A New Hybrid System Capable of Efficient Lentiviral Vector Production and Stable Gene Transfer Mediated by a Single Helper-Dependent Adenoviral Vector

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Received 9 September 2002/Accepted 3 December 2002

To achieve efficient and sustained gene expression, we developed a new lentivirus/adenovirus hybrid vector (LA vector) that encodes sequences required for production of a human immunodeficiency virus-based lentiviral vector (i.e., a lentiviral vector, a gag/pol/rev expression cassette, a tetracycline-inducible envelope cassette, and the tetracycline-inducible transcriptional activator cassette) in a single helper-dependent adenovirus vector backbone. Via either transfection or infection, human cell lines transduced with the LA vector produced a lentiviral vector in a doxycycline-dependent manner at titers up to $10^5$ to $10^6$ green fluorescent protein transducing units per ml, which are comparable to the titers obtained by conventional multiple plasmid transfection methods. Efficient spread and persistent expression of the transgene were observed in cells maintained in long-term culture that had been infected with the LA vector. Furthermore, when cocultured with adherent cells infected with the LA vector, the human T-cell leukemia cell line was successfully transduced with a marker gene. This LA vector possesses the advantages of efficient gene transfer from an adenoviral vector and stable integration from a lentiviral vector; therefore, it might have potential for a variety of gene therapy applications.

Efficient gene delivery and sustained gene expression are required for successful human gene therapy (4, 63). Although viral vectors are considered the most efficient vehicles for gene transfer, currently available viral vectors have not fully achieved these two requirements. Retroviral vectors (RVs) and lentiviral vectors (LVs) can integrate into host chromosomes, allowing long-term gene expression, but are still limited to in vitro or ex vivo gene delivery because of their relatively low titers and restricted insertional capacities (39, 58, 62, 64, 65). Adeno-associated virus vectors can be prepared at high titers and can integrate into a chromosome of nondividing cells; however, their small cloning capacities (up to 5 kb) curtail wider applications (29, 36). Adenoviral vectors (AdVs) have particular advantages for use as in vivo gene transfer vehicles, including a broad host range, the ability to infect both dividing and nondividing cells, and ease of high-titer purification (5, 29, 66). However, AdVs rarely integrate into the host genome (20), so the proportion of transduced cells decreases with each cell division. It is therefore desirable to develop a vector that can both integrate into the host genome to achieve sustained gene expression and be easily grown to titers sufficient for clinical use.

Recently, development of several AdV/RV hybrid vectors has been reported (6, 9, 12, 15, 68). The purpose of developing these hybrid vectors was to overcome the limitations of transient retention of an AdV in infected cells by introducing integrating machinery mediated by RVs. Two AdVs with E1 deleted, one carrying retroviral genes and the other carrying an RV construct, were used for coinfection to efficiently produce fully assembled and functional RVs in vitro (12, 68) and in vivo (9, 15) by converting initially infected cells into retrovirus-producing cells. These AdV/RVs take advantage of the favorable aspects of both vector systems, whereby the nonintegrative high-titer AdV is able to deliver DNA that becomes integrated into neighboring cells by the RV intermediary. However, the efficacy of this viral vector system was low and substantially limited because neighboring cells must be dividing for the second stage of infection by the RV to occur. To overcome this limitation, it would be desirable to use an LV instead of an RV. Because both adenovirus and lentivirus can infect both dividing and nondividing cells (40, 49, 55), this type of hybrid vector should allow efficient integration of a transgene into nondividing cells in vivo.

Here we report development of a lentivirus/adenovirus hybrid vector (LA vector), in which a helper-dependent AdV (HDAEv) (28, 34, 46, 53) was used as a carrier of the LV production machinery. By using the large cloning capacity of HDAEv, both lentiviral genes and a lentivirus transfer vector construct were contained within a single AdV carrier as an all-in-one vector, thereby enhancing the efficiency of second-stage LV production. The LA vector transduces cells by a two-stage mechanism similar to that of AdV/RVs but additionally transduces both dividing and nondividing cells. Furthermore, an HDAEv with all adenoviral structural genes deleted offers the potential benefits of reduced cytotoxicity and reduced cellular immune response against vector-transduced cells in vivo, which may prolong second-stage LV production. As a safety precaution, we introduced a tetracycline (TET)-
inducible transcriptional regulator (18) into our vector to produce LVs that can be regulated.

In this study, we show the abilities of the LA vector to produce LVs in a TET-inducible manner by both transfection and infection in various human cell lines and to increase the number of transduced cells by continuous production of LVs in long-term culture. We also demonstrate that coculture with adherent cells infected with the LA vector is an efficient method to transduce a human T-cell leukemia cell line. The potential applications of the vector as a new gene delivery tool for gene therapy are discussed.

MATERIALS AND METHODS

Cells. Cell lines including 293T (11), the HeLa human cervical adenocarcinoma cell line, and the Gli36 human glioma cell line (56) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal calf serum (FCS) (Omega, Tanzana, Calif.). Hep3B and HepG2 human hepatocellular carcinoma cells were cultured in Eagle’s minimum essential medium supplemented with 10% FCS, 1 mM sodium pyruvate, and nonessential amino acids. L87 and L88 human bone marrow stroma cells (59) were cultured in Freederick’s modified Dulbecco’s medium supplemented with 15% FCS, 50 μM β-mercaptoethanol, and 1 mM hydrocortisone. The CEM T-lymphoblastoid cell line was cultured in RPMI 1640 supplemented with 10% FCS. Human peripheral blood mononuclear cells were obtained from the UCLA Center for AIDS Research. For TET-inducible production of LV, TET-approved FCS was used (Clontech, Palo Alto, Calif.).

Construction of vectors. For a human immunodeficiency virus (HIV)-based LV, we used a self-inactivating lentiviral transfer vector, pRRLCMVGFPSin, which encodes the enhanced green fluorescent protein (GFP) gene driven by the human cytomegalovirus (CMV) promoter (13). A DNA fragment of the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) (69) was cloned into pRRLCMVGFPSin downstream of the GFP expression cassette to generate pSINWcG. A central purpyrimidine tract and termination sequences cloned by PCR were then inserted into pSINWcG upstream of the GFP expression cassette to generate pSINPWcG, as previously described by Follenzi et al. (16).

To regulate LV production, a TET-inducible system was introduced into an expression cassette for use of the vesicular stomatitis virus G envelope glycoprotein (VSV-G) for pseudotyping LV (2, 40, 49). To construct a TET-inducible envelope plasmid (pTRE-VSV-G), a VSV-G fragment from pMD.G (13, 35, 70) was subcloned into pRevTRE (Clontech), which has a TRE-responsive element (TRE). A DNA fragment encoding an HIV type 1 (HIV-1) gag/pol/rev expression cassette with the CMV promoter was isolated from pCMV.ΔARV.93 (a multiply deleted HIV-1 packaging plasmid) (70) and subcloned into pTRE-VSV-G to create gpGR TRE-VSV-G.

An HDAdV plasmid, pSTK120 (52), and pSTK68, which is a parental plasmid of pSTK120, contain inverted terminal repeats (ITRs; replication origin of viral DNA) and the packaging signal of human adenovirus type 5. To construct a complete LA vector plasmid, a red fluorescent protein (RFP) expression cassette from pDsRed N1 (Clontech) was cloned into pSTK68 to generate pSK-R. A reverse TET-controlled transactivator expression cassette (rTATA) from pRevTetOn (Clontech) was subcloned into pSK-R to create pSK-RT. The gag/pol/rev and VSV-G expression cassette of pGPR TRE-VSV-G were subcloned into pSK-RT to produce pSK-RT. Finally, an LV fragment from pSINWcG was subcloned into pSK-RT, forming the complete LA vector plasmid, pLAcG, which is approximately 31.1 kb in size as a plasmid and 28.2 kb in size as a linear adenovirus genome. For a control HDAdV that expresses GFP, a GFP expression cassette of pEGFP N1 (Clontech) was subcloned into pSTK120 to create pHAdG. A detailed description of this subcloning can be provided by the authors on request.

Generation and titration of an LV by transfection. HIV-based LV stocks were generated by transient plasmid transfection into 293T cells as described previously (51). At 8 h posttransfection of 293T cells with plasmids by the calcium phosphate method, the medium was replaced, and at 36 h posttransfection, the virus-containing medium was harvested, centrifuged, and filtered through a 0.45-μm-pore-size filter. For titration of an LA vector, 105 293T cells in a six-well plate were transfected with 8 μg of pLAcG and cultured in the presence of various concentrations of dодеxycline (DOX), a TET analogue. For a control LV, LVcG, 2 μg of pSINPWcG, 3 μg of pCMV.ΔARV.93, and 1 μg of pMD.G were transfected into 293T cells. The vectors were titrated on 293 cells in the presence of Polybrene (4 μg/ml) for GFP expression, using a FACScalibur flow cytometer (Becton Dickinson, San Jose, Calif.), on day 2 postinfection, with the results being defined as transducing units per milliliter (TU/ml). The amount of p24 viral core antigen in the medium was measured by an enzyme-linked immunosorbent assay (ELISA).

Generation of helper-dependent adenovirus. The LA vector and a control HDAdV, HDAdGc, were prepared by using the Cre/loxP helper virus system, as previously described (41, 46, 52). Briefly, linearized vector constructs were transfected into 293Cre4 cells in the presence of helper AdLCl86 (adenovirus with E1 deleted), propagated serially, and purified by CsCl ultracentrifugation. The GFP titers of the LA vector on 293T cells was 4.1 × 107 TU/ml. The vector stock contained 0.71% helper virus contamination as determined by Southern hybridization using a probe for the adenoviral packaging signal (data not shown).

Lentiviral production after infection with the LA vector virus. To confirm production of LV in cells infected by the LA vector, 4 × 105 cells were infected with LAcG at various multiplicities of infection (MOIs). At 48 h postinfection, the infected cells were washed three times with phosphate-buffered saline (PBS) and incubated in growth medium in the presence or absence of DOX (1 μg/ml). At 48 h postinfection, the medium was harvested and used for titration on 293 cells.

To test inhibitors of lentiviral and adenoviral infection, 293 cells were infected with the virus supernatant in the presence or absence of zidovudine (AZT; 5 μM; Sigma), an anti-VSV-G monoclonal antibody (32), or rabbit anti-adenovirus type 5 (Ad5) serum.

To investigate the kinetics of LA vector production after infection, 4 × 105 cells were infected with LAcG at an MOI of 1 or 10 in six-well plates. The medium was collected at different time points and titrated on 293 cells as described above.

The absence of replication-competent HIV-based virus was assayed by monitoring the p24 antigen in the culture medium of human peripheral blood mononuclear cells by ELISA for 6 weeks after infection with each vector batch (13, 35, 44, 70).

Long-term culture. Gli36 cells (2 × 105) were infected with the LA vector, LAcG, at an MOI of 10 and incubated in the presence of AZT. The following day, the cells were split, mixed with uninfected Gli36 cells at a ratio of 1:4, and cultivated in the presence of DOX, with or without AZT, on a 10-cm-diameter dish. The cells were split at a ratio of 1:20 once a week, and expression of GFP was examined by fluorescence-activated cell sorter (FACS). At each passage, DNA was extracted from a portion of the cells and analyzed for proviral integration by Southern hybridization.

Coculture of LA vector-transduced cells with human hematopoietic cells. Gli36 cells (4 × 105) were infected with the LA vector, LAcG, at an MOI of 10 on a six-well plate. At 4 h postinfection, the infected cells were washed three times with PBS and cultivated in the presence or absence of DOX and AZT. At 24 h postinfection, 105 CEM cells were added for cocultivation. At 3 days postinfection, nonadherent cells were transferred into a six-well plate and incubated for three days. Additional inductive processes do not reduce the number of detached Gli36 cells in the nonadherent cell fraction and also enhances GFP expression from the LV-infected CEM cells. Finally, the cells were harvested, treated with an anti-human CD4 monoclonal antibody conjugated with allophycocyanin (eBioscience, San Diego, Calif.), and analyzed by FACS.

RESULTS

Construction and functional testing of the LA vector. An LA vector plasmid, pLAcG, was created by subcloning all of the expression cassettes required for production of LV into a single HDAdV plasmid encoding only the ITRs and the packaging signal as adenoviral sequences (Fig. 1A). The LA vector encodes a third-generation self-inactivating HIV-1 vector and an expression cassette of only three of the nine HIV genes (gag, pol, and rev). The VSV-G expression cassette was placed under control of the TET-inducible promoter so that production of LV could be regulated by DOX. The LA vector has two marker genes, GFP in the LV and RFP in the HDAdV backbone, to distinguish between cells transduced with the LA vector alone and those with secondarily produced LV.

To test inducible and functional LV production by the LA vector, 293T cells were transfected with the LA vector plasmid, pLAcG, and control plasmids and incubated with various con-
centrations of DOX. Production of LV by a single round of pLAcG transfection was dose dependent and reached a maximum titer \((1.5 \times 10^6 \text{ TU/ml})\) at a DOX concentration of 1 \(\mu\text{g/ml}\) (Fig. 2). This titer was at a level comparable to that of the positive control \((1.1 \times 10^6 \text{ TU/ml})\) obtained by using the conventional three-plasmid cotransfection protocol (Fig. 2) \((13, 40, 70)\). Production of LV was independent of the levels of p24, which are expressed constitutively from the vector, suggesting that production of LV depends on the amount of VSV-G envelope, whose expression is regulated by the TET-inducible system. The 293 cells infected with the resulting supernatant were positive for GFP only, although the transfected 293T cells were positive for both GFP and RFP (data not shown). These data confirmed that the LA vector plasmid was functional in its ability to produce LV in a DOX-dependent manner.

**Infection of cells with LA vector results in production of functional LV.** We next investigated whether the LA vector can produce infectious LV particles after infection. As LV producers, Gli36 cells were infected with the LA vector at an MOI of 1 or 10 in the absence or presence of DOX. 293 cells infected with supernatant from the LA vector-infected Gli36 cells showed expression of GFP in a DOX- and MOI-dependent manner (Fig. 3A).

To differentiate the LV-infected cells from the LA-infected

**FIG. 1.** Outline for the LA vector system. (A) Schematic structure of the LA vector. An LA vector is an HDAdV encoding expression cassettes for production of an LV. The LA vector has two ITRs (Ad-ITR) and the packaging signal (Ad-pac) of human adenovirus type 5 and encodes five gene expression cassettes: (i) a self-inactivating LV that contains the central polypurine tract, the WPRE, and the enhanced GFP gene driven by the human CMV promoter as a marker; (ii) gag/pol/rev from HIV-1; (iii) the VSV-G envelope driven by the TRE for pseudotyping of the lentivirus; (iv) the reverse TET-controlled transactivator (rtTA); and (v) the RFP as a marker of the adenoviral backbone. (B) Two-stage transduction with the LA vector. The LA vector infects the initial target cells efficiently as an AdV and produces an LV in situ in the presence of DOX. The LV then infects surrounding secondary target cells and integrates into chromosomes for stable gene expression.

**FIG. 2.** Production of LV following LA vector transfection. An LA vector plasmid, pLAcG, and control plasmids were transfected into 293T cells by the calcium phosphate coprecipitation method. Two days posttransfection, the medium was collected for titration of GFP expression on 293 cells and for quantification of p24 expression by ELISA. The titers obtained by transfection of pLAcG were comparable to those obtained by conventional cotransfection of control plasmids (pSINPWcG, pCMVΔR8.93, and pMD.G). Data shown are average titers and standard deviations from three experiments performed in triplicate.
cells, we attempted to compensate RFP and GFP signals by FACS (Fig. 3B). Among the LA-infected Gli36 cells in the presence of AZT, most GFP-positive cells were also RFP positive. However, the LA-infected Gli36 cells in the presence of DOX consisted of two populations positive for GFP, GFP/RFP double-positive and GFP single-positive cells, suggesting LV spread from the LA-infected cells. 293 cells transduced with the LV-containing supernatant from the LA-infected cells were GFP single positive, suggesting that the observed GFP expression was caused by LV infection rather than contaminating LA in the supernatant.

To confirm that the observed GFP expression was due to LV infection, 293 cells were infected with supernatant from the LA vector in the presence of reverse transcriptase inhibitor, AZT, an anti-VSV-G monoclonal antibody, or anti-Ad5 serum (Fig. 4). As in the case of control LV infection, treatment of the supernatant from the LA-infected cells with AZT or an anti-VSV-G antibody but not an anti-Ad5 serum markedly eliminated GFP-positive cells (Fig. 4). These data confirmed that GFP expression in secondary target cells was mediated by LV but not by AdV or pseudotransduction of the overexpressed GFP protein.

Several human cell lines were screened for their ability to produce LV following infection by the LA vector. The Gli36, Hep3B, HepG2, HeLa, L87, and L88 cell lines were transduced with LAcG at an MOI of 1 or 10. Of these cell lines, Gli36 and Hep3B produced the highest titers of LV (2.7 × 10^5 to 1.3 × 10^6 and 5.0 × 10^5 to 6.7 × 10^5 TU/ml, respectively) (Fig. 5), which correlated with the high levels of p24 production (236 and 316 ng/ml, respectively). In all of the cell lines tested, LV production was DOX dependent, although the titers of LV produced by two cell lines, Gli36 and L88, infected with the LA vector at an MOI of 10 were relatively high, even in the absence of DOX. Taken together, these findings indicate that the LA vector produced infectious LV particles from infected cells after DOX induction, and the yield was dependent upon the MOI and the target cell type.

**Kinetics of lentiviral production after LA vector infection.**
To determine how long cells can produce LV following infection by the LA vector. The Gli36, Hep3B, HepG2, HeLa, L87, and L88 cell lines were transduced with LAcG at an MOI of 1 or 10. These cell lines, Gli36 and Hep3B produced the highest titers of LV (2.7 × 10^5 to 1.3 × 10^6 and 5.0 × 10^5 to 6.7 × 10^5 TU/ml, respectively) (Fig. 5), which correlated with the high levels of p24 production (236 and 316 ng/ml, respectively). In all of the cell lines tested, LV production was DOX dependent, although the titers of LV produced by two cell lines, Gli36 and L88, infected with the LA vector at an MOI of 10 were relatively high, even in the absence of DOX. Taken together, these findings indicate that the LA vector produced infectious LV particles from infected cells after DOX induction, and the yield was dependent upon the MOI and the target cell type.
creased (Fig. 6). Even at day 7, LV production was still observed at a titer of $3.5 \times 10^4$ (detection limit, $10^4$ TU/ml). Thus, LA-infected Gli36 cells continued to sustain LV production for at least 7 days postinfection.

**In vitro spread of LV-transduced cells following LA infection.** The spread of lentivirus in the long-term culture of infected Gli36 cells was analyzed. In the LA-infected AZT-untreated sample [LA-infected/AZT(−)], the percentage of GFP-positive cells increased more than 2.3-fold (from 15.3 to 35.4%) during the first week and remained at higher levels throughout the study (Fig. 7). Among the AZT-treated cells [LA-infected/AZT(+)] and cells infected with the control HDAdV, HDAdcG, the number of GFP-positive cells significantly decreased over time. At 6 weeks postinfection, the percentage of GFP-positive cells was still 30% in the LA-infected/AZT(−) sample but less than 2% in the LA-infected/AZT(+) sample and the control HDAdV sample. Southern hybridization using high-molecular-weight DNA extracted from the cells at week 6 confirmed proviral integration and demonstrated that GFP expression correlated with the copy numbers of the integrated GFP transgene (data not shown), indicating that the spread of GFP-positive cells in the LA-infected/AZT(−) sample was due to production of LV by the LA vector.

**Transduction of human hematopoietic cells via LA vector-transduced adherent cells in coculture.** To investigate a potential application of the LA vector, we attempted to transduce the human T-cell leukemia cell line (CEM) in coculture with adherent cells infected by the LA vector. We expected highly efficient transduction of CEM cells by cocultivation, since previous studies using RVs have shown that hematopoietic progenitor cells were infected more efficiently in coculture with virus-producing cells than via infection with the virus-containing supernatant (31, 54).

In this experiment, Gli36 cells infected with the LA vector were used as LV producers and CEM cells were used as targets. To confirm that the nonadherent cell population in the Gli36-CEM coculture consisted of CEM cells (CD4 positive)
but not detached Gli36 cells (CD4 negative), harvested cells were stained with anti-human CD4-APC antibody. CD4-positive cells were then gated and analyzed for RFP and GFP expression by FACS. More than 60% (63.7%) of the human CD4-positive CEM cells became GFP positive after 3 days in coculture with Gli36 cells infected with the LA vector in the presence of DOX. GFP expression was also observed in the absence of DOX (29.1%). That the transduction of CEM cells was mediated by LV is supported by the following: (i) the percentage of GFP-positive cells was reduced in the presence of AZT (below 1%), and (ii) no RFP expression was observed in CD4-positive CEM cells (data not shown). The titer of LV in the medium on day 3 was 5.0 \times 10^4 TU/ml and the estimated titer based on the transduced CEM cells in the coculture (6.4 \times 10^4 TU/ml) suggests that transduction in this coculture system is more efficient than infection with supernatant containing LV, as previously reported (31, 54).

**DISCUSSION**

To achieve efficient and sustained gene expression, we developed an LA vector system and demonstrated that it is able to produce LV from a variety of infected cells and to stably deliver a marker gene into neighboring cells, which is mediated by the LV machinery. Efficient two-stage transduction (Fig. 1B) in vitro was shown by spread of LV-transduced cells in Gli36 cells and by transduction of nonadherent CEM cells in coculture with adherent cells transduced with the LA vector.

AdVs have been successfully used in vivo to transduce postmitotic cells such as hepatocytes (24, 33, 38, 57), myotubes (48), and neurons (3). However, gene expression from an AdV is transient due to immune rejection and lack of integration. Immune rejection is caused mainly by leaky expression of viral genes retained in the vector, resulting in a cytotoxic T lymphocyte (CTL)-mediated immune response by the host against the virus-transduced cells (66). The HDAdV system can overcome this CTL response and achieve long-term expression (37, 42); however, even an HDAdV cannot overcome the limited duration of expression due to dilution of viral DNA as cells divide. Only through chromosomal integration or episomal replication of vector DNA can permanent expression be achieved, which is necessary for treatment of hereditary diseases.

Recently, several AdV/RV hybrid vectors based on AdVs with E1 deleted have been reported (6, 9, 12, 15, 68), and the in vivo efficacy of these hybrid vectors was investigated by using mouse models. In one study, inoculation of AdV/RVs, one encoding an RV with the GFP and the other encoding gag/pol and the 4070A amphotropic env, into a human ovarian cancer cell line, SKOV3, in mouse xenografts achieved stable transduction in 10 to 15% of cells at 16 days postinfection (15). In another study, a set of four AdV/RVs encoding gag/pol, VSV-G, an RV expressing the neomycin resistance gene, and the TET-inducible transcriptional activator to control expression of gag/pol and env were coadministrated intratumorally into human A375 melanoma xenografts in nude mice in vivo (9). The frequency of G418 colonies recovered from tumors transduced with all four hybrid vectors increased with time to as much as 7.2% 4 weeks after administration. In these in vivo situations, the LA vector system would have more advantages than currently available AdV/RVs. First, the LA vector system can infect nondividing cells both in the adenoviral stage and in the second LV stage, thereby enhancing the transduction efficiency of nondividing cells. Second, compared with the currently available AdV/RVs based on multiple AdVs with E1 deleted, the LA vector is an all-in-one vector, which precludes the need to simultaneously deliver multiple vectors that encode the structural genomic components into each target cell. Based on this multimodal design feature, this LA vector should mediate more efficient second-stage LV production in vivo. The increased efficiency of the hybrid system, coupled with an input vector that is an HDAdV that elicits the host CTL response, should result in reduced rates of immune clearance of LV-producing cells in vivo, although the CTL response against other transgene products, including LV structural proteins, remains. Therefore, the LA vector features several key advantages over currently available viral vectors for efficient and stable in vivo gene therapy.

One potential target for LA vector-based in vivo gene therapy is the liver. Most AdVs accumulate in the liver after intravenous injection (22, 27, 30) and transduce hepatocytes efficiently (nearly 100%) (33). In contrast, due to limited titers, direct injection of LVs in vivo has yielded limited transduction efficiencies (less than 10%) (25, 44, 60, 61) in the absence of stimulation by growth factors (7, 43, 47) or hepatectomy (45). Therefore, injection with the LA vector in the AdV stage would be greatly beneficial for transduction of hepatocytes as the first target cells in vivo. In addition, continuous production of LV in situ should contribute to efficient and permanent transduction of neighboring hepatocytes by second-stage LV-mediated integration. The data from this study showing that liver-derived Hep3B cells can efficiently produce LV (Fig. 5) suggest a potential application of the LA vector for hepatic gene therapy.

For our LA vector system, an HDAdV was used because of its large cloning capacity (up to 35 kb) (28). Therefore, five gene expression cassettes could be inserted into the same AdV carrier as an all-in-one vector (Fig. 1A). As described in this study, all of these cassettes were expressed at functional levels to produce infectious LVs, which demonstrates for the first time that a single HDAdV can deliver as many as five genes. As a safety precaution, a TET-inducible system was introduced into the LA vector to regulate LV production. The leaky production of LV observed in this study is known to be one of the drawbacks of the TET-regulatable system, as reported previously (1, 17, 21). High levels of background production of LV at a high MOI in this study coincided with the results of a previous study of an AdV/RV hybrid vector (9). To overcome this drawback, a different system that can be more tightly regulated in vivo as well as in vitro experiments might be introduced (e.g., rapamycin and mifepristone) (8, 50, 67). Incorporation of insulator sequences might also help to protect the regulatable cassette from the effects of other enhancers and/or promoters on the vector and thus reduce leaky expression of the lentiviral proteins (10, 23).

HIV-based LV stocks with titers of 10^6 to 10^7 TU/ml can be obtained through conventional transient cotransfection of three or four plasmids into 293T cells (13, 40, 70). The titers can be increased to 10^9 TU/ml following concentration, but
this still might not be sufficient for efficient in vivo transduction into some target tissues. Attempts to develop LV-packaging cell lines have been reported (14, 26), although the titers of LV preparations are still limited. As shown in this study, transfection of the LA vector plasmid can produce LVs at levels comparable to the positive control of conventional three-plasmid cotransfections (Fig. 2). Therefore, our all-in-one LA vector plasmid could be used as a new method to produce an LV as a single plasmid transfection. Moreover, because an LA vector is an AdV that can infect many adherent cell types efficiently, it was possible to screen a variety of cell lines for efficient production of LV. For example, among the six cell lines tested, Gli36 and Hep3B produced the highest titers of LV. By identifying more cell lines that can produce LVs, the LA vector might be utilized as an alternative tool to produce high-titer LV preparations or to prepare virus-packaging cell lines.

In conclusion, this new LA vector has great potential in a variety of applications of in vivo gene transfer to achieve efficient gene delivery and sustained gene expression via in situ generation of an LV.

ACKNOWLEDGMENTS

We thank Merck Research Laboratories for the HDAdV system; Didier Trono for lentiviral constructs; Stefan Kochanek for the STK plasmids; Yoshinaga Suoki for the Gli36 cells; Leo Lefrancois for an anti-VSV-G antibody; Tom Hope for the WPRE; David Rawlings for the L87 and L88 cell lines and helpful discussions; Dong Sung An for lentiviral constructs; Stefan Kochanek for the STK plasmids; Didier Trono for lentiviral constructs; Stefan Kochanek for the STK plasmids; and helps of critical discussion; Koki Morizono for technical assistance and discussion; Graeme Dougherty and Bill McBride for allowing us to use the FACScalibur flow cytometer; Oliver Dorigo, Lily Wu, and Jerome Zack for critical reading of the manuscript; Arnie Berk for the anti-AdV serum and critical discussion; and Wendy Aft for preparation of the manuscript. We also thank the members of our laboratory, Katie Huang, Michael Balamotis, Felicia Hernandez, and Arturo Diaz, for helpful discussions.

This work was supported by the UCLA Gene Medicine Program.

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