nor AdS-LacZ transduction was inhibited by cell cycle arrest caused by mitomycin C (Figure 5g).

We next examined levels of expression of the various protein components of the third-generation HDAd-HIV vectors, in comparison to plasmid transfection. Mean fluorescence intensities of eGFP expression in the 293T producers after HDAd-FG12 transduction was similar to those after plasmid transfection (Figure 6a). These near-equivalent levels of eGFP expression were confirmed by immunoblotting (Figure 6b). Cellular levels of HIV proteins, however, were much higher after HDAd-HIV-PV transduction of 293T cells compared to plasmid transfection (Figure 6c), as were secreted levels of HIV Capsid or CA (Figure 6d). In contrast, cell-associated levels of VSV G after HDAd-VSVG1 transduction were lower, compared to those after either plasmid transfection or transduction using the second-generation HDAd-HIV-1 (Figure 7a,b). This was also true of HDAd-VSVG2 (data not shown), in which the expression cassette was inserted into the HDAd in the opposite orientation (Figure 1). Because of this, we decided to construct two additional HDAd-VSVG plasmids, with the 2.7 kb SRe-VSV G CDNA cassette inserted into a different location of pα28E4LacZ, in both orientations (Figure 1). Both HDAds (HDAd-VSVG3 and HDAd-VSVG4) were amplified to titers of ~5 × 1012 vp/ml and used to transduce 293Ts at increasing MOIs, in conjunction with HDAd-FG12 and HDAd-HIV-PV. As shown in Figure 8a, expression of VSV G in the 293T producers was now roughly equivalent to that of cells transduced with HDAd-HIV-1. In addition, resultant LV titers using both of the new HDAd-VSVG vectors had increased to ~2 × 107 IU/ml, ~20-fold higher than that of plasmid transfection (and roughly half that of the resultant titers from HDAd-HIV-1 in this experiment) (Figure 8b). Because the refeeding supernatant volumes were greater by 1.6-fold (5 versus 3 ml), from a single six-well the total LV IU was 1.0 × 108, thus a conservative estimate of the increase in total number of IU was closer to 30-fold, compared to plasmid transfection of 293T cells (which would be ~3.0 × 106 total IU of LV produced from a single six-well).

In order to test scalability of adherent cells, ~108 293T cells on a single 15-cm plate were transduced with all three HDAds (HIV-PV, FG12, and VSVG3) at an MOI of ~1,000 and after extensive washing at 16 hours were refed in a total volume of 105 ml. At 48 hours, culture supernatant was harvested and resultant end-point eGFP titers on target HOS cells were ~1.5 × 107 IU/ml. Thus, total IU were

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DISCUSSION

We describe here a two-step method for production of third generation, high-titer VSV G-pseudotyped replication-defective LV supernatants based upon HIV. The first step is to produce high titer HDAds that separately encode the three components of HIV vector system, including VSV G, packaging vector, and transfer vector. Step two is to transduce 293T cells with the HDAds at high MOI and, after extensive washing to remove any leftover, contaminating HDAd, recover replication-defective, VSV G-pseudotyped HIV vector in the cell culture supernatants. The pseudotyped particles produced appear bona fide in that cellular transduction was absolutely dependent upon the presence of all LV components; however, it was inhibited by the antiretroviral efavirenz, it was insensitive to neutralizing adenoviral serum, it was independent of cell cycle status, and it was stable and long-lasting. HIV LV preparations did contain trace amounts of the HDAds. After cell transduction and extended passage, HIV CA was undetectable in culture supernatants, suggesting that preparations were not contaminated with replication-competent lentivirus, as would be expected given that the individual components were of third-generation with very limited shared homologous sequences.

Compared to standard calcium phosphate-mediated plasmid transfection of cells, using this two-step method overall LV IU production on a per cell basis per unit time was ~30-fold greater. This is likely due to the remarkable consistency of HDAd transduction of 293T cells compared to plasmid transfection, and also to higher levels of HIV structural and enzymatic protein expression, as the increased amount of secreted structural protein CA paralleled the number of IU. CA:IU ratio was remarkably similar to that of plasmid transfection. Titters achieved here were greatly superior compared to that previously reported for a similar system, likely because the HDAd vector titters obtained here were orders-of-magnitude higher and gene expression was not tetracycline-inducible.

Once the HDAds are available, production of HIV is facile since it only involves transduction of 293T cells, which we have shown is scalable and can be accomplished using adherent cells on stacked large plates (cell factory) or suspension cells in spinner flasks. In addition, once the HDAd stocks are produced, it is uncomplicated to generate more HDAd using HIV, without any additional vector transfection or manipulation. The HIV vector preparations contain a trace amount of HDAd, it is easily quantifiable and should pose no infectious or biohazardous risk since it cannot replicate, even in 293 cells. It is unlikely that any replication-competent adenovirus is present within these LV preparations, but absence of replication-competent adenovirus would need to be formally shown prior to any clinical use. Although we have not attempted to quantify the amounts of HIV present, the amount of contaminating HDAd is trace. Since this is a novel LV production system, in addition to standard test article assessment, in accordance with guidance documents issued by the Food and Drug Administration (FDA) regarding cell and gene therapy vector products (http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/default.htm), consultation with the FDA will be required in order to determine allowable amounts of contaminating HV and HDAd. While we know of no facile physical or chemical method to purify VSV G-pseudotyped LV from contaminating adenovirus, if necessary, any remaining adenovirus of any form could be neutralized or removed using antiserum against serotype 5 adenovirus (see Figure 5d,e). HIV could be excluded from being packaged by using cells expressing Cre recombinase, which occurs in 293-derived 116 cells. VSV G pseudotyped LV particles could be purified away from any adenovirus by heparin affinity chromatography, if necessary. This HDAd production method should be applicable to any of a number of medically relevant transgenes by appropriately modifying the HIV TV in a single cloning reaction, and it could also be used to prepare other types of replication-defective retroviral/LVs, including those based upon murine leukemia and equine infectious anemia viruses.

It should be straightforward to adapt this method for GMP scale-up for clinical trial use. Although we recognize that production of the multiple HDAds used may require good manufacturing practices and would be more expensive than amplifying plasmids, because of the ~20-fold increase in LV titer and ~30-fold increase in overall infectious units, this production method may be more cost-effective and likely more reproducible than standard calcium phosphate cotransfection of adherent 293T cells. How it compares in terms of safety, ease of production, and overall cost to optimized bioreactor systems of suspension 293 cells will require further study.

MATERIALS AND METHODS

Cells and plasmids

293-based cells were propagated and transfected using calcium phosphate method as described. Adherent 293T cells were propagated in Dulbecco’s modified Eagle’s medium as described, with the addition of 20 μg/ml ciprofloxacin. Suspension 293Ts were adapted to growth in spinner flasks over the course of 2–3 weeks in the same medium and then maintained in mid-log phase at 1–5 × 10⁶ cells/ml. For all HDAd constructions, the base plasmid was pA28E4LacZ (~31 kb in size). HDAd-VSVG1 and HDAd-VSVG2 were both constructed by inserting a 2.7 kb Sphr-VSV G cDNA cassette into the unique Nhe I site of the unique SnaB1 site of pΔ28E4LacZ (~31 kb in size). HDAd-VSVG4 were similarly constructed by inserting the same cassette into the unique Nhe I site (opposite orientations) whereas HDAd-VSVG3 and HDAd-VSVG4 were similarly constructed by inserting the same cassette into the unique SnaB1 site. HDAd-HIV-PV was constructed by inserting a derivative of HIV-PV™ into the unique SnaB1 site; HDAd-HIV-TV by inserting a 6 kb MluI+Pme1 DNA fragment from HIV transfer vector pFG12 (ref. 58) into the unique SnaB1 site (termed HDAd-FG12); HDAd-HIV-1 by inserting both the 2.7 kb Sphr-VSV G cDNA cassette into an Xba 1 site and an 11.0 kb HIV-CMV-lacZ eGFP self-inactivating construct into the unique SnaB1 site of a 20 kb derivative of pA28E4LacZ. Correct structure of all plasmid constructions were confirmed by both restriction endonuclease digests and functional analyses, by both immunoblotting and titering on target cells after plasmid DNA transfection of 293T cells, and DNA sequencing where necessary.

HDAd and HIV vector production

HDAds were amplified using HIV first on plates, then in spinner flasks, and purified using two sequential CsCl gradients as described. Absence of replication-competent HDAd was determined by restriction endonuclease digest of purified genomic DNA; presence of contaminating levels of HV was determined as described; presence of RCL was determined by passaging...
transduced cells for several weeks and testing for presence of HIV Capsid (CA) in culture supernatant. To produce HIV vector supernatant from adherent cells, poly-L-lysine coated plates were used. Increasing amounts of HDAd were added overnight to confluent 293T cells, and cells were extensively washed the next day. Typically, vector supernatant was harvested 48–60 hours later and titered by end-point dilution on HOS cell targets, with titering efficiency based on flow cytometric analysis of eGFP+ cells 48–72 hours later (at least 10,000 events collected). LaCZ staining was performed on formaldehyde-glutaraldehyde fixed HOS cells, using X-gal as a substrate, as described.30 Protein expression was analyzed by immunoblotting of RIPA cell lysates after sodium dodecyl sulfate–polyacrylamide gel electrophoresis, using anti-eGFP (Clontech, Mountain View, CA), anti-VSV G (clone P5D4) cell lysates after sodium dodecyl sulfate–polyacrylamide gel electrophoresis, using anti-eGFP (Clontech, Mountain View, CA), anti-VSV G (clone P5D4) as primary antibodies and appropriate, species-matched horseradish peroxidase–conjugated secondary antibodies (from SIGMA-Aldrich), with light visualization using enhanced chemiluminescence (GE Healthcare Biosciences, Pittsburgh, PA) and autoradiographic film (Carestream Health, Rochester, NY). Vector supernatant HIV CA levels were quantified by enzyme-linked immunosorbent assay as described.30

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