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# Requirements for Efficient Production and Transduction of Human Immunodeficiency Virus Type 1-Based Vectors

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**A number of human immunodeficiency type 1 (HIV-1)-based vectors have recently been shown to transduce nondividing cells in vivo as well as in vitro. However, if these vectors are to be considered for eventual clinical use, a major consideration is to reduce the probability of unintended generation of replication-competent virus. This can be achieved by eliminating viral genetic elements involved in the generation of replication-competent virus without impairing vector production. We have designed a system to transiently produce HIV-1-based vectors by using expression plasmids encoding Gag, Pol, and Tat of HIV-1 under the control of the cytomegalovirus immediate-early promoter. Our data show that the best vector yield is achieved in the presence of the Rev/Rev-responsive element (RRE) system. However, the constitutive transport element of Mason-Pfizer monkey virus can substitute for RRE and Rev at least to some extent, whereas the posttranscriptional regulatory element of human hepatitis B virus appeared to be inefficient. In addition, we show that high-titer virus preparations can be obtained in the presence of sodium butyrate, which activates the expression of both the packaging construct and the vector genome. Finally, our results suggest that efficient infectivity of vectors defective in the accessory proteins Vif, Vpr, Vpu, and Nef depends on the nature of the target cells.**

Vectors derived from Moloney murine leukemia virus (MLV) are widely used in gene delivery and human gene therapy studies. Most mammalian cells express the MLV amphotropic receptor on the cell surface, allowing vector entry (8). However, the nuclear entry of the vector preintegration complex depends on cell mitosis, probably due to nuclear membrane breakdown (25). Thus, MLV-based vectors efficiently infect only proliferating cells and not quiescent cells. This property severely limits the general use of retroviral vectors for direct gene delivery in vivo, since a majority of the cells either are terminally differentiated or remain in the quiescent state without stimulation. In contrast to MLV, lentiviruses can infect and integrate their genomes into the chromosomes of nondividing cells. In the case of human immunodeficiency virus type 1 (HIV-1), the capability of infecting quiescent cells maps to three viral proteins present in the HIV-1 preintegration complex, namely, the matrix (MA) portion of the Gag protein, the integrase, and the Vpr protein (7, 11–13, 18). To date, the relative importance of these apparently redundant functions is unknown. Recently, HIV-1-based vectors pseudotyped with heterologous envelopes, notably the G protein of vesicular stomatitis virus (VSV), have been shown to infect a wide array of quiescent cell types, including fibroblasts and primary monocyte-derived macrophages in culture as well as hepatocytes, myocytes, photoreceptor cells in retina, and neuronal cells in brain in vivo (5, 22, 29, 30, 46). MLV vectors, under similar conditions, fail to infect these cell types efficiently. These observations demonstrate the potential advantages of using lentivirus vectors for direct in vivo gene delivery.

Most of the HIV-1-based vector production systems reported to date consist of the cotransfection into 293T cells of three plasmid constructs: (i) a packaging construct containing all HIV genes except the *env* gene, (ii) an expression plasmid for the VSV G protein to confer a broad host tropism to the vector, and (iii) an HIV vector containing the gene of interest. When considering using HIV-1-based vectors for gene therapy, one needs to address the possibility of generating replication-competent HIV-1 during vector production. The major route for generating replication-competent retrovirus with such a system would be through homologous recombination events occurring among these plasmid constructs during transfection. Safety would be greatly improved by deleting regions of the viral genome that are not absolutely required for vector production or for efficient infection of the target cells. In addition, elimination of genes nonessential for HIV vector production and infectivity will facilitate the establishment of stable packaging cell lines for HIV vectors.

Apart from the *env* gene, which is inactivated since the particles are pseudotyped with the VSV G protein, the obvious target sequences to be eliminated are the HIV-1 genes encoding the accessory proteins. The HIV-1 genome encodes two regulatory proteins, Tat and Rev, as well as four accessory proteins, namely, Vif, Vpr, Vpu, and Nef. Although transcription initiation from the HIV long terminal repeat (LTR) depends on Tat, insertion of an enhancer from the cytomegalovirus (CMV) immediate-early gene bypasses the Tat requirement for HIV gene expression (23). However, recent data demonstrates that Tat is essential for the HIV life cycle at a postentry step in the target cell, suggesting that Tat expression in the packaging cell lines not only can increase vector titers but also may enhance infection of the target cells (17, 20). Rev binds the Rev-responsive element (RRE) within the *env* gene in the viral mRNAs and thereby increases transportation of unspliced or singly spliced HIV-specific mRNA from the nucleus into the cytoplasm (9, 26). Although this is an essential function for HIV-1 replication, other RNA transport elements have been shown to substitute for the RRE function (6, 19). Most importantly, the function of these elements relies on endogenous factors rather than on Rev. Bypassing the RRE and Rev requirement may thus eliminate the need for the stable expression of HIV Rev in the packaging cell lines. The Vif, Vpr, Vpu,

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and Nef proteins have been shown to be dispensable for HIV replication in immortalized cell lines (28). In addition, it has recently been shown that none of the accessory proteins is required for efficient HIV-based vector production from transfected 293T cells (22, 23, 46).

In the present study, we evaluated more precisely the requirement for Rev and the accessory proteins, Vif, Vpr, Vpu, and Nef, for the production of high-titer HIV-1-based vectors and efficient transduction of target cells. We have also tested heterologous mRNA transport elements derived from Mason-Pfizer monkey virus (MPMV) and hepatitis B virus (HBV) for their ability to substitute for the function of Rev and RRE. Our results show that while the absence of the accessory proteins has little effect on vector production, the presence of sodium butyrate, which activates the CMV enhancer and the HIV LTR promoter function (24, 39), can significantly increase vector titers from the transfected 293T cells. While the MPMV constitutive transport element (CTE) can substitute for the function of RRE and Rev, the vector can be generated most efficiently only from a packaging plasmid containing the RRE sequence. Finally, while the absence of accessory proteins in the vector shows little effect on infectivity in most cell types, these proteins enhance significantly the infectivity of HIV vector in quiescent primary human skin fibroblasts. Our studies should facilitate the establishment of stable packaging cell lines for the production of high-titer, helper-free HIV vectors for human gene therapy.

## MATERIALS AND METHODS

**Plasmid construction.** To construct pCMV-HIV-1, the 0.7-kb *Bam*HI-*Sph*I fragment with a 19-bp deletion in the putative packaging signal of pCMVΔP1ΔenvpA (31) was fused with the 8-kb *Sph*I-*Hin*dIII (from position 1447 to 9606) fragment of pNL4-3 (1) and the 4-kb *Sal*I-*Eco*RI fragment from pCMV-G (45). In addition, a deletion of the 580-bp *Bgl*II (position 7031 in pNL4-3)-*Bgl*II (position 7611 in pNL4-3) fragment was created in the HIV-1 Env-coding region to eliminate the expression of this protein and reduce the potential of generating helper virus during vector production. To generate pCMV-HIVnef(−), the sequence between the *Hpa*I site at position 8650 in pNL4-3 and the *Hin*dIII site at position 9606 in pNL4-3 of pCMV-HIV-1 was deleted. To generate pCMV-HIVvif(−), pCMV-HIV-1 was digested with *Nde*I (position 5123 in pNL4-3) and repaired with the Klenow fragment to create a 2-bp insertion in the coding region of the *vif* gene. To generate pCMV-HIV-vpu(−), the initiation codon of Vpu was mutated by site-directed mutagenesis (Mutagene kit; Bio-Rad, Hercules, Calif.) with the oligonucleotide 5′TGCTAC TATTATAGGTTGTACATGTACTACTTACTG3′. To generate pCMV-HIV-vpr(−), pCMV-HIV-1 was digested with *Eco*RI (position 5747 in pNL4-3) and repaired with the Klenow fragment to generate a 4-bp insertion in the coding region of the *vpr* gene. The pCMV-HIVvpr(−)nef(−) double mutant was generated by digesting pCMV-HIVnef(−) with *Eco*RI and repaired with the Klenow fragment as described above.

To generate pCH-GP-1, the 0.66-kb fragment between position 766 and the *Sph*I site at position 1447 in pNL4-3 was amplified by PCR with the oligonucleotides Gag5′ (5′GAGGATCCTAGAAGGAGAGAGATGGGT3′) and Gag3′ (5′GAGGATCCAATAGGCCCTGCATGCACTG3′). The resulting fragment was ligated with the 3.7-kb *Sph*I-*Nde*I fragment from pNL4-3 and the 4-kb *Sal*I-*Eco*RI fragment from pCMV-G. To generate pCHGP-2, the RRE (between positions 7754 and 8013 in HXB-2 [33]) was amplified by PCR from pv653RSN (31) by using the oligonucleotides RRE5 (5′GCAAGCTTCTGCAGAGCAGT GGGAATAGG3′) and RRE3 (5′GCAAGCTTACCCCAAATCCCCAGGAG CTG3′) and cloned immediately after the *gag-pol* gene in pCHGP-1. To generate pCHGP-3, the 0.65-kb *Stu*I-*Hin*dIII fragment from pCCAT-1 (44) was cloned after the *gag-pol* gene in pCHGP-1. To generate pCHGP-4, the MPMV CTE (between positions 8020 and 8240 in MPMV [40]) was amplified by PCR from pGem7 fz(−) MPMV(8001-8240) (6) and cloned behind the *gag-pol* gene in pCHGP-1.

To generate pv653CMVβ-gal, a CMV β-galactosidase (β-gal) cassette was first constructed by ligating the 0.75-kb *Xba*I-*Sal*I fragment containing the CMV promoter from pCMV-G to the 3.1-kb *Xba*I-*Sma*I fragment containing the β-gal gene from pSP6-β-gal (32) with pBluescript SK(−) (Stratagene, La Jolla, Calif.) to generate pCMVβ-gal. The 3.8-kb *Not*I-*Sma*I fragment containing the CMV β-gal cassette from pCMVβ-gal was ligated with the 8-kb fragment from *Bam*HI-digested pv653RSN. To generate pCMV-Tat, pCMV-G was digested with *Xho*I, and the 4.7-kb fragment containing the CMV promoter was ligated with the 0.36-kb *Sal*I-*Bam*HI fragment containing the Tat-coding region from pCV1 (4). To generate pCMV-env, the 2.7-kb *Xba*I fragment from pCMVEnv-amDra containing the amphotropic envelope coding region was ligated with the 4.7-kb *Bam*HI fragment from pCMV-G. The construction of pCMV-G has been described previously (45). pCMV-rev was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program.

**Cells.** HeLa, HT1080, and 293T cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (FCS). SupT1 cells were maintained in RPMI 1640 medium supplemented with 10% FCS. Primary human fibroblast CCD 1059sk cells (obtained from the American Type Culture Collection; no. CRL2072) were grown in Eagle's minimum essential medium supplemented with 10% FCS. Quiescent CCD 1059sk cells were obtained by growing confluent cells for 5 to 10 days in minimum essential medium with 10% FCS, resulting in a population of cells in growth arrest at the $G_0/G_1$ phase as determined by flow cytometry analysis. Quiescent HeLa cells were obtained by plating $2 \times 10^5$ cells in each well of a 12-well plate 24 h prior to gamma irradiation. The cells were subjected to gamma irradiation at a dose of 4,000 rads, and approximately 90% of the cells were arrested at $G_2$ phase 3 days after irradiation as determined by flow cytometry analysis.

**Vector production and infection of target cells.** To generate infectious HIV vectors, 293T cells were seeded at a density of $4 \times 10^6$ cells per 10-cm-diameter culture dish. The infectious vector with all of the accessory proteins was generated by cotransfecting 10 μg of pCMV-HIV-1, 10 μg of pCMV-G, and 20 μg of pv653CMVβ-gal by the calcium phosphate coprecipitation method (16). The culture medium was replaced 6 to 8 h later, and the culture supernatant was collected 18 h after transfection, filtered through 0.45-μm-pore-size filters, and stored at −80°C. To generate the vector without any accessory protein, 293T cells were cotransfected with 8 μg of pCHGP plasmid series, 8 μg of pCMV-G, 16 μg of pv653CMVβ-gal, 4 μg of pCMV-Tat, and 4 μg of pCMV-Rev. 293GP/LCZL cells containing an MLV-based provirus with the CMV β-gal cassette were used in this study. The VSV G protein-pseudotyped MLV vector was generated as described before (45).

To determine the vector titer, $5 \times 10^4$ HT1080 cells were plated in a 12-well plate in the presence of 8 μg of Polybrene per ml 24 h prior to infection. The cells were infected overnight with various dilutions of the vector and assayed for β-gal activity 48 h after infection.

To assay for β-gal activity, cells were washed once with phosphate-buffered saline, fixed in 1.25% glutaraldehyde for 15 min, and stained for 4 h at 37°C in a solution containing 5 mM potassium ferriferrocyanide, 400 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (GBT, St. Louis, Mo.) per ml, and 1 mM $MgCl_2$.

**Helper virus assays.** To detect helper virus, SupT1 cells were infected with $6 \times 10^6$ infectious vector particles containing all accessory proteins or $6 \times 10^5$ vector particles containing no accessory proteins in the presence of 8 μg of Polybrene per ml in T75 flasks. Passage of the infected cells was allowed to continue for 10 weeks. During each passage, the culture supernatant was harvested and stored at −80°C. The p24 level in the supernatant was determined by an enzyme-linked immunosorbent assay (Coulter Corporation, Miami, Fla.). No replication-competent virus was detected after 10 weeks of culture.

## RESULTS

**Effect of accessory proteins on vector production.** To generate an infectious HIV-1-based vector containing all accessory proteins, 293T cells were cotransfected with the three constructs shown in Fig. 1A. As shown in Table 1, the three-plasmid cotransfection resulted in the production of a vector titer of $3.9 \times 10^6$ infectious units (IU)/ml. To determine whether the accessory proteins have any effect on vector production, the gene encoding each of the four accessory proteins in pCMV-HIV-1 was mutated. In addition, a combination of both Vpr and Nef mutations was introduced into pCMV-HIV-1. As shown in Table 1, mutations in the accessory proteins had very little effect on vector production from transiently transfected 293T cells. After 10 weeks of culture of cells transduced with high-titer vector, no helper virus could be detected in any experiment by screening for p24 expression.

**Effect of RNA transport elements on vector production.** To address the question of whether other RNA transport elements can substitute for the RRE and Rev function, a series of pCHGP plasmids were constructed (Fig. 1B). pCHGP-1 contains the HIV-1 *gag* and *pol* genes under the control of the CMV promoter. The HIV RRE was inserted immediately downstream of the *gag-pol* gene to generate pCHGP-2 to facilitate the *gag-pol* RNA exit from the nucleus. In pCHGP-3 and pCHGP-4, the posttranscriptional regulatory element

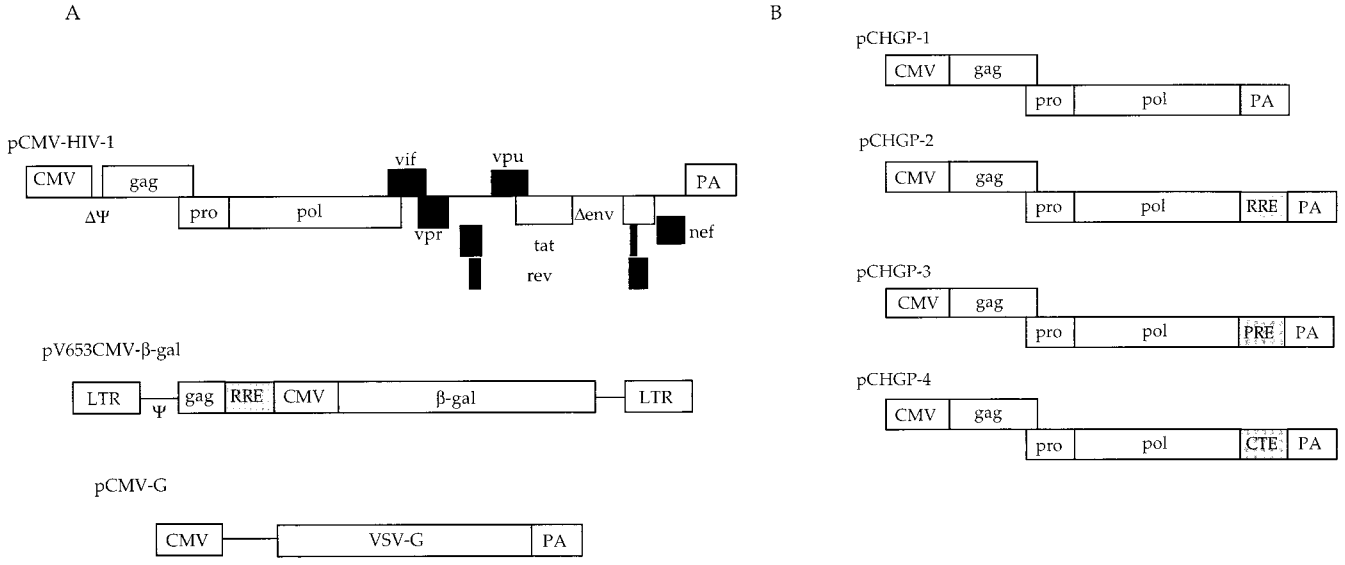


FIG. 1. Structures of expression plasmids of the HIV-1-based vector production system. (A) Packaging system; (B) minimal *gag-pol* constructs. Expression of packaging and VSV G protein (VSV-G) envelope constructs is driven by the CMV immediate-early promoter (CMV). The polyadenylation signal (PA) is derived from the rabbit β-globin gene. pCMV-HIV-1 contains all the HIV-1 genes except a 19-bp deletion in the packaging signal (ΔΨ) and a 600-bp deletion in the *env* open reading frame (Δenv). Accessory-protein open reading frames are shown as solid boxes. The RNA transport elements are presented as shaded boxes. The vector genome pv653CMV-β-gal consists of the HIV-1 LTRs flanking the RRE and the reporter gene (β-gal) driven by the CMV promoter.

(PRE) from HBV and the CTE from MPMV, respectively, were inserted immediately downstream of the *gag-pol* gene.

To test whether these constructs produce HIV *gag-pol* particles, they were cotransfected into 293T cells with or without pCMV-Rev, and the p24 level in the culture supernatant was determined 48 h after transfection. As shown in Table 2, pCHGP-1 generates a low level of p24 with or without Rev, consistent with the observation that the RRE is required for efficient transportation of the HIV *gag-pol* transcript from the nucleus into the cytoplasm. The presence of the RRE in pCHGP-2 results in 30-fold stimulation of p24 production when Rev is coexpressed. The HBV PRE transport element did not have any stimulating effect on p24 expression; however, in the presence of Rev, a 3.5-fold increase in the p24 level relative to that for pCHGP-1 was observed. Finally, the presence of the CTE from MPMV increases the p24 level seven- to ninefold relative to that of pCHGP-1-transfected cells. As expected, the stimulation of p24 production by the CTE is not Rev dependent. Therefore, the CTE is able, to some extent, to substitute for the Rev and RRE requirement in our system.

To test whether these packaging plasmids generate infectious vectors in the absence of accessory protein, each of the constructs from the pCHGP series was cotransfected with pCMV-G, pv653CMVβ-gal, and pCMV-Tat, which is required for the efficient expression of the genomic RNA derived from pv653CMVβ-gal. In addition, pCMV-Rev was either included or not included in each cotransfection experiment to determine the effect of Rev on the vector titer. Vector particles generated 24 to 48 h after transfection were harvested, and titers were determined in HT1080 cells by X-Gal staining and counting of positive blue cells.

As shown in Table 3, pCMV-HIV-1 generated a titer of about $10^7$ IU/ml, and the titer was not affected by cotransfection of pCMV-Rev. Cells transfected with pCHGP-1 generated low titers with or without Rev. In contrast, cells cotransfected with pCHGP-2 and pCMV-Rev generated a titer which was at least 3 orders of magnitude higher than that without pCMV-Rev. For pCHGP-3, a very low vector titer was obtained from the transfected cells, consistent with the low p24 level derived from pCHGP-3-transfected cells (Table 2). Despite the small effect of Rev on the p24 level generated from pCHGP-4-transfected cells (Table 2), the vector titer derived from this construct increased more than 50-fold with cotransfection of pCMV-Rev. This increase in titer with Rev probably reflects the fact that Rev stimulates the transportation of the vector genomic RNA derived from pv653CMVβ-gal into the cytoplasm, thereby allowing more viral RNA to be packaged into virions. Overall, the vector titer in Table 3 corresponds to the p24 level generated by each packaging construct shown in Table 2. These results demonstrate that the CTE from MPMV can substitute for the function of RRE and Rev to facilitate HIV gene expression, whereas the PRE from HBV fails to perform this function. However, a combination of the RRE and Rev is able to generate a vector titer which is at least 10-fold higher than that generated by the CTE (compare vector titers of pCHGP-2 and pCHGP-4 in Table 3).

**Sodium butyrate stimulates vector production from 293T cells.** Sodium butyrate has been shown to stimulate the activity of the HIV-1 LTR and the CMV immediate-early promoter (24, 39). To determine whether sodium butyrate had any effect on vector production, vector particles were generated from v653CMVβ-gal in the presence of various concentrations of sodium butyrate. As shown in Fig. 2, addition of sodium bu-

TABLE 1. Effect of HIV-1 accessory proteins on vector production

| pCMV-HIV | Titer (IU/ml)[a] |
|---|---|
| Wild type | $(3.9 \pm 0.7) \times 10^6$ |
| vpr(−) | $(8.3 \pm 2.0) \times 10^6$ |
| vpu(−) | $(4.8 \pm 1.0) \times 10^6$ |
| vif(−) | $(6.0 \pm 0.7) \times 10^6$ |
| nef(−) | $(1.1 \pm 0.1) \times 10^7$ |
| nef(−)vpr(−) | $(3.1 \pm 0.3) \times 10^6$ |

[a] The vectors were harvested 24 h after transfection, and the titer was determined in HT1080 cells by counting of blue cells after X-Gal staining. The numbers are the averages from triplicate experiments ± standard deviations.

TABLE 2. Expression of p24 in pCHGP-transfected cells[a]

| Plasmid construct | pCMV-Rev | p24 (ng/ml)[b] |
|---|---|---|
| pCHGP-1 | − | 1.4 ± 0.2 |
| | + | 1.8 ± 0.1 |
| pCHGP-2 | − | 3.5 ± 0.2 |
| | + | 98.8 ± 14.6 |
| pCHGP-3 | − | 0.4 ± 0.1 |
| | + | 6.2 ± 0.7 |
| pCHGP-4 | − | 10.2 ± 0.8 |
| | + | 16.4 ± 1.0 |
| pCMV-HIV-1 | − | 6,958 ± 349 |
| | + | 7,471 ± 287 |

[a] 293T cells were transfected in a 10-cm-diameter culture dish with 20 μg of pCHGP as indicated and 10 μg of either pCMV-Rev (+) or pBluescript (−). Forty-eight hours after transfection, the culture medium was assayed for p24 protein expression.

[b] Averages from triplicate experiments ± standard deviations.

TABLE 3. Vector production from pCHGP-transfected 293T cells

| Plasmid construct | pCMV-Rev | Titer (IU/ml)[a] |
|---|---|---|
| pCHGP-1 | − | $(1.4 \pm 0.3) \times 10^2$ |
| | + | $(2.5 \pm 1.1) \times 10^2$ |
| pCHGP-2 | − | $(1.0 \pm 0.6) \times 10^2$ |
| | + | $(6.0 \pm 0.6) \times 10^5$ |
| pCHGP-3 | − | $(1.1 \pm 0.4) \times 10^1$ |
| | + | $(3.6 \pm 0.2) \times 10^2$ |
| pCHGP-4 | − | $(8.4 \pm 1.2) \times 10^2$ |
| | + | $(5.3 \pm 1.7) \times 10^4$ |
| pCMV-HIV-1 | − | $(1.2 \pm 0.4) \times 10^7$ |
| | + | $(1.1 \pm 0.1) \times 10^7$ |

[a] The infectious vector was generated as described in Materials and Methods. Titers were determined in HT1080 cells. The numbers are the averages from triplicate experiments ± standard deviations.

tyrate had little effect on the vector titer generated from pCMV-HIV-1-transfected cells, whereas the titer generated from pCHGP-2-transfected cells increased approximately 15-fold in the presence of 4 mM sodium butyrate. To determine the possible reason for the titer increase, the p24 level in the culture supernatant of transfected 293T cells was determined. The p24 level of the pCMV-HIV-1-transfected cells increased slightly, from 6.6 μg/ml, with a titer of $6.0 \times 10^6$ IU/ml, in the absence of sodium butyrate to 9.9 μg/ml, with a titer of $1.1 \times 10^7$ IU/ml, in the presence of 4 mM sodium butyrate. However, the p24 level of the pCHGP-2 transfected cells increased from 0.1 μg/ml, with a titer of $4.4 \times 10^5$ IU/ml, in the absence of sodium butyrate to 1.1 μg/ml, with a titer of $6.4 \times 10^6$ IU/ml, in the presence of 4 mM sodium butyrate. Thus, the 10-fold increase in p24 production in pCHGP-2-transfected cells in the presence of sodium butyrate can account for the observed 15-fold increase in the vector titer shown in Fig. 2. The lack of stimulation in pCMV-HIV-1-transfected cells may reflect the extremely high level of p24 already generated by this construct in the absence of sodium butyrate.

**Effect of accessory proteins on vector infectivity.** To study the ability of the HIV vector to infect quiescent cells and the effect of the accessory proteins on infectivity, HeLa cells were exposed to gamma irradiation to arrest cells at the $G_2$ phase of the cell cycle. Proliferating or growth-arrested HeLa cells were transduced with either MLV-β-gal, a β-gal gene-containing MLV vector; the HIV-1-based vector v653CMVβ-gal(+), containing all four accessory proteins; or v653CMVβ-gal(−), containing no accessory protein. Positive cells were scored by X-Gal staining 2 days after transduction. Results for HeLa cells were expressed as the percentages of titers observed with the same virus preparations in growing HT1080 cells. As shown in Fig. 3, no significant difference in titer was observed in proliferating or quiescent HeLa cells transduced with either the v653CMVβ-gal(+) or the v653CMVβ-gal(−) vector. In contrast, the transduction efficiency of the MLV vector in quiescent cells was reduced more than 2,000-fold. Similar results were obtained with irradiated HT1080 cells transduced with the three vectors (data not shown). To demonstrate that the observed β-gal activity is not due to pseudotransduction of the β-gal activity present in the vector preparation, proliferating HeLa cells transduced with the vector were treated with increasing concentrations of 3′-azido-3′-deoxythymidine. Both the number of blue cells and the β-gal activity in cell extracts decreased with increasing concentrations of 3′-azido-3′-deoxythymidine (data not shown). These results demonstrate that, in contrast to the MLV vector, HIV-1-based vectors can transduce quiescent cells efficiently and the HIV-1-encoded accessory proteins are not required to transduce these cells.

To test the infectivity of HIV-1-based vectors in other cell types, primary human skin fibroblasts were allowed either to proliferate or to grow to confluency and then were infected with the three retroviral vectors described above. Fibroblasts grown to confluency become contact inhibited and arrested in the $G_0/G_1$ phase of the cell cycle (data not shown). As shown in Fig. 4, the three vectors exhibit similar transduction efficiencies in dividing fibroblasts. However, in quiescent cells, MLV-β-gal vector transduction dropped to barely detectable levels. The capacity of v653CMVβ-gal(+) remained unchanged. In contrast, v653CMVβ-gal(−), which is defective for the HIV-1 accessory proteins, showed a four- to sevenfold-decreased level of efficiency in transducing the contact-inhibited fibroblasts relative to the v653CMVβ-gal(+) vector. These results suggest that the requirement for accessory proteins for efficient transduction by HIV-1-based vectors is cell type dependent.

## DISCUSSION

The ability of HIV-based vectors to infect terminally differentiated cells and quiescent cells makes them suitable vectors for direct in vivo gene delivery. However, large-scale production and purification of HIV vectors require the establishment of stable packaging cell lines. Since HIV-1 encodes at least nine proteins and stable expression of some of these proteins, such as Vpr, is known to be extremely toxic to the cells (21, 34), selective expression of only those proteins absolutely essential for vector production and infectivity is important.

Consistent with previous studies, transient transfection into 293T cells of all the required components for HIV production resulted in high-titer vector production in the present study. Mutation of the genes encoding HIV-1 accessory proteins has little effect on vector production. Efficient HIV-1 infection requires the presence of the Nef protein, which appears to facilitate virus capsid disassembly upon infection (3, 38). The requirement for Nef for virus uncoating can be bypassed if the virus is pseudotyped with VSV G protein (2), which may explain why there was no significant titer reduction in the present study. Vpu has been shown to facilitate the release of budding

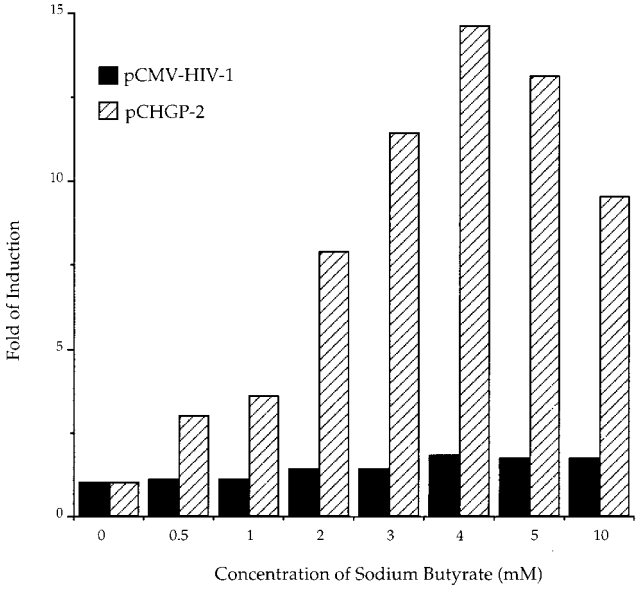


FIG. 2. Stimulation of vector production by sodium butyrate. Vectors derived from either pCMV-HIV-1 or pCHGP-2 were generated in 293T cells in the presence of various concentrations of sodium butyrate as indicated. Titers were determined in HT1080 cells as described in Materials and Methods. Values are the ratios of titers with sodium butyrate to titers without sodium butyrate for each vector. This experiment was repeated once, and similar results were obtained (data not shown).

virus particles from the surfaces of infected cells (42). The enhancement of capsid release is cell type dependent and is not limited to HIV; it can also facilitate the release of visna virus and MLV from infected cells (15, 37). The Vpu-deficient vector is generated from 293T cells by transient transfection. Since 293T has an extremely high transfection efficiency, this procedure presumably produces excessive amounts of the vector

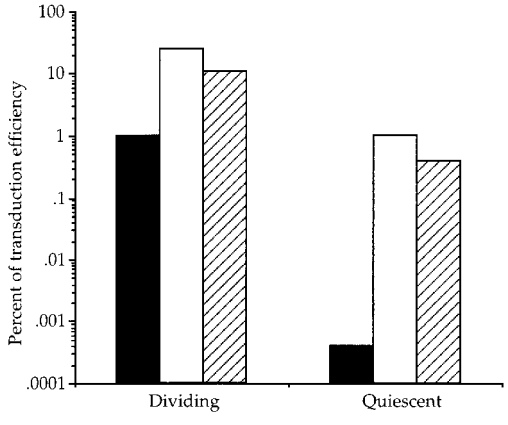


FIG. 3. Transduction efficiency in HeLa cells. Three hundred microliters of MLV-β-gal with a titer of $2.8 \times 10^6$ IU/ml (■), v653CMVβ-gal(−) with a titer of $1.4 \times 10^6$ IU/ml (▨), or v653CMVβ-gal(+) with a titer of $3.7 \times 10^6$ IU/ml (□) was used to transduce actively dividing or growth-arrested HeLa cells in 12-well plates. The cells were harvested 2 days after transduction, and the total β-gal activity was determined by blue-cell count after X-Gal staining. The results are presented as the percentage of the vector titer observed in HT1080 cells for each viral preparation ([titer in dividing or quiescent HeLa cells/titer in HT1080 cells] × 100). The experiment was repeated with 100 and 30 μl of the same vector stocks, and similar results were observed. Transduction of the HeLa cells with different vector stocks was repeated at least twice, and similar results were obtained (data not shown).

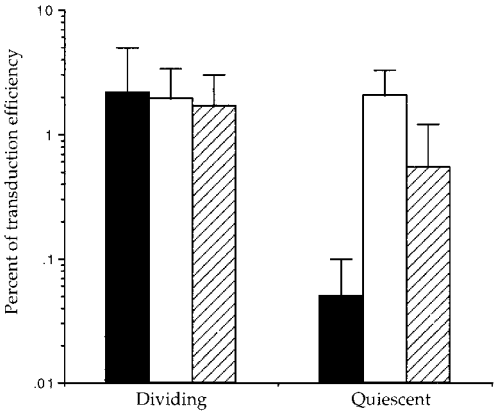


FIG. 4. Transduction efficiency in human skin fibroblasts. Ten microliters of MLV-β-gal (■), v653CMVβ-gal(−) (▨), or v653CMVβ-gal(+) (□) was used to infect dividing and quiescent fibroblasts in a 12-well plate. Two days after transduction, the titer was determined by blue-cell counting after X-Gal staining. Data for transduction of quiescent and dividing fibroblasts are presented as the percentage of the titer observed in growing HT1080 cells for each viral preparation ([titer in growing or quiescent fibroblasts/titer in HT1080 cells] × 100). The values are averages from four experiments, with standard deviations.

genome as well as HIV-encoded proteins, which may make the Vpu effect negligible. Alternatively, Vpu may have no effect on virus release from 293T cells, as observed for a number of other cell lines (36). The Vpr protein, like HIV MA and integrase, contains a nucleophilic determinant that permits nuclear localization of viral nuclear capsid and replication in nondividing cells (18). Mutation of Vpr, however, has no effect on the virus infectivity in proliferating cells such as the growing HT1080 cells used to determine the titer of the Vpr-deficient vector. Vif acts during virus assembly to make the virus particle competent for subsequent infection (43). However, this effect is dependent on the cell type from which the virus is generated (10). The absence of a titer decrease for the Vif-deficient vector may reflect the permissiveness of 293T cells to complement the Vif defect.

When the minimal pCHGP-2 packaging construct is used, our results showed, as predicted, that p24 expression from transfected cells is strongly dependent on the presence of Rev. Interestingly, when the HBV PRE transport element was used, its mRNA-transporting activity appeared to be strongly increased when the Rev protein was coexpressed, although it was still considerably lower than that of the RRE-Rev system. This transactivating property was not observed when the RevM10 (27) transdominant negative mutant was used (14). In addition, it has been suggested that HBV and RRE mRNA transport from the nucleus to the cytoplasm could involve the same pathway (35). Together, these data suggest that Rev could enhance PRE transport activity directly by binding the PRE sequence or indirectly by recruiting cellular factors involved in the nuclear export mechanism. Insertion of the HBV PRE into the second intron of HIV-1 has been shown to increase the accumulation of the unspliced RNA in the cytoplasm, demonstrating that this element can facilitate RNA exit from the nuclei (19). However, the present study demonstrates that the PRE from HBV slightly increases p24 expression or vector production from the transfected cells only when Rev is expressed. Therefore, PRE, as a transport element, does not appear to be useful for our purpose to bypass the HIV-1 Rev protein requirement.

In contrast, pCHGP-4, which contains the CTE derived from the MPMV genome, enables Rev-independent expres-

sion of p24, as it is not stimulated significantly by cotransfection of pCMV-Rev. However, the p24 level derived from cells transfected with pCHGP-4 is at least fivefold lower than that with pCHGP-2 in the presence of Rev. In these experiments, pCHGP-2 was cotransfected with pCMV-Rev, and as a result, large amounts of Rev may be available in the transfected cells to efficiently transport RRE-containing transcripts from nuclei into the cytoplasm. In contrast, the CTE in pCHGP-4 interacts with endogenous factors which may be in limited supply compared with Rev generated from transient transfection. One such factor interacting with the CTE of MPMV has recently been identified as ATP-dependent RNA helicase A (41). Thus, a difference in the endogenous levels of the protein factors required for efficient transportation of either RRE- or CTE-containing transcripts may account for the observed difference in p24 expression of these two constructs. Alternatively, the RRE and Rev may be intrinsically more efficient than the CTE to transport the HIV-encoded messages. The efficiency of transporting HIV-encoded messages by either the RRE or CTE can be elucidated with cell lines stably expressing the HIV Rev protein.

Our results demonstrate that HIV-derived vectors transduce nonproliferating cells efficiently, whereas the MLV vector fails to give detectable transduction. This observation is consistent with the fact that at least three nuclear localization signal-containing proteins, including MA, Vpr, and integrase, are present in the lentivirus particle, and these proteins facilitate active transport of the nucleocapsid from the cytoplasm into the nuclei of the infected cells. The functional redundancy of these proteins may explain why accessory protein-deficient vector particles can still infect growth-arrested HeLa cells at the same efficiency as vector particles containing all of the accessory proteins. These results are consistent with the recent observations reported by others that the accessory proteins are not required for efficient infection of growth-arrested 293T cells (23, 46). However, the infectivity of the accessory protein-deficient vector in contact-inhibited primary human skin fibroblasts is reduced approximately threefold compared with that of the vector containing all of the accessory proteins. Our results suggest that for efficient infection of this cell type, the accessory proteins, either alone or in combination, are beneficial. We conclude that the effect of HIV accessory proteins on infectivity is dependent on the cell type or cell proliferation state. This conclusion is in agreement with the results reported by Zufferey et al. (46) and Kafri et al. (22) that for efficient infection of human macrophages in culture and adult mouse livers in vivo, the presence of accessory proteins enhances infectivity, whereas they are dispensable for efficient infection of neuronal cells in vivo.

High-titer HIV-1 vectors have so far been generated from 293T cells by transient transfection. Although this method is convenient, it is not suitable for mass vector production for clinical application. Establishment of stable packaging cell lines for HIV vectors not only overcomes the mass production problem, but it will also make helper virus generation unlikely because multiple homologous recombination events are required for such an event to occur. Such events occur more frequently before stable transfection and integration take place. Our results suggest that while the CTE can partially alleviate the requirement for stable Rev expression in the packaging cell lines, a combination of Rev and the RRE still generates significantly higher vector titers than the CTE. The HIV-1-encoded accessory proteins are not required for efficient vector production from 293T cells, and the infectivity of the resulting vector is similar to that of the vector containing all accessory proteins except in the case of primary human skin fibroblasts. Recent results from Kim et al. (23) suggest that the requirement for Tat can be bypassed by using an HIV vector containing a hybrid HIV LTR with the U3 regions replaced with the CMV promoter. Based on these studies, the indispensable components of the packaging cell lines for HIV vectors should therefore be only *gag*, *pol*, Rev, and VSV G protein. To efficiently infect cell types such as quiescent skin fibroblasts and hepatocytes, some of the accessory proteins will have to be expressed in the packaging cell lines. The present study should help to facilitate the successful establishment of packaging cell lines for HIV-1-derived vectors.

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