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Factors Influencing the Titer and Infectivity of Lentiviral Vectors

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ABSTRACT

Lentiviral vectors have undergone several generations of design improvement to enhance their biosafety and expression characteristics, and have been approved for use in human clinical studies. Most preclinical studies with these vectors have employed easily assayed marker genes for the purpose of determining vector titers and transduction efficiencies. Naturally, the adaptation of these vector systems to clinical use will increasingly involve the transfer of genes whose products may not be easily measured, meaning that the determination of vector titer will be more complicated. One method for determining vector titer that can be universally employed on all human immunodeficiency virus type 1-based lentiviral vector supernatants involves the measurement of Gag (p24) protein concentration in vector supernatants by immunoassay. We have studied the effects that manipulation of several variables involved in vector design and production by transient transfection have on vector titer and infectivity. We have determined that manipulation of the amount of transfer vector, packaging, and envelope plasmids used to transfect the packaging cells does not alter vector infectivity, but does influence vector titer. We also found that modifications to the transfer vector construct, such as replacing the internal promoter or transgene, do not generally alter vector infectivity, whereas inclusion of the central polypurine tract in the transfer vector increases vector infectivity on HEK293 cells and human umbilical cord blood CD34$^+$ hematopoietic progenitor cells (HPCs). The infectivities of vector supernatants can also be increased by harvesting at early time points after the initiation of vector production, collection in serum-free medium, and concentration by ultracentrifugation. For the transduction of CD34$^+$ HPCs, we found that the simplest method of increasing vector infectivity is to pseudotype vector particles with the RD114 envelope instead of vesicular stomatitis virus G glycoprotein (VSV-G).

OVERVIEW SUMMARY

We have analyzed several variables involved in the design and production of lentiviral vectors for the effect they have on vector titer and infectivity. Wide variability in the amounts of input transfer vector, packaging, and envelope plasmids, as well as changes to the transfer vector construct, are tolerated with no significant change in vector infectivity. We found that inclusion of the central polypurine tract, concentration by ultracentrifugation, and collection in serum-free medium increase the infectivity of vectors on HEK293 cells and human umbilical cord blood CD34$^+$ HPCs. In addition, use of the RD114 envelope instead of VSV-G dramatically increases the infectivity of lentiviral vectors when they are used to transduce CD34$^+$ HPCs, but the titers and infectivities of RD114-pseudotyped vectors as determined on HEK293 cells is misleading, because of poor performance of the RD114 envelope on these cells.

INTRODUCTION

LENTIVIRAL VECTORS (lentivectors) have been the subject of many years of preclinical study, and have been approved for therapeutic and cell-marking studies in humans. As these vectors enter clinical use, it will become increasingly common for investigators to use them to deliver genes whose products are not as easily measured as those of marker genes that have

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been used in most studies heretofore. This will create some difficulties with respect to the measurement of vector titers, because this has generally been performed by measuring marker transgene expression. Titering by analysis of gene expression may provide the most useful data for predicting the performance of vector supernatants in various cell types; however, two studies have shown that this method likely underestimates the number of vector genomes transferred into target cells, because variability in gene expression will lead some marked cells to be counted as unmarked (Sastry *et al*., 2002; Lizée *et al*., 2003). Consequently, a more accurate determination of the total number of infectious units may be achieved by analyzing gene transfer by quantitative polymerase chain reaction (PCR) (Sastry *et al*., 2002; Lizée *et al*., 2003). The same groups also demonstrated that titering lentivectors by determining the concentration of vector genome RNA in the supernatant overestimates the vector titer by 1000- to 10,000-fold, owing to the fact that such analysis counts defective or otherwise noninfectious particles as well as free-floating vector genomes in the supernatant (Sastry *et al*., 2002; Lizée *et al*., 2003).

Another method for titering human immunodeficiency virus type 1 (HIV-1)-based lentivectors involves measuring the amount of Gag (p24) protein in vector supernatants. We sought to determine the reliability of this assay when used to titer lentivector supernatants and to determine whether any of the variables involved in lentivector construction or production by transient transfection influence vector titer and infectivity. We define "infectivity" as the number of infectious units (IU) per unit of p24 in the supernatant. We determined vector titer (IU/ml) by a standard limiting dilution assay on HEK293 cells, whereas the p24 content was determined by enzyme-linked immunosorbent assay (ELISA). For the purpose of examining the effects on titer and infectivity of minor changes to the vector preparation protocol, we performed our analyses on HEK293 cells. Because it is also our goal to produce lentivector supernatants with high infectivities for the gene modification of primitive human hematopoietic progenitor and stem cells, we selected some of the variables of lentivector construction and preparation for analysis of their effects on infectivity in both HEK293 cells and human umbilical cord blood $CD34^+$ hematopoietic progenitor cells (HPCs).

Previous studies have shown that lentiviral vectors can transduce human $CD34^+$ HPCs more efficiently than vectors derived from the Moloney murine leukemia virus (Uchida *et al*., 1998; Case *et al*., 1999; Evans *et al*., 1999; Miyoshi *et al*., 1999); however, we and others have found that the transduction of human HPCs is strictly dependent on the use of high-concentration supernatants (Sutton *et al*., 1999; Haas *et al*., 2000; Salmon *et al*., 2000). Because it is difficult to produce some lentivectors at high titer, such as those bearing large expression cassettes, techniques for increasing the infectivity of lentivectors on this clinically important cell type are highly desirable. In this work, we demonstrate that the infectivities of lentivectors on $CD34^+$ HPCs can be increased by including the central polypurine tract (cPPT) in the transfer vector, collecting the supernatant at early time points, collecting the supernatant under serum-free conditions, ultraconcentrating the supernatant, and replacing the vesicular stomatitis virus G glycoprotein (VSV-G) with the RD114 feline endogenous virus envelope.

MATERIALS AND METHODS

Cell culture

HEK293 cells (henceforth denoted 293 cells) and 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 m*M* L-glutamine. Primary human $CD34^+$ hematopoietic progenitor cells (HPCs) were isolated from umbilical cord blood by Ficoll-Hypaque density gradient centrifugation followed by passage through two Miltenyi MidiMACS CD34 separation columns (Miltenyi Biotec, Auburn, CA). After isolation, $CD34^+$ HPCs were plated at 1×10^5 cells per well on 24-well plates coated with fibronectin fragment CH-296 (TaKaRa Bio, Otsu, Shiga, Japan) and prestimulated for 20 hr in X-VIVO 15 medium containing Flt-3 ligand (50 ng/ml) (R&D Systems, Minneapolis, MN), c-Kit ligand (50 ng/ml) (Biosource International, Camarillo, CA), and thrombopoietin (50 ng/ml; R&D Systems). Human samples were obtained and processed in accordance with protocols approved by the Committee on Clinical Investigations at Children's Hospital Los Angeles (Los Angeles, CA).

Lentiviral vector construction

Figure 1 depicts a general schematic for the transfer vectors used in this study. The vector plasmid pHR′-hCMV-eGFP (Naldini *et al*., 1996) was the kind gift of I. Verma (Salk Institute, San Diego, CA). The self-inactivating vector plasmid pCCL-hCMV-eGFP (Dull *et al*., 1998) was the kind gift of L. Naldini (Cell Genesys, South San Francisco, CA). Vectors carrying different internal promoters were made by replacing the human cytomegalovirus (hCMV) promoter in this vector construct. The MNDU3 promoter (the U3 region of the MND oncoretroviral

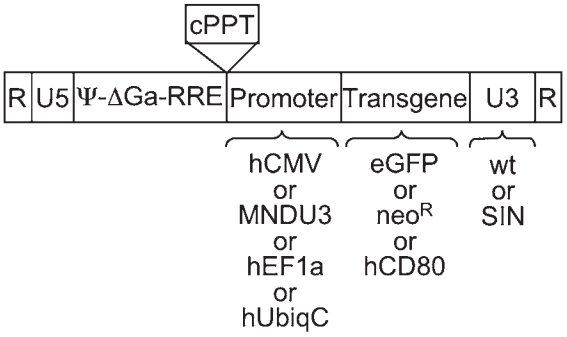


FIG. 1. Lentiviral transfer vectors used in this study, shown in the form of the vector genome. R, U5, and U3, LTR components; ψ-ΔGa, encapsidation determinant; RRE, Rev-responsive element. When added to the transfer vector, the central polypurine tract (cPPT) was inserted upstream of the internal promoter. One of four internal promoters was used: hCMV, human cytomegalovirus promoter; MNDU3, promoter from the U3 region of the MND retroviral vector; hEF1α, human elongation factor 1a promoter; hUbiqC, human ubiquitin C promoter. One of three reporter transgenes was used: eGFP, enhanced green fluorescent protein; hCD80, human CD80; *neo*^{R}, neomycin resistance gene. Vectors with wild-type (wt) or self-inactivating (SIN) U3 regions were used.

vector, which is derived from a variant of the myeloproliferative sarcoma virus with the negative control region of the long terminal repeat [LTR] removed) (Challita *et al*., 1995), the human elongation factor 1a (hEF1α) promoter, or the human ubiquitin C (hUbiqC) promoter was inserted in place of the hCMV promoter as previously described (Haas *et al*., 2003). The vector plasmid pCCL-hCMV-hCD80 was constructed by recovering the hCD80 sequence from the plasmid pIC20H-hCD80 (Stripecke *et al*., 2000) by digestion with *Nco*I and *Eco*RI and ligating it and a synthetic *Bam*HI-to-*Nco*I linker to pCCL-hCMV-eGFP, which had been digested with *Bam*HI and *Eco*RI. The vector plasmid pCCL-hCMV-neo was constructed by recovering the *neo* sequence from LNSX (Robbins *et al*., 1997) by digestion with *Eco*RI and *Bam*HI and cloning it into pIC20H digested with the same enzymes, followed by release of the *neo* sequence from pIC20H-neo, using *Bgl*II and *Sal*I, and ligating it to pCCL-hCMV-X digested with *Bam*HI and *Sal*I. The plasmid pCCL-hCMV-X had previously been constructed by digesting pCCL-hCMV-eGFP with *Bam*HI and *Sal*I and inserting a multiple cloning site. Finally, the human immunodeficiency virus type 1 (HIV-1) central polypurine tract was recovered from pNL4-3 (Adachi *et al*., 1986) by PCR with the primers 5′-GTCGTCGAATTCACAAATGGCAGTATTCAT-CC-3′ and 5′-GTCGAATTCCCAAACTGGATCTCTGCTGT-CC-3′, which add *Eco*RI sites to each end, and ligated into pBluescript that had been digested with *Eco*RI. The cPPT was then removed from this plasmid by *Eco*RI, blunted with *PfuTurbo* polymerase (Stratagene, La Jolla, CA), and ligated upstream of the hCMV promoter in pCCL-hCMV-eGFP, which had been digested with *Cla*I and blunted with *PfuTurbo* polymerase, to generate the plasmid pCCL-cPPT-hCMV-eGFP.

Vector supernatant preparation

Vector supernatants were prepared in duplicate by calcium phosphate-mediated cotransfection (calcium phosphate transfection kit; Invitrogen Life Technologies, Carlsbad, CA) of 293T cells as previously described (Soneoka *et al*., 1995). 293T cells were plated on poly-L-lysine-coated 10-cm plates at 6.5×10^6 cells per plate in DMEM with 10% FBS (D10) and allowed to adhere for 6 hr before transfection with 10 μg of transfer vector plasmid, 10 μg of pCMVΔR8.9 packaging plasmid (Zufferey *et al*., 1997), and 2 μg of pMD.G(VSV-G) envelope plasmid (Naldini *et al*., 1996), unless noted otherwise. In some experiments, the amount of each of these plasmids was titrated in different amounts into the transfections, using amounts indicated in the appropriate section of this article. In one set of experiments, vector particles were pseudotyped with the envelope of the feline endogenous retrovirus RD114 by transfecting with pCMV-RD114/TR (Sandrin *et al*., 2002) in place of the VSV-G-expressing plasmid. Twelve hours after application of the DNA precipitate, the cells were rinsed three times with Dulbecco's phosphate-buffered saline (PBS), and then subjected to induction with 10 m*M* sodium butyrate (Sigma Scientific, Brighton, MI) in D10. After 8–12 hr, the cells were rinsed once with PBS and then refed with fresh D10. In experiments in which we wished to collect vector particles under serum-free conditions, the packaging cells were fed with UltraCULTURE medium (Cambrex, East Rutherford, NJ) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 m*M* L-glutamine after the sodium butyrate induction. In most experiments, vector supernatants were collected 24 hr after application of fresh medium and then filtered through a 0.2-μm syringe filter and stored at -80°C. In one set of experiments, we compared unconcentrated and concentrated vector supernatants, in which case the supernatant was concentrated by ultracentrifugation at $50{,}000 \times g$ for 2 hr, as previously described (Burns *et al*., 1993).

Cell transduction with lentiviral vectors

To determine the titers of the lentiviral vectors, 293 cells were plated at 2×10^5 cells per well in six-well plates and allowed to adhere for 6 hr, at which time the medium was removed and replaced with 1 ml of vector diluted 1:100, 1:1000, and 1:10,000 and supplemented with Polybrene (8 μg/ml; Sigma, St. Louis, MO). At the time of vector addition, the number of cells per well was counted from three wells and the average was used to calculate the vector titer. Vector supernatants remained on the cells overnight and were then replaced with fresh medium. All titers were determined at 72 hr after the addition of vector by harvesting the cells with trypsin–EDTA, washing them with PBS, and analyzing them for enhanced green fluorescent protein (eGFP) expression, using a FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA). Vector titers (IU/ml) were calculated according to the equation [(% eGFP positive)/100] $\times$ dilution factor $\times$ cell number. To ensure maximum accuracy, we calculated the titers only from transductions with dilution factors that resulted in fewer than 10% of the cells becoming eGFP positive, and all titers were performed in duplicate.

When vectors expressing hCD80 were used, a similar titering protocol was performed except that 72 hr after transduction, the 293 cells were harvested with enzyme-free cell dissociation buffer (Invitrogen Life Technologies), washed with PBS, suspended in 10% mouse serum immunoglobulin (MSIg; Beckman Coulter, Fullerton, CA) blocking solution, and exposed to a biotin-conjugated antibody to CD80 for 15 min at 4°C, followed by exposure to streptavidin–phycoerythrin (PE) for 10 min at 4°C. After washing three times with PBS, the cells were analyzed for PE labeling, using a flow cytometer. For vectors expressing the neomycin resistance gene (*neo*^{R}), titering was performed by applying 1:100,000 dilutions of vector to 293 cells for 24 hr, followed by application of D10 containing G418 (1 mg/ml; Sigma) for 14 days with D10–G418 replacement every 3–4 days. At the end of this period, the cells were washed extensively with PBS and colonies remaining attached to the plate were stained with crystal violet and counted. Each colony was counted as one transduced cell for the purpose of calculating titer.

Human CD34$^+$ HPCs were transduced after 1 day of prestimulation as described above by adding lentivector supernatants to the cells in the presence of Polybrene (4 μg/ml). Twenty-four hours after transduction, the medium was carefully removed and replaced with Iscove's modification of Dulbecco's medium (IMDM), containing 20% FBS, 0.5% bovine serum albumin, interleukin 3 (IL-3, 5 ng/ml; Biosource International), IL-6 (10 ng/ml; Biosource International), and c-Kit ligand (25 ng/ml). Cells were maintained in culture for 1 week after application of the lentivector supernatant, at which time the cells

were harvested, washed once with PBS, and analyzed for eGFP expression with a flow cytometer.

HIV-1 Gag (p24) measurement

The p24 concentration in lentivector supernatants was determined by a diagnostically certified p24 immunoassay (Beckman Coulter) according to the manufacturer's instructions. To ensure that the measured p24 concentrations were within the linear range of the assay, vector supernatants were diluted 1:1000, 1:5000, and 1:10,000. All p24 assays were performed in duplicate.

RESULTS

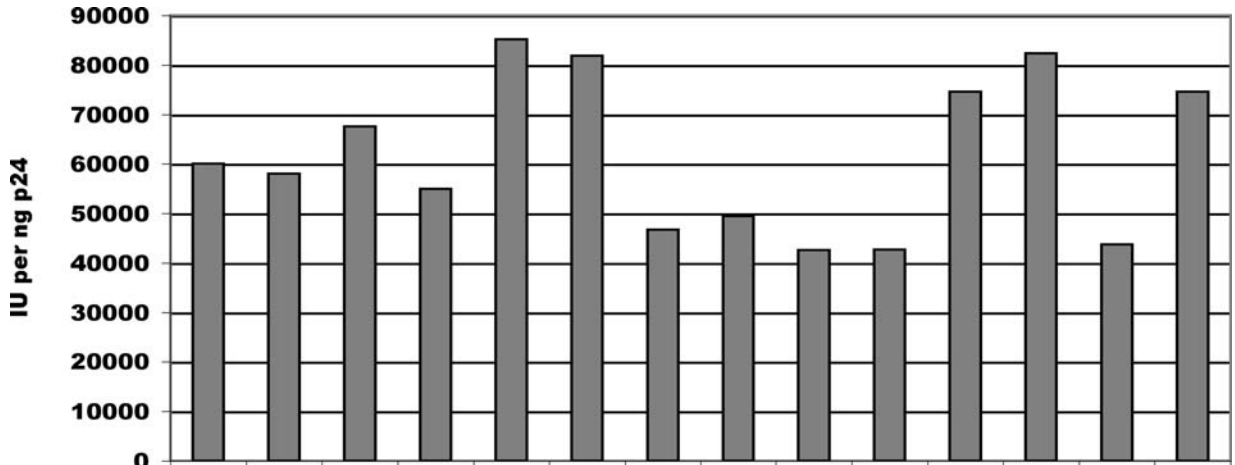
Range of lentivector infectivities

To assess the effects of altering certain variables in the production of lentiviral vectors by transient transfection, we first determined the normal range of infectivities yielded by vector supernatants produced by this method. To that end, we evaluated 14 independent preparations of a vector that had been prepared at different times, but each of which was the product of similar transfection procedures. In this series, all supernatants represent a single collection 24 hr after the packaging 293T cells had been transfected with 10 μg of pHR′-hCMV-eGFP, 10 μg of pCMVΔR8.9, and 2 μg of pMD.G(VSV-G), and induced with sodium butyrate. Although the absolute titers of these supernatants ranged over 10-fold, the infectivities of all of them fell within a p24 range of 42,000 to 85,000 IU/ng (Fig. 2). Because there is a small amount of error inherent in the determination of both titer and p24 content by limiting dilution, we consider this range to be the best achievable accuracy from combining the data from these two assays. Consequently, for the remainder of this work, we consider all infectivities falling in this range to be normal.

Effect of plasmid amount transfected into packaging cells

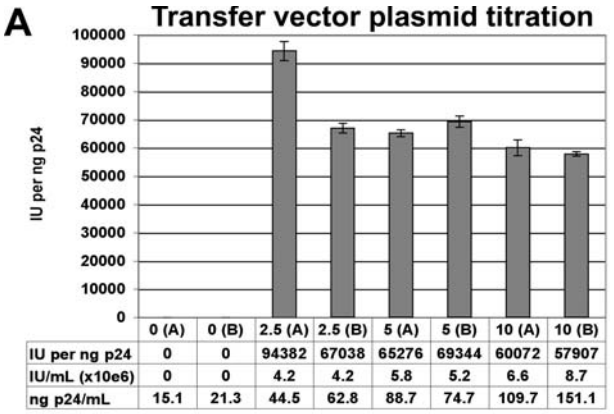
We next evaluated the effects of changing the amount of each plasmid used in the transfections to produce the vector supernatants. In the three-plasmid transfection system we used to package our vectors, it is customary to use 10 μg of transfer vector plasmid to transfect roughly 5–10 $\times$ 10^6 293T cells adherent to a poly-L-lysine-coated 10-cm plate (Soneoka *et al*., 1995). We thus evaluated the effect of using a range of 0 to 10 μg of the pHR′-hCMV-eGFP transfer vector plasmid while holding the amount of the packaging plasmid pCMVΔR8.9 and the envelope plasmid pMD.G(VSV-G) constant at 10 and 2 μg, respectively. Each condition was prepared twice in separate 10-cm dishes. Supernatants were collected 24 hr after the termination of sodium butyrate induction. Each supernatant was then titered in duplicate by eGFP expression and assessed in duplicate for p24 concentration. From these assays, we determined that although the vector titer increases in parallel with the amount of transfer vector plasmid used to transfect the packaging cells (Fig. 3A), there is no significant difference in the infectivities of vectors produced under any of the different conditions. All preparations that included any amount of transfer vector plasmid yielded supernatants with infectivities in the range of 58,000 to 94,000 IU/ng p24, with no significant differences between any of the conditions (Fig. 3A).

Similar to the analysis of the effect of using different amounts of transfer vector plasmid in the packaging cell transfection, we also evaluated independent manipulation of either pCMVΔR8.9 or pMD.G(VSV-G). It is customary to use 10 μg of

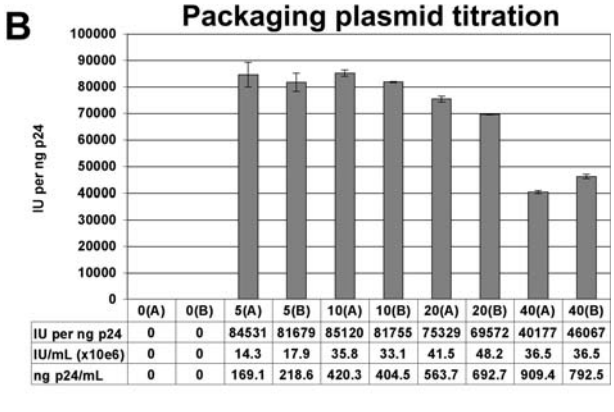


	1	2	3	4	5	6	7	8	9	10	11	12	13	14
IU per ng p24	60073	57909	67458	54898	85120	81755	46667	49364	42609	42645	74564	82272	43701	74585
IU/mL (x10e6)	6.6	8.8	4.6	4.4	35.8	33.1	15.7	13.4	24.4	24.9	28.2	15.5	14.5	21.2
ng p24/mL	109.7	151.1	67.4	79.8	420.3	404.5	335.8	270.8	572.2	583.7	378.2	188.4	331.8	283.8

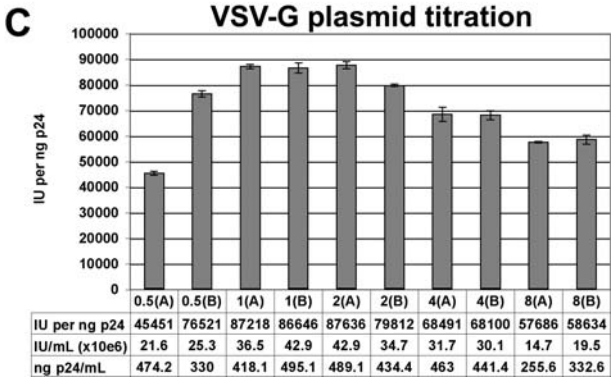
FIG. 2. Analysis of titers and infectivities from 14 vector supernatants prepared similarly. Each supernatant was produced by transfection with 10 μg of pHR′-hCMV-eGFP, 10 μg of pCMVΔR8.9, and 2 μg of pMD.G(VSV-G) and collected at 24 hr. The graph depicts the infectivity (infectious units per nanogram of p24) for each independent supernatant based on the determination of vector titer by limiting dilution on 293 cells and measurement of p24 concentrations by ELISA; both assays were performed in duplicate for each supernatant.



	0 (A)	0 (B)	2.5 (A)	2.5 (B)	5 (A)	5 (B)	10 (A)	10 (B)
IU per ng p24	0	0	94382	67038	65276	69344	60072	57907
IU/mL (x10e6)	0	0	4.2	4.2	5.8	5.2	6.6	8.7
ng p24/mL	15.1	21.3	44.5	62.8	88.7	74.7	109.7	151.1



	0(A)	0(B)	5(A)	5(B)	10(A)	10(B)	20(A)	20(B)	40(A)	40(B)
IU per ng p24	0	0	84531	81679	85120	81755	75329	69572	40177	46067
IU/mL (x10e6)	0	0	14.3	17.9	35.8	33.1	41.5	48.2	36.5	36.5
ng p24/mL	0	0	169.1	218.6	420.3	404.5	563.7	692.7	909.4	792.5



	0.5(A)	0.5(B)	1(A)	1(B)	2(A)	2(B)	4(A)	4(B)	8(A)	8(B)
IU per ng p24	45451	76521	87218	86646	87636	79812	68491	68100	57686	58634
IU/mL (x10e6)	21.6	25.3	36.5	42.9	42.9	34.7	31.7	30.1	14.7	19.5
ng p24/mL	474.2	330	418.1	495.1	489.1	434.4	463	441.4	255.6	332.6

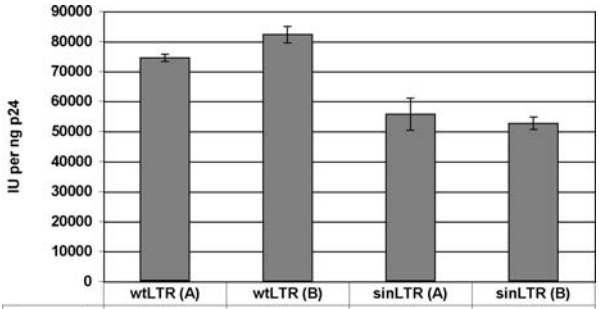
FIG. 3. Independent titration of plasmids used to produce lentiviral vectors by transient transfection. The graphs depict the infectivity (infectious units per nanogram of p24) when the transfer vector plasmid pHR′-hCMV-eGFP (**A**), packaging plasmid pCMVΔR8.9 (**B**), and pMD.G(VSV-G) envelope plasmid (**C**) were titrated into transfections in the amounts indicated on the *x* axis of each graph (in micrograms) while the other two plasmids were held constant (see Materials and Methods). Each condition was prepared and analyzed in duplicate, as indicated by the letters *A* and *B*. Error bars indicate the standard deviation.

pCMVΔR8.9 for a 10-cm plate (Zufferey *et al*., 1997), and because it is this plasmid that expresses the p24 Gag protein, we tested a wide range of input pCMVΔR8.9 to determine whether the concentration of p24 in the supernatant would fluctuate in parallel. We thus performed transfections using between 0 and 40 μg of pCMVΔR8.9 and analyzed vector titers and p24 concentrations in duplicate. As expected, cells not transfected with pCMVΔR8.9 produced no detectable p24 and thus no vector titer. High-titer vectors were produced with 5, 10, 20, and 40 μg of pCMVΔR8.9; however, we found that the highest titer supernatants were produced with 20 μg of this plasmid (Fig. 3B). Infectivities, on the other hand, were not significantly different with any of the pCMVΔR8.9 amounts used, although there was a trend toward lower infectivities when 40 μg was used (where infectivities were close to 40,000 IU/ng p24), as compared with amounts in the range of 5–20 μg, which produced vectors with infectivities in the range of 69,000 to 85,000 IU/ng p24.

Finally, it is customary to use 2 μg of pMD.G(VSV-G) in this vector preparation system (Naldini *et al*., 1996), so we evaluated a range of 0.5 to 8 μg, while holding the amount of transfer vector and packaging plasmids each to 10 μg. High-titer vectors were produced at all tested amounts of pMD.G(VSV-G), and each of these supernatants had infectivities in the normal range. Here, infectivities ranged from 45,000 to 88,000 IU/ng p24 (Fig. 3C).

Effect of the transfer vector LTR type

Although the previous analyses were made using a transfer vector with a wild-type LTR (pHR′-hCMV-eGFP), most of the lentivectors in current use have a modification in the LTR to increase their biosafety, called the self-inactivating or SIN deletion. The SIN deletion removes 400 bp from the U3 region of the LTR to reduce the promoter activity from the LTR (Miyoshi *et al*., 1998; Zufferey *et al*., 1998). We demonstrated that the inactivation of transcription from LTRs with this deletion (the sinLTR) is not complete (Logan *et al*., 2004), but it is significantly decreased from wild type. Because SIN lentivectors will likely be favorable for most clinical applications, we compared the supernatants produced by packaging either 10 μg of pHR′-hCMV-eGFP or 10 μg of a SIN transfer vector plasmid, pCCL-hCMV-eGFP, with standard amounts of pCMVΔR8.9 and pMD.G(VSV-G). We found that both vectors are produced at high titers in excess of 1×10^7 and that the infectivities of both vectors fall into the normal range (Fig. 4).



	wtLTR (A)	wtLTR (B)	sinLTR (A)	sinLTR (B)
IU per ng p24	74564	82272	55802	52734
IU/mL (x10e6)	28.2	15.5	12.6	13.5
ng p24/mL	378.2	188.4	225.8	256

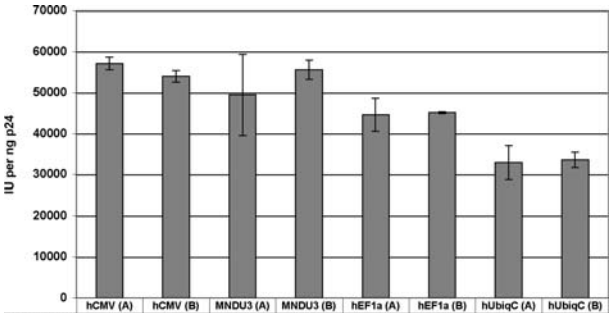
FIG. 4. Comparison of titers and infectivities with wtLTR (pHR′-hCMV-eGFP) and sinLTR (pCCL-hCMV-eGFP) vectors prepared in similar fashion. Both vectors were prepared in duplicate, as indicated by the letters *A* and *B*. Error bars indicate the standard deviation.

Effect of the transfer vector internal promoter

In the past, we have noticed that different transfer vector constructs tend to yield rather different titers. It has also been demonstrated that transfer vectors carrying different sequences can yield titers ranging more than 100-fold and vector infectivities ranging more than 4-fold (Kumar *et al*., 2001). Consequently, we are interested in the possibility that making changes to the transfer vector, such as replacing the internal hCMV promoter that has been most frequently used in vectors to date with the sequences for other promoters, may alter the infectivities of the resultant vector supernatants. We thus constructed SIN lentivectors that were the same in sequence except for the internal promoter. We measured the infectivities of vectors carrying internal hCMV, MNDU3, hEF1α, and hUbiqC promoters. All yielded infectivities in the normal range, with the exception of those carrying the hUbiqC promoter, which yielded slightly lower infectivities on average (~33,000 IU/ng p24) (Fig. 5).

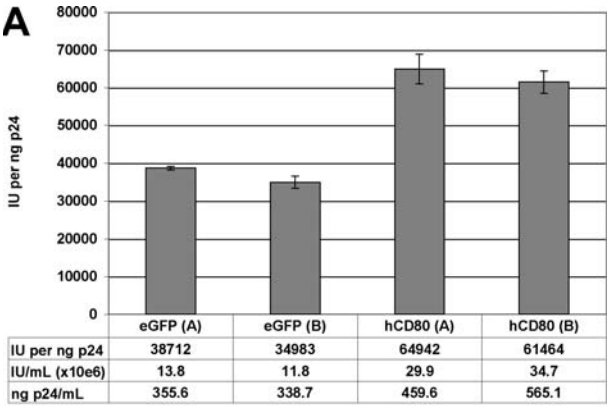
Effect of the transfer vector transgene

We next compared SIN lentivectors that had the same sequences with the exception of the reporter transgene, which was expressed from an internal hCMV promoter. When we compared vectors expressing eGFP with those expressing human CD80 (hCD80), a transmembrane protein involved in the costimulation of T cells, we found that the titers and infectivities of both vectors were in the normal range (Fig. 6A). eGFP was detected by direct analysis with a flow cytometer, whereas detection of CD80 required staining the cells with a biotin-conjugated antibody to CD80 followed by labeling with streptavidin–PE, so we believe that analysis of titer by both methods is highly sensitive and accurate. We also compared vectors expressing eGFP with those expressing the neomycin resistance gene (*neo*^{R}) and found that those expressing *neo*^{R} had infectivities lower than the normal range (~25,000 IU/ng p24) based on colony survival in G418-supplemented medium (Fig. 6B).

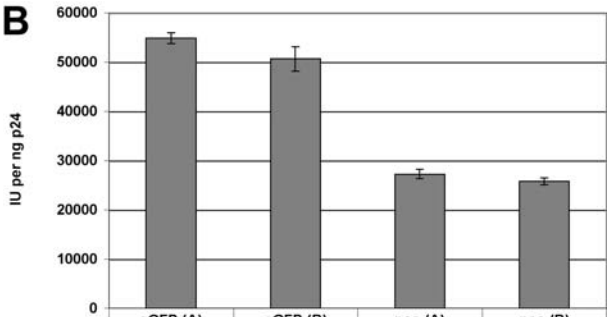


	hCMV (A)	hCMV (B)	MNDU3 (A)	MNDU3 (B)	hEF1a (A)	hEF1a (B)	hUbiqC (A)	hUbiqC (B)
IU per ng p24	57106	53951	49410	55594	44568	45083	32880	33562
IU/mL (x10e6)	7.5	8.7	3	3.3	2.1	2.7	1.8	2.9
ng p24/mL	131.7	160.7	61.5	59.3	46.5	59.4	53.8	85

FIG. 5. Titers and infectivities of lentivectors bearing different internal promoters. Transfer vectors were of the same design except for the promoter they carried, which was one of the following: the hCMV promoter, the MNDU3 promoter, the hEF1α promoter, or the hUbiqC promoter. Each vector was prepared and analyzed in duplicate, as indicated by the letters *A* and *B*. Error bars indicate the standard deviation.



	eGFP (A)	eGFP (B)	hCD80 (A)	hCD80 (B)
IU per ng p24	38712	34983	64942	61464
IU/mL (x10e6)	13.8	11.8	29.9	34.7
ng p24/mL	355.6	338.7	459.6	565.1



	eGFP (A)	eGFP (B)	neo (A)	neo (B)
IU per ng p24	54865	50685	27242	25771
IU/mL (x10e6)	4.7	4.3	5.9	5
ng p24/mL	86.1	85.2	214.9	193.9

FIG. 6. Titers and infectivities of lentivectors bearing different transgenes. (**A**) Comparison of vectors carrying either the eGFP or hCD80 gene. (**B**) Comparison of vectors carrying the eGFP and *neo*^{R} genes. Each vector was prepared and analyzed in duplicate, as indicated by the letters *A* and *B*. Error bars indicate the standard deviation.

Effect of cPPT inclusion in the transfer vector

The central polypurine tract (cPPT) has been demonstrated by some investigators to increase the transduction of several cell types (Follenzi *et al*., 2000; Sirven *et al*., 2000; Zennou *et al*., 2000; VandenDriessche *et al*., 2002; Van Maele *et al*., 2003), but has been found by other investigators not to affect the transduction of other cell types (Dvorin *et al*., 2002; Limón *et al*., 2002). Here, we evaluated the effect that adding the cPPT to the transfer vector has on vector titer and infectivity. We found that addition of the cPPT leads to a significant increase in both vector titer (increased 6.7-fold) and vector infectivity (increased 5.4-fold) when analyzed on 293 cells (Fig. 7A). Whereas vectors lacking the cPPT had infectivities within the normal range, vectors carrying the cPPT exhibited infectivities of roughly 240,000 IU/ng p24.

As was observed on 293 cells, lentivectors carrying the cPPT also had higher infectivities on CD34$^+$ HPCs, exhibited by their ability to transduce twice as many cells as the vector lacking the cPPT when they were matched by p24 concentration (Fig. 7B). When the two vector types were matched by their infectious unit titers on 293 cells, there was little difference in the resulting transduction efficiencies on HPCs (Fig. 7C).

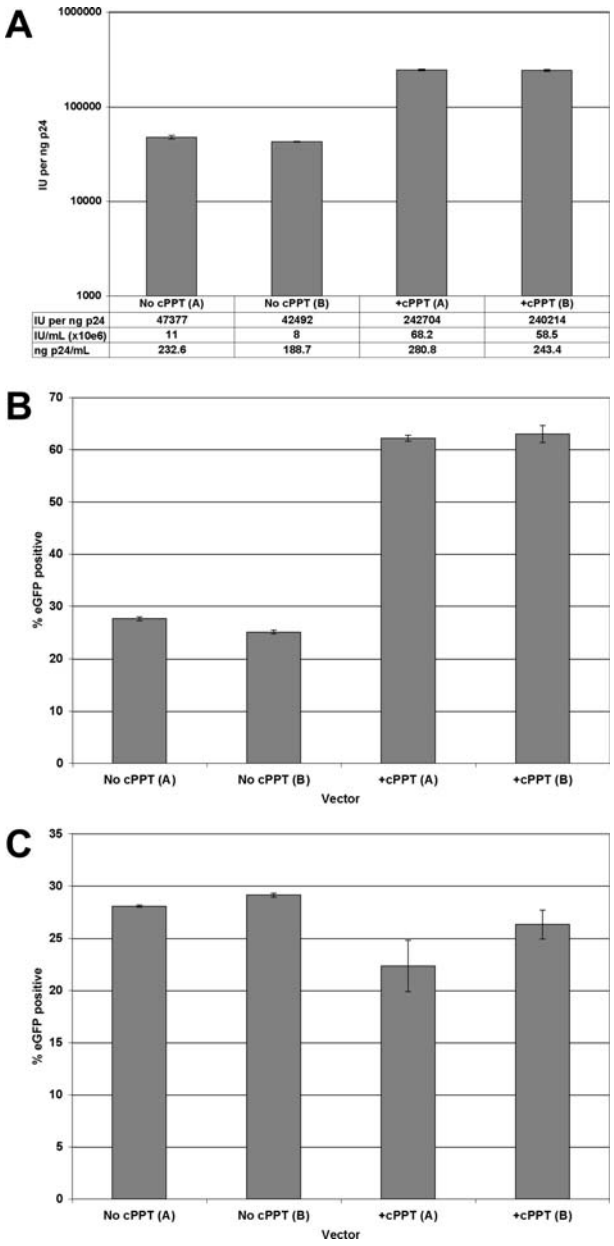


FIG. 7. Analysis of the effect on titer and infectivity of inclusion of the central polypurine tract (cPPT) in the transfer vector construct. (**A**) Transfer vectors were of similar construction except for the absence (No cPPT) or presence (+cPPT) of the cPPT upstream of the internal promoter. Each vector was prepared and analyzed in duplicate, as indicated by the letters *A* and *B*. (**B** and **C**) Transduction of human cord blood CD34$^+$ HPCs with lentivectors lacking or carrying the cPPT. Cells were transduced in duplicate with 124.3 ng of p24/ml equivalent (**B**) or with 8.5×10^6 IU/ml (**C**) of each vector and analyzed for eGFP expression by flow cytometry after 1 week. Error bars indicate the standard deviation.

Effect of vector supernatant collection time

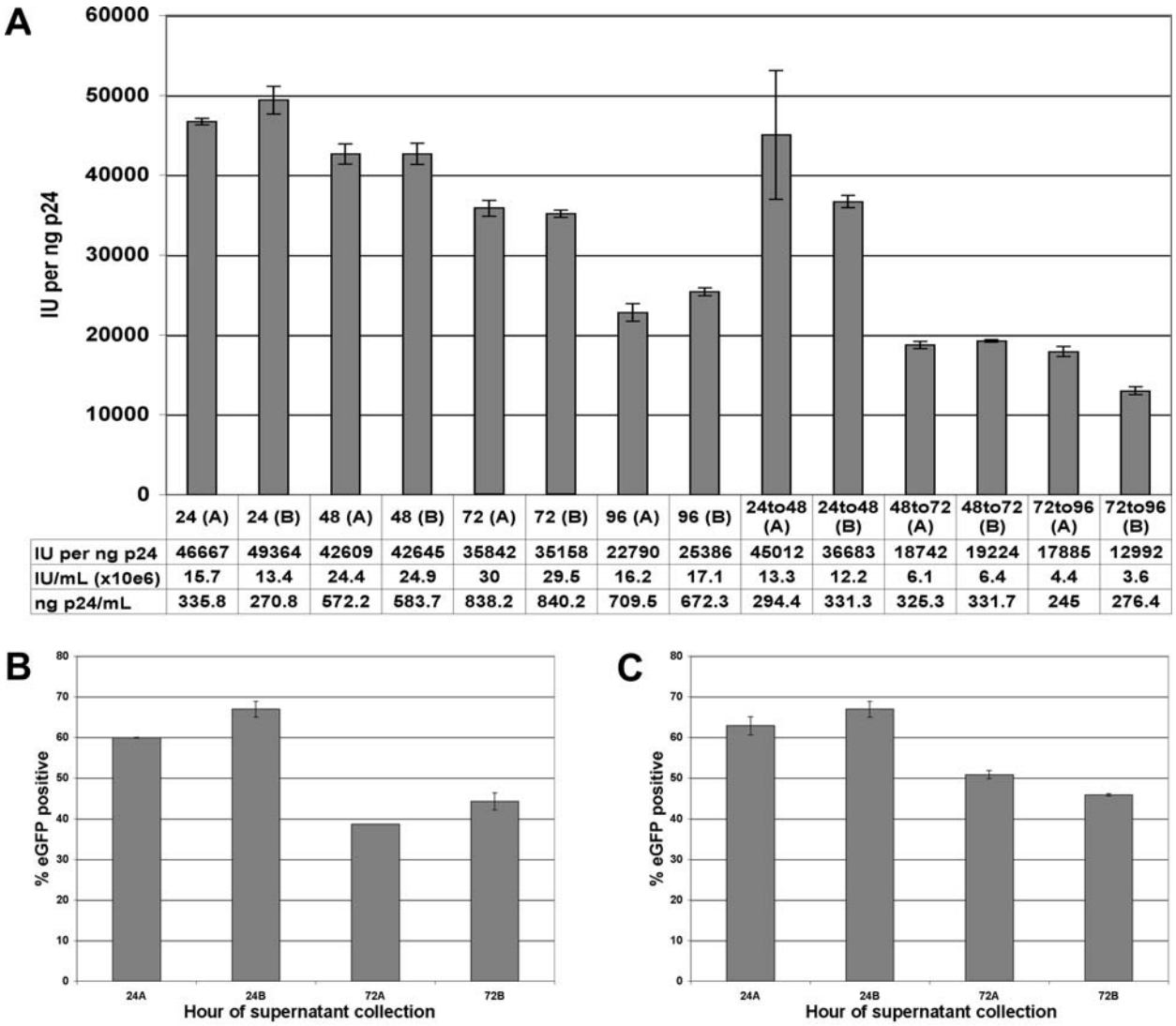
When producing vectors by transient transfection, there is a great deal of flexibility with respect to how long the packaging cells can be incubated after sodium butyrate induction. Some laboratories collect supernatant successively at each 12 or 24 hr of incubation, whereas other laboratories allow the transfected cells to incubate for longer periods of time for a single supernatant collection. We evaluated the effect of the length of time that the medium was conditioned by the packaging cells by analyzing the titers and infectivities of vector supernatants made by single collections at 24, 48, 72, or 96 hr or by successive collections after the passage of each 24-hr period up to 96 hr. When comparing single collections at the different time points, we determined that the titer of the supernatants continued to increase up to the 72-hr time point, but started to decline after that time (Fig. 8A). The infectivities of the vectors collected at the different time points were within the normal range up to the 48-hr collections, but there was a trend toward lower infectivity vector supernatants with the 72- and 96-hr collections, both of which were below 40,000 IU/ng p24 (Fig. 8A).

When successive collections were made at each 24-hr time point up to 96 hr, we also found that the vector infectivities were within the normal range only up to the 24- to 48-hr collections, and the two subsequent collections both had significantly lower titers and infectivities (Fig. 8A). It can be concluded from the successive vector collections that there is little advantage to collecting and refreshing the medium each 24 hr with respect to the total number of vector particles that can be collected, because roughly the same number of vector particles was collected in a single 72-hr collection as was found in the sum of the 24-hr, 24- to 48-hr, and 48- to 72-hr collections (Fig. 8A). The output of vector particles drops noticeably after roughly 48 hr, which is expected in a transient transfection system, wherein transfected plasmids are expected to be expressed at high levels for less than 72 hr.

Because we demonstrated that a single vector collection at 72 hr contains almost all the vector particles the producer cells are capable of producing during that period of time, and that the supernatant has a slightly decreased infectivity on 293 cells, we wanted to determine whether 72-hr supernatant could also transduce CD34$^+$ HPCs efficiently. We performed transductions wherein we matched supernatants collected at 24 and 72 hr by p24 content (Fig. 8B) or by infectious units (Fig. 8C). In both cases, the supernatants collected at 72 hr transduced the HPCs less efficiently than the 24-hr supernatants, with the 24-hr supernatants providing a roughly 50% higher level of transduction than the 72-hr supernatants when they were matched by p24 content (Fig. 8B).

Effect of vector concentration by ultracentrifugation

To achieve the high titers necessary for the transduction of certain cell types, it is frequently necessary to concentrate the vector particles present in a supernatant into a smaller volume. The most common way to achieve this type of concentration involves ultracentrifugation of the vector supernatant at extremely high *g* forces (Burns *et al.*, 1993). We thus produced a large volume of a vector supernatant and then concentrated a portion of it in order to determine whether the infectivity of the concentrated portion differed from that of the unconcentrated (raw) portion. After spinning the supernatant at $50{,}000 \times g$ for 2 hr, we were able to achieve a 144-fold decrease in supernatant volume, which exhibited an infectious unit titer 125-fold concentrated over the raw supernatant, for a recovery rate of 87%. When the concentrated vector was analyzed for infectivity (by



	24 (A)	24 (B)	48 (A)	48 (B)	72 (A)	72 (B)	96 (A)	96 (B)	24to48 (A)	24to48 (B)	48to72 (A)	48to72 (B)	72to96 (A)	72to96 (B)
IU per ng p24	46667	49364	42609	42645	35842	35158	22790	25386	45012	36683	18742	19224	17885	12992
IU/mL (x10e6)	15.7	13.4	24.4	24.9	30	29.5	16.2	17.1	13.3	12.2	6.1	6.4	4.4	3.6
ng p24/mL	335.8	270.8	572.2	583.7	838.2	840.2	709.5	672.3	294.4	331.3	325.3	331.7	245	276.4

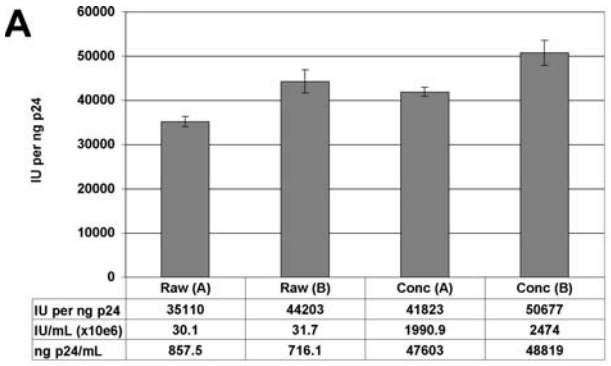
FIG. 8. Effect of vector collection time on titer and infectivity. (**A**) After transfection and sodium butyrate induction of the packaging cells, the supernatant was collected either once at 24, 48, 72, or 96 hr or at each subsequent 24-hr period, indicated as 24 to 48, 48 to 72, or 72 to 96. Each condition was prepared and analyzed in duplicate, as indicated by the letters *A* and *B*. (**B** and **C**) Transduction of $CD34^+$ HPCs with lentivector supernatants collected at 24 or 72 hr. Cells were transduced in duplicate with 135.4 ng of p24/ml equivalent (**B**) or with 6.7×10^6 IU/ml (**C**) of each vector and analyzed after 1 week for eGFP expression. Error bars indicate the standard deviation.

rediluting it in D10 for the infectious unit and p24 determinations), we found it to have a slightly greater than normal infectivity, in the range of 82,000 to 96,000 IU/ng p24 (Fig. 9A).

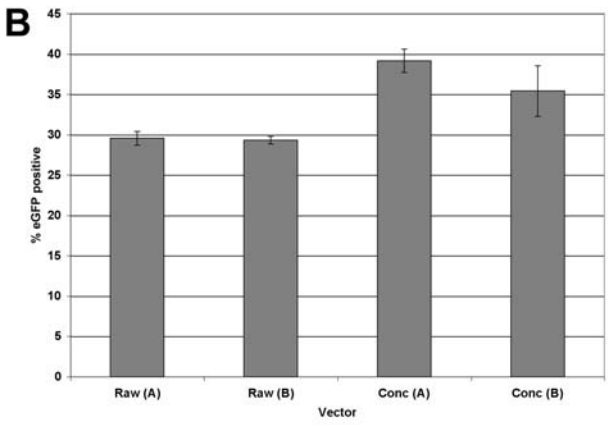
The trend toward higher infectivity of concentrated supernatants was also observed on $CD34^+$ HPCs when equal amounts of vector based on p24 concentration were used (Fig. 9B). When raw and concentrated vectors were matched by their 293 infectious unit titers, they exhibited similar transduction efficiencies on HPCs (Fig. 9C). Because we had to dilute the concentrated vector significantly in order to match either its p24 level or titer with that of the raw supernatant, we also determined what level of transduction was achievable with the concentrated vector in its undiluted form. After ultracentrifugation, the supernatant achieved high levels of transduction of HPCs, exceeding 85% (data not shown), which is roughly the upper limit of achievable transduction in $CD34^+$ HPCs from cord blood (Amsellem *et al*., 2002).

Effect of serum-containing versus serum-free vector production

Reducing the concentration of serum present is advantageous for the transduction of some cell types and, consequently, serum-free replacements for standard media have been developed for use in vector supernatant production. We produced a vector collected either in standard medium (D10) or in serum-free medium (SFM). To determine whether the presence of serum is more important during vector production or during



	Raw (A)	Raw (B)	Conc (A)	Conc (B)
IU per ng p24	35110	44203	41823	50677
IU/mL (x10e6)	30.1	31.7	1990.9	2474
ng p24/mL	857.5	716.1	47603	48819



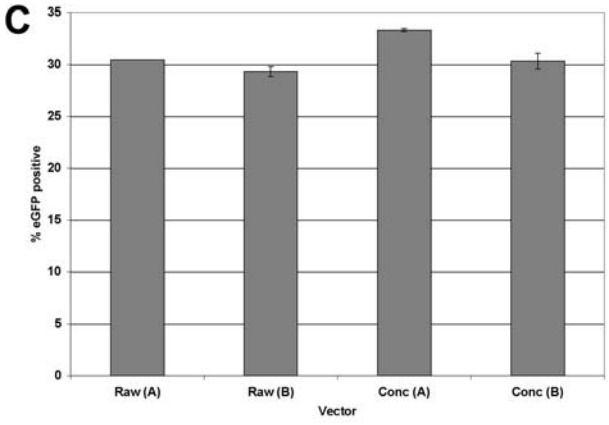


FIG. 9. Effect of vector concentration by ultracentrifugation on titer and infectivity. (**A**) Duplicate batches of a lentivector were prepared (as indicated by the letters *A* and *B*). A small portion of each vector supernatant was reserved for analysis of preconcentration titers, whereas the majority was concentrated by centrifugation at 50,000 × *g* for 2 hr to achieve a 144-fold reduction in volume. Represented in the graph are the titers and infectivities of the unconcentrated (Raw) and concentrated (Conc) supernatants. (**B** and **C**) Transduction of $CD34^+$ HPCs with raw and concentrated lentivector supernatants. Cells were transduced in duplicate (as indicated by the letters *A* and *B*) with 291.2 ng of p24/ml equivalent (**B**) or with 1.7×10^7 IU/ml (**C**) of each vector and analyzed after 1 week for eGFP expression. Error bars indicate the standard deviation.

transduction, we titered both vectors diluted in D10 and SFM. We found that the infectivity of vectors produced in D10 was within the normal range whether it was diluted in D10 or SFM for the titer procedure; however, the measured titer tended to be slightly lower when diluted in SFM (Fig. 10A). In contrast, vector supernatant produced in SFM had a higher titer and a higher than normal infectivity, particularly when diluted in D10 for the titering procedure (Fig. 10A). We conclude that vector production is superior when the producer cells are incubated in SFM, but that transduction of 293 cells for the titer is more efficient when serum is present. A slightly elevated infectivity of vector produced in serum-free medium was also observed on HPCs when either p24 levels (Fig. 10B) or infectious units (Fig. 10C) were matched.

Effect of RD114/TR envelope use

Much interest has been demonstrated in using envelope proteins other than VSV-G to pseudotype lentivectors. In particular, the envelope of the RD114 feline endogenous virus has been used to pseudotype lentivectors in both transient transfections and from stable producer cell lines (Hanawa *et al*., 2002; Sandrin *et al*., 2002; Strang *et al*., 2004). Because of the attractiveness of the RD114 envelope, particularly for use in engineering stable packaging cell lines for large-scale lentivector production for clinical use, we were interested in the infectivity of vector particles pseudotyped by this envelope. Sandrin and colleagues (2002) have demonstrated that an RD114 protein that has had its cytoplasmic tail replaced with that of the amphotrophic variant of MMLV (RD114/TR) produces higher titers than unmodified RD114 protein. We thus evaluated supernatants produced by transfecting the producer cells with a range of RD114/TR-expressing plasmid (pCMV-RD114/TR) from 5 to 20 μg. Vector infectivities were significantly lower than the normal range at all levels of input RD114/TR plasmid. Although we could produce vectors with a titer of $\sim 7 \times 10^6$, using 10 μg of RD114/TR plasmid, the infectivity of these supernatants was within the range of 22,000 to 24,000 IU/ng p24 on 293 cells (Fig. 11A).

Interestingly, contrary to our finding that the RD114/TR envelope produced vectors with lower than normal infectivities on 293 cells, we found that this envelope is exceedingly well suited for use in transduction of human cord blood $CD34^+$ HPCs. Indeed, neither the titers nor the infectivities of RD114/TR-pseudotyped vectors determined on 293 cells are predictive of the ability of these vectors to transduce HPCs. When we transduced HPCs with vectors packaged with either 2 μg of pMD.G(VSV-G) or 10 μg of pCMV-RD114/TR at matched p24 levels (Fig. 11B) and infectious units (Fig. 11C), we observed significantly higher levels of transduction in both cases when using the RD114/TR pseudotype.

DISCUSSION

We have studied variables involved in lentivector production by transient transfection and the influence they have on

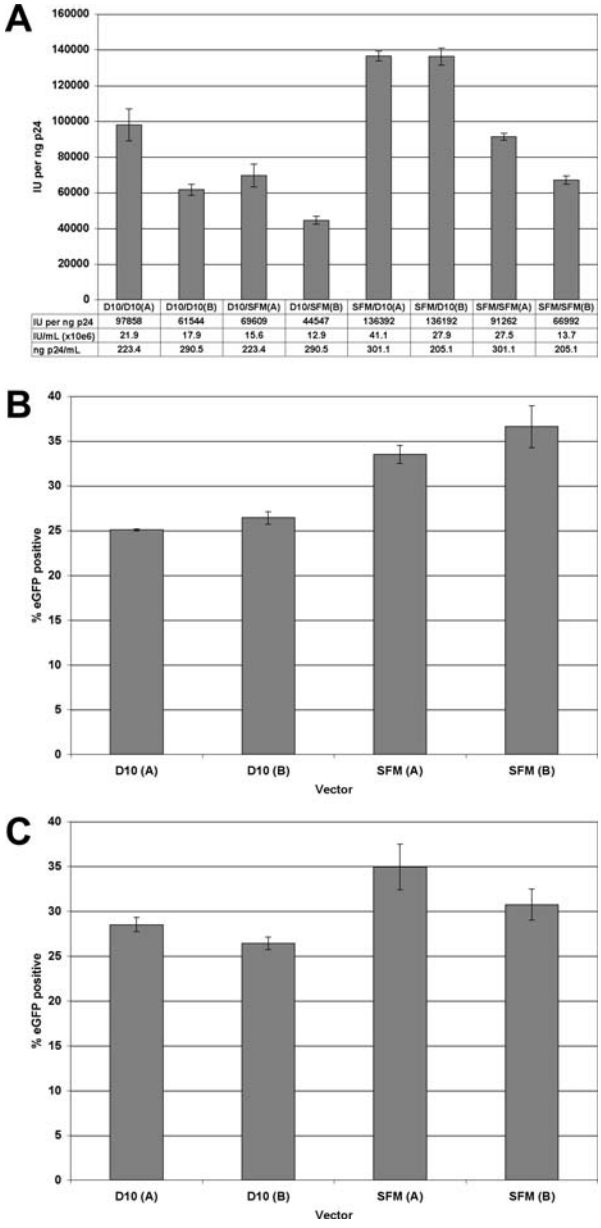


FIG. 10. Effect of vector collection in serum-containing and serum-free medium. (**A**) Titers and infectivities of lentivectors produced in serum-containing medium (D10 is DMEM with 10% [v/v] fetal bovine serum) versus serum-free medium (SFM). D10/D10 was produced in D10 and diluted in D10 for titering; D10/SFM was produced in D10 and diluted in SFM; SFM/SFM was produced in SFM and diluted in SFM; SFM/D10 was produced in SFM and diluted in D10. (**B** and **C**) Transduction of $CD34^+$ cells with supernatant collected in D10 or SFM. Cells were transduced in duplicate (as indicated by the letters *A* and *B*) with 231.3 ng of p24/ml equivalent (**B**) or with 1.8×10^7 IU/ml (**C**) of each vector and analyzed after 1 week for eGFP expression. Error bars indicate the standard deviation.

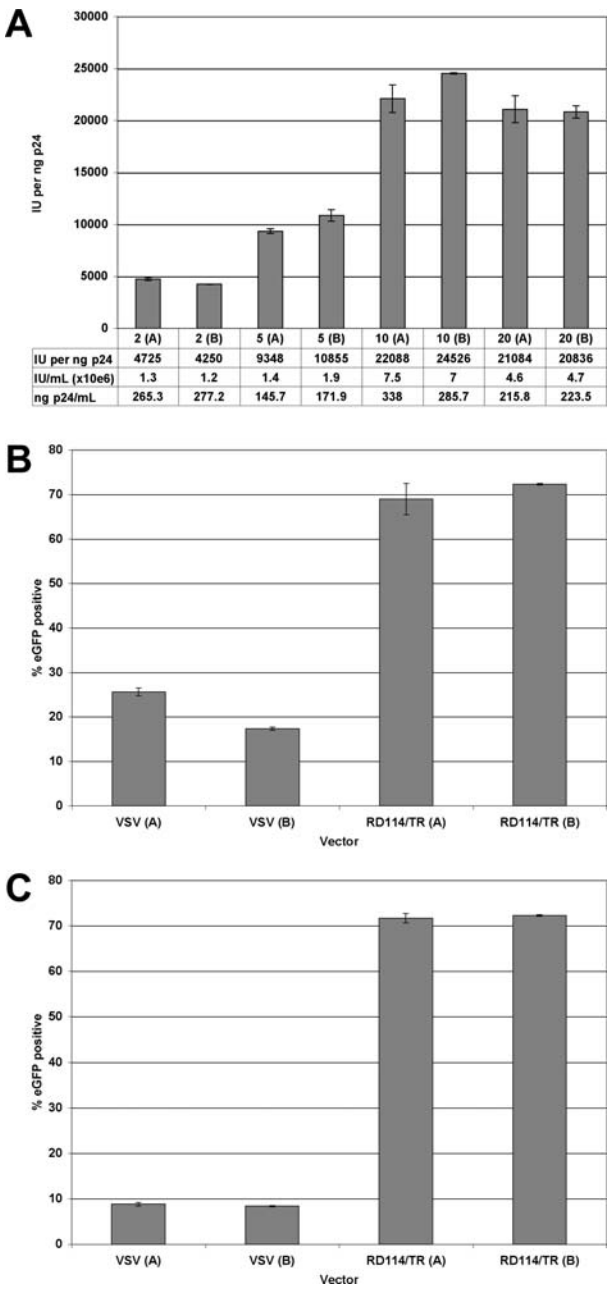


FIG. 11. Effect of RD114/TR envelope use on vector titer and infectivity. (**A**) Titration of pCMV-RD114/TR envelope plasmid. Transfer vector and packaging plasmid amounts were held constant while the amount of pCMV-RD114/TR envelope plasmid was varied as indicated on the *x* axis (in micrograms). (**B** and **C**) Transduction of $CD34^+$ HPCs with lentivectors pseudotyped with VSV-G or RD114/TR. Cells were transduced in duplicate (as indicated by the letters *A* and *B*) with 142.9 ng of p24/ml equivalent (**B**) or with 3.5×10^6 IU/ml (**C**) of each vector and analyzed after 1 week for eGFP expression. Error bars indicate the standard deviation.

vector titer and infectivity. To put our infectivity findings into context, we first evaluated the titer and infectivity of 14 preparations of the same lentivector prepared in the same manner to determine the normal range of infectivities for lentivectors produced by transient transfection. From this survey, we concluded that the normal infectivity range is roughly 40,000 to 85,000 IU/ng p24.

In using this methodology, we have determined that the

amount of any of the three plasmids used to produce lentivectors by transient transfection—the transfer vector plasmid (Fig. 3A), the packaging plasmid (Fig. 3B), and the envelope plasmid (Fig. 3C)—does not affect the infectivity, but does influence the titer of the supernatant. We were somewhat surprised that increasing the amount of packaging plasmid to four times its normal amount did not greatly diminish vector infectivity (Fig. 3B). We believe that this indicates that non-vector-associated p24 does not enter the supernatant in large quantities, or that the ability of 293T cells to produce p24 is already largely saturated at a 10-μg input of packaging plasmid.

While analyzing another variable of the vector production protocol, we determined that the timing of vector supernatant collection does not significantly alter infectivity up to some time between 48 and 72 hr, but that longer incubations produce supernatants with lower infectivities (Fig. 8A). Interestingly, supernatant collected at 24 hr displays roughly 50% higher infectivity on $CD34^+$ HPCs than does 72-hr supernatant, indicating that factors inhibiting transduction of these cells may accumulate in the supernatant over time. Related to this issue, we also have determined that concentration of vector supernatants by ultracentrifugation modestly increases the infectivity of lentivectors on $CD34^+$ HPCs (Fig. 9B and C). This finding may be significant, because it indicates that if there are producer cell-derived molecules in the vector supernatant that negatively affect infectivity, they may be partially removed by ultracentrifugation. In addition, ultracentrifugation has been shown to remove defective particles from vector supernatants, which also can explain the increase in vector infectivity (Higashikawa and Chang, 2001). We also have shown that the production of vectors in serum-free medium moderately increases vector infectivity (Fig. 10A and B), suggesting that the presence of serum during vector production may be involved in the generation of negative regulators of transduction.

In addition to assessing all variables involved in the production of lentivectors by transient transfection as described above, we also analyzed the effects on titer and infectivity of altering the transfer vector construct. To begin, we determined that neither the type of long terminal repeat (Fig. 4) nor the internal promoter (Fig. 5) included in the vector significantly alters titer or infectivity. We also have demonstrated that lentivectors carrying three different transgene sequences have normal infectivities, with the exception that neo^R-expressing vectors may have slightly decreased infectivities (Fig. 6), although it is possible that the latter finding is attributable to a lower sensitivity of the G418 resistance assay compared with direct detection of eGFP or hCD80 expression. These findings are in accord with those of Lizée *et al*. (2003), who found that vectors expressing eGFP, tyrosinase, or neo-poly(A) polymerase all had roughly the same infectivities.

The only component of transfer vector design that we have identified as having a significant effect on the infectivity of lentivectors is inclusion of the central polypurine tract (cPPT), which increases vector infectivity by about 6-fold on 293 cells and more than 2-fold on $CD34^+$ HPCs. The cPPT is normally found in the *pol* region of the HIV-1 genome and, in conjunction with the central termination sequence, serves the purpose of directing the formation of a DNA flap during reverse transcription (Follenzi *et al*., 2000). It has been suggested that the DNA flap interacts with host factors to favor translocation of the vector preintegration complex across the nuclear membrane (Zennou *et al*., 2000). Our finding of increased lentivector infectivity when the cPPT is present in the transfer vector is in agreement with the work of Sirven *et al*. (2000), who demonstrated that the presence of the cPPT in lentivector constructs increased the transduction of $CD34^+$ HPCs by up to 4-fold. It should be noted, however, that when we matched the non-cPPT-carrying and cPPT-carrying vector supernatants by infectious unit titer instead of p24 content, we saw no difference in the level of $CD34^+$ HPC transduction, indicating that the relative transduction efficiencies of the two vector types are similar in both cell types.

Finally, we also evaluated the infectivity of lentivectors pseudotyped with the RD114/TR feline endogenous virus envelope. We found that vectors bearing the RD114/TR pseudotype had infectivities on 293 cells that were below the normal range that we established for VSV-G-pseudotyped vectors. Others have also mentioned in their work that RD114-pseudotyped vectors appeared to have lower infectivities when compared with those made with the VSV-G envelope in standard titering assays on adherent fibroblast or epithelial cell lines (Strang *et al*., 2004). It has previously been demonstrated, however, that the RD114 pseudotype is superior to VSV-G for the transduction of human umbilical cord blood $CD34^+$ HPCs. This is true for both MMLV (Kelly *et al*., 2001) and HIV-1 (Hanawa *et al*., 2002; Sandrin *et al*., 2002) vectors bearing the RD114 pseudotype. Unlike VSV-G, which binds to a ubiquitous lipoprotein component in the cell membrane (Mastromarino *et al*., 1987), RD114 must interact with a specific receptor protein in order for vectors bearing this envelope to gain entry into cells. The specific receptor for RD114 is the human neutral amino acid transporter (hACT0, also known as hASCT-2 or RDR), which was originally shown by Tailor *et al*. (1999) to be expressed in nearly all tissues, and more recently has been characterized with a specific antibody to be expressed on most tissues but at widely disparate levels among individuals (Green *et al*., 2004). Thus, we hypothesize that our finding of low infectivity of RD114/TR-pseudotyped lentivectors, when titered on 293 cells, is most likely a result of low expression of the specific receptor protein hASCT-2 on these cells. Cord blood $CD34^+$ HPCs, on the other hand, are likely easily transduced by RD114/TR-pseudotyped vectors because of a high level of expression of hASCT-2, although, we have not specifically verified that hypothesis.

In conclusion, titering vectors by measuring transgene expression remains the most useful method for characterizing the performance of a given vector, but may not always be possible. Quantitative PCR (q-PCR) methods for titering vectors are also useful when the appropriate equipment and primer sets are available (Sastry *et al*., 2002; Lizée *et al*., 2003); however, the instrumentation expense and difficulty of establishing effective primer sets for different vector constructs may deter some investigators from using this method. In addition, discrepancies between gene transfer titers as determined by q-PCR and titers determined by gene expression will have to be kept in mind. We have shown that titering HIV-1-based lentivectors by p24 immunoassay offers a reasonable degree of accuracy (within a roughly 2-fold range) provided that vectors are of similar design in terms of carrying or lacking the cPPT. In addition, the p24 immunoassay is not adversely affected by fluctuations in the vec-

tor preparation protocol or most transfer vector design modifications, making it an expedient method for titering lentivectors of similar design for use in preclinical gene therapy studies.

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