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Identification of a Human Immunodeficiency Virus Type 2 (HIV-2) Encapsidation Determinant and Transduction of Nondividing Human Cells by HIV-2-Based Lentivirus Vectors

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Although previous lentivirus vector systems have used human immunodeficiency virus type 1 (HIV-1), HIV-2 is less pathogenic in humans and is amenable to pathogenicity testing in a primate model. In this study, an HIV-2 molecular clone that is infectious but apathogenic in macaques was used to first define *cis*-acting regions that can be deleted to prevent HIV-2 genomic encapsidation and replication without inhibiting viral gene expression. Lentivirus encapsidation determinants are complex and incompletely defined; for HIV-2, some deletions between the major 5′ splice donor and the *gag* open reading frame have been shown to minimally affect encapsidation and replication. We find that a larger deletion (61 to 75 nucleotides) abrogates encapsidation and replication but does not diminish mRNA expression. This deletion was incorporated into a replication-defective, envelope-pseudotyped, three-plasmid HIV-2 lentivirus vector system that supplies HIV-2 Gag/Pol and accessory proteins in *trans* from an HIV-2 packaging plasmid. The HIV-2 vectors efficiently transduced marker genes into human T and monocytoid cell lines and, in contrast to a murine leukemia virus-based vector, into growth-arrested HeLa cells and terminally differentiated human macrophages and NTN2 neurons. Vector DNA could be detected in HIV-2 vector-transduced nondividing $CD34^+$ $CD38^-$ human hematopoietic progenitor cells but not in those cells transduced with murine vectors. However, stable integration and expression of the reporter gene could not be detected in these hematopoietic progenitors, leaving open the question of the accessibility of these cells to stable lentivirus transduction.

Replication-defective retrovirus vectors are advantageous for gene transfer because they permit permanent chromosomal integration and stable gene expression. Following entry into target cells, however, murine retrovirus and retrovirus vectors require mitosis-dependent dissolution of the nuclear envelope to achieve integration (36, 43). Therefore, these vectors can stably transduce dividing cells but possess limited utility for gene delivery to quiescent or postmitotic cells that are important targets for gene therapy.

In contrast, lentiviruses infect nondividing cells (36). For human immunodeficiency virus type 1 (HIV-1), this property has been mapped to establishment of a stable preintegration complex and to virion proteins that mediate transport of the preintegration complex across an intact nuclear envelope (6, 17, 18, 22, 55). Accordingly, retrovirus vectors derived from HIV-1 (35) can transduce growth-arrested and terminally differentiated, postmitotic cells. Naldini et al. (45, 46) established that these lentivirus-specific biological properties hold for HIV-1-derived vectors and showed their capacity for in vitro and in vivo gene delivery.

HIV-1 vectors have now been engineered to reduce both the risks for recombination and the complement of genes needed for transduction of neuronal cells (65). However, some safety concerns remain incompletely explored, since the determinants of the severe pathogenicity of HIV-1 in humans remain uncertain and no animal model amenable to testing of disease causation by the parental lentivirus exists (16). In addition, some of the accessory genes of HIV-1 appear to be required for targeting of some tissues in vivo—*vif* and *vpr* for hepatocytes, for example (25). In this regard, simian immunodeficiency virus (SIV) with multiple nonstructural gene deletions has been shown to cause disease in infant primates (2) and more recently even in adult animals (51).

Safety of HIV-2 vectors can be tested in primates susceptible to HIV-2/SIV pathogenicity (50). In addition, HIV-2 accounts for less than 1% of human HIV infections worldwide and has now been documented to be both less transmissible sexually and less pathogenic in longitudinally studied West African human populations (26, 39). In the most comprehensive, prospective natural history study, all indices of virulence, including HIV-related morbidity and $CD4^+$ lymphocyte depletion, were much lower for HIV-2-infected than HIV-1-infected subjects in the same West African population; 5-year AIDS-free survival was 100% in the HIV-2 cohort (39). These considerations prompted us to study the feasibility of a replication-defective lentivirus vector system derived from HIV-2. To further enhance safety potential, we have used $HIV\text{-}2_{KR}$ (57), a molecular clone that was apathogenic following infection established by high-dose intravenous challenge in pig-tailed macaques and either delayed or prevented disease induction by subsequent challenge with highly virulent $HIV\text{-}2_{EHO}$ (38).

Genomic regions that determine mRNA encapsidation are crucial to retrovirus vector system design but have received very limited study for HIV-2. In the present work, we initially focused on studying the effects of deleting the region between the major splice donor (SD) and the *gag* start codon (following convention, in this paper this segment of retroviral genomes is designated "ψ"). In murine oncoretroviruses such as Moloney murine leukemia virus (Mo-MuLV), ψ is relatively long (351

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nucleotides [nt]), and deletions in ψ markedly attenuate genomic mRNA encapsidation. In addition, attachment of ψ (and more optimally, the ψ′ segment, which includes ψ plus a portion of *gag*) to heterologous test RNAs confers nearly wild-type levels of encapsidation (3, 37). In HIV-1, ψ is considerably shorter (44 nt), and a 21-nt deletion in the region also suffices to greatly reduce or prevent HIV-1 encapsidation (34). However, requirements for HIV-1 encapsidation appear more complex than for murine retroviruses: an important distinction is that neither ψ nor ψ′ from HIV-1 can confer efficient encapsidation when attached to heterologous test RNAs; involvement of regions outside of ψ and ψ′ in HIV-1 RNA packaging have been suggested (4, 5, 10, 20, 27, 35, 40, 62). A complete description of packaging determinants has not been achieved for any lentivirus: interaction of multiple regions distributed widely within the HIV-1 genome has been proposed (5).

HIV-2 packaging determinants are potentially even more complex. For example, deletions in HIV-2 ψ were reported to variably increase or decrease HIV-2 genome encapsidation without inhibiting infectivity and to produce an increase in HIV-2 LTR expression in a transient proviral transfection assay (19). In one study of the closely related lentivirus SIV_{mac}, the leader sequence upstream of the major 5′ SD was reported to be the principal packaging determinant (52). Recently, deletions within the ψ region of HIV-2 were reported to have minimal effects on encapsidation or replication, while regions in the 5′ leader (that are also present in all spliced RNAs) severely reduced *gag*/*pol* mRNA packaging (41); this finding implies the existence of other genomic encapsidation signals, as some means of discriminating the full-length mRNA from spliced viral messages must be available to the packaging machinery. Another level of complexity stems from the functional intron within the HIV-2 long terminal repeat (LTR) (9, 12, 61, 63), a situation which is unique in retroviruses and which provides another potential means of distinguishing genomic from subgenomic mRNAs. In this study, regions of the HIV-2 genome that, when deleted, prevent genomic encapsidation and replication but not viral protein expression were identified.

These data were then used to construct an HIV-2-based retrovirus vector system, and vectors were tested for the ability to transduce dividing, growth-arrested, and terminally differentiated human cells. Aphidicolin-arrested cells, monocyte-derived macrophages, and a terminally differentiated postmitotic human neuronal cell culture model (NTN2 neurons) were transduced efficiently with these vectors. NTN2 neurons are a polarized human neuronal cell system derived from NT2 teratocarcinoma cells by a 6-week process using retinoic acid and several mitotic inhibitors (13, 14). Third-replate cells, used in this study, are irreversibly postmitotic, morphologically resemble primary neurons, express a number of neuron-specific markers, and can be maintained on a basement membrane matrix as clumps of neurons that elaborate functional axons and dendrites (13, 14, 29, 48, 49).

The ability to lentivirus vectors to transduce quiescent hematopoietic cells or their pluripotent precursors remains uncertain. Indeed, a large body of literature suggests that resting T cells (cells in G_0, which represent the majority of peripheral blood T lymphocytes) cannot be productively infected with HIV; arrest of reverse transcription at various stages and variable rescuability of such intermediates by subsequent cycling have been reported (55, 56, 58, 64). We compared the abilities of HIV-2 vectors and Mo-MuLV vectors to target primitive ($CD34^+$ $CD38^-$) hematopoietic progenitor cells that are not actively cycling. These cells have proven elusive in transduction experiments using murine retrovirus vectors (1, 47). Hematopoietic stem cells possess dual properties of self-renewal and multilineage differentiation (44). These rare cells are largely quiescent and divide stochastically in vivo. Since no specific assay exists for true stem cells, they have been operationally defined by the CD34 antigen (expressed by 1 to 3% of bone marrow cells) and lack of expression of the CD38 antigen in combination with other lineage markers. A preponderance of studies have indicated that human pluripotent marrow-repopulating ability resides within the small fraction of total $CD34^+$ cells that are $CD38^-$ (23, 44, 47). A consensus stem cell phenotype has been suggested by numerous studies: $CD34^+$ $CD38^-$ $CD33^-$ HLA-DR^- Thy-1^{lo} Lin^- $CD45RO^+$ rhodamine-123^{dull} (44, 47, 60). Since ex vivo manipulation and retrovirus vector transduction may trigger cell cycling and differentiation of $CD34^+$ cells, we performed multiparameter flow cytometry after transduction to sort these cells according to surface expression of CD34 and CD38 as well as cell division history and then assayed sorted subsets for the marker gene. The results showed a preference of HIV-2 vectors over murine vectors to establish proviral DNA in this cell population. However, the integration status of the vector DNA remains to be determined.

MATERIALS AND METHODS

Plasmid construction. pE32, an infectious $HIV\text{-}2_{KR}$ molecular clone, was constructed by a series of ligations combining portions of the subgenomic viral plasmids KTM2 (57) and RTΔSAC (a modification of RTSAC 57 which eliminates an extra *Sac*I site flanking the 3′ LTR) with pRc/CMV (Invitrogen). Briefly, a *Not*I site was introduced at nt 165 of the HIV-2 LTR within KTM2 by PCR-based mutagenesis. The *Not*I-*Sac*I fragment of this plasmid (containing the 5′ half of the HIV-2 genome), the *Sac*I-*Xba*I fragment of RTΔSAC (containing the 3′ half of the genome), and the *Xba*I-*Not*I fragment of pRc/CMV were combined in one plasmid by three-part ligation; a second three-part ligation with KTM2 restored the full 5′ LTR, generating a full-length, infectious provirus. pE32Δψ was derived by overlapping PCR-based deletion mutagenesis of the illustrated 61 nt from KTM2, followed by substitution of the appropriate fragment into pE32 to generate pE32Δψ. DNA sequencing verified the deletion.

We deleted 771 nt in the HIV-2 *env* gene that encompass the V3 loop by excising the two contiguous *Nsi*I fragments in *env* from RTΔSAC. In addition, PCR-based mutagenesis was used to terminate HIV-2 sequences with the stop codon of the *nef* gene (introducing an *Xba*I site that was joined in a separate ligation to the *Xba*I site of pRc/CMV), thereby replacing the HIV-2 3′ LTR with the bovine growth hormone polyadenylation signal. The *Sac*I-*Pvu*I fragment of this construct was then joined in a three-part ligation with pE32Δψ to create pE41, an HIV-2 packaging plasmid that has deletions of the ψ region, *env*, and the 3′ LTR. pE40 is identical to pE41 except that *env* and a portion of the 3′ U3 elements are intact.

lacZ vector L15.7 contains, 5′ to 3′, the 5′ LTR, the leader and ψ, the first 373 nt of *gag*, the HIV-2 Rev response element (RRE), a simian virus (Sv40)-promoted *lacZ* gene cassette derived from pCH110 (Pharmacia), and the 3′ LTR. To construct the *nef*/*gfp* fusion in vector pLGFP, an *Mlu*I linker was inserted at a unique *Nco* site in *nef*, followed by an in-frame insertion of a PCR-generated copy of the S65T mutant of green fluorescent protein (GFP) (21). Vector LACG is deleted in viral genes in the same manner as vector L15.7 but contains an internally promoted S65T mutant GFP reporter in reverse orientation to the HIV-2 LTR.

RNase protection assays. A 290-nt *Eco*RI-*Nhe*I fragment of $HIV\text{-}2_{KR}$ *gag* was cloned in antisense orientation to the T3 promoter, and a riboprobe was in vitro transcribed with 6.25 μM [^{32}P]UTP (800 Ci/mmol), 250 μM each of the three other ribonucleoside triphosphates (rNTPs), placental RNase inhibitor, dithiothreitol (5 mM), 0.5 ng of plasmid template linearized at the *Sal*I site, and T3 polymerase (see Fig. 3A). Virions were isolated for RNase protection by clearing supernatants with two low-speed centrifugations at 450 and 1,200 × *g*, followed by passage through a 0.2-μm-pore-size filter and ultracentrifugation at 50,000 × *g* for 90 min at 4°C. Riboprobes were mixed with various portions of RNase-free DNase-treated total cellular RNA or virion RNA from each transfection; in each case, the fractions of cellular and virion RNA used were equal. RNA was isolated by the guanidium isothiocyanate method. Riboprobes and RNAs were co-ethanol precipitated, heat denatured at 95°C for 5 min and annealed at 68°C for 10 to 20 min in a thermal block, digested at 37°C for 45 min with a mixture of RNase A and RNase T_1, reprecipitated, and electrophoresed in a denaturing 8 M urea–5% polyacrylamide gel. The assay was linear over 5 orders of magnitude.

PCR and Southern blotting for detection of HIV-2 *pol* sequences. Genomic DNA from heavily transduced cells was prepared by proteinase K digestion and organic extraction. One microgram of this genomic DNA was added to all tubes except for the PCR mix negative control, with or without added genomic DNA from HIV-2-infected T cells as internal standards. Reactions were subjected to

25 cycles of amplification in 100-μl PCR mixtures with *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 200 μM dNTPs, and outer HIV-2 *pol* primers (cctacttctagagaagcct ggg and gtgcccatatatatcctgattcc), followed by transfer of 10 μl of this reaction mixture to a 100-μl PCR mixture containing inner (cttaaggccccacctcctgagg and cttcttgccagattcccctcc) primers and 40 cycles of amplification; 10 μl of each product was subjected to electrophoresis in 1.5% agarose followed by overnight alkaline Southern transfer to a nylon membrane and hybridization to a randomly primed, internal ^{32}P-labeled HIV-2 *pol* probe.

Transfections and production of pseudotyped vectors. Lipofection of T-cell lines was performed with DOTAP (Boehringer Mannheim), using 30 μg of proviral DNA. COS-1 cells were electroporated at 250 V in a Bio-Rad Gene-Pulser. 293-T cells seeded the day before in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), glutamine, penicillin, and streptomycin were transfected by calcium phosphate coprecipitation using a 2:3:1 weight ratio of HIV-2 packaging, HIV-2 vector, and VSV-G expression plasmids with a total of 35 to 50 μg of DNA per 75-cm^2 flask. Medium was replaced 8 to 16 h after transfection, and supernatant was harvested once or twice between 48 and 96 h. LZRNL vector was prepared similarly by calcium phosphate transfection of pHCMV-G in 293GPLZRNL cells (7). HIV-2 and LZRNL vector supernatants were precleared by two 10-min centrifugations at 435 and 1,200 × *g* and filtered through a 0.45-μm-pore-size filter. p26 antigen in unconcentrated supernatants measured by antigen capture enzyme-linked immunosorbent assay (Coulter) averaged 90 to 120 ng, with peak values greater than 400 ng/ml. To produce concentrated stocks of vesicular stomatitis virus G protein (VSV-G)-pseudotyped vectors, cleared, filtered supernatants were ultracentrifuged at 50,000 × *g* for 90 to 120 min at 4°C. The viral pellet was resuspended 4 h to overnight at 4°C in 50 mM Tris (pH 7.8)–130 mM NaCl–1 mM EDTA or DMEM with 1% fetal bovine serum. DNase treatment of vectors was performed with 50 U of RNase-free DNase I (Boehringer Mannheim) per ml for 2 h at 37°C.

Transductions and vector titrations. HeLa cells (5×10^4 per well) were seeded in 12-well plates and incubated for 4 to 16 h with vector supernatants supplemented with 4 to 6 μg of Polybrene per ml. The medium was replaced, and staining was performed at 48 to 60 h by fixing cells in 1% formaldehyde–0.2% glutaraldehyde in phosphate-buffered saline (PBS) for 5 min, washing them twice with PBS, and quenching aldehydes with a 0.1 M glycine rinse, followed by incubation for 2 to 8 h in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining medium (0.4 mg of X-Gal per ml, 2 mM $MgCl_2$, 6 mM potassium ferrocyanide, and 6 mM potassium ferricyanide in PBS). Titers were calculated as the number of blue-staining foci divided by the dilution factor. For transduction of U937 cells, 4 μg of Polybrene per ml was used, and flow cytometric analysis of GFP expression was performed at 48 h (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, Calif.).

Growth arrest. HeLa cells were arrested in G_1/S phase with the DNA polymerase α/δ inhibitor aphidicolin (24), which was maintained at 20 μg/ml during transduction and replenished daily until staining for β-galactosidase expression. Fluorescence-activated cell sorting (FACS) analysis of propidium iodide-stained cells at 24 h confirmed complete G_1/S arrest of the aphidicolin-treated cells.

NTN2 neurons. Third-replate NTN2 neurons (Stratagene) were plated at 10^4 per well in 48-well plates (precoated with Matrigel basement membrane matrix) for 10 days to 2 weeks in neuron-conditioned medium containing mitotic inhibitors (1 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, and 10 μM uridine), which was replenished every 2 days. The cells displayed prominent neurite extension by 24 to 36 h and remained viable for over 4 weeks. Vectors were diluted for titration in medium containing the mitotic inhibitors plus Polybrene (4 μg/ml). Cells were stained for 4 h with X-Gal at 72 h after transduction. No background X-Gal staining of untransduced NTN2 cells was seen, even if cells were stained for 1 week. Heat treatment (56°C, 40 min), leaving the VSV-G expression plasmid pHCMV-G out of the producer cell transfection, or addition of zidovudine (10 μM) eliminated transduction.

Monocyte-derived macrophages. Primary human macrophages were prepared from Ficoll-purified human peripheral blood mononuclear cells from normal donors by adherence to plastic as described previously (30). After initial adherence to fibronectin, cells were plated at 10^5 per ml in plastic chamber slides (Nunc) in RPMI with 15% FCS and 5% autologous donor serum. The chambers were washed four times by vigorous pipetting with PBS to remove all nonadherent cells on days 4, 6, and 7 after plating and before transduction on day 10. The cells were >99% nonspecific esterase positive (Sigma kit 90-A1); 48 h after transduction with serial dilutions of GFP-encoding vector in the presence of Polybrene (4 μg/ml), slides were washed with PBS and scored by epifluorescence microscopy.

$CD34^+$ cell isolation, purification, PKH26-GL staining, and transduction. Human $CD34^+$ cells were isolated from growth factor-mobilized peripheral blood as described previously (31). Briefly, five daily injections of granulocyte-macrophage and granulocyte colony-stimulating factors (GM-CSF and G-CSF; each at 5 μg/kg/day) were administered; leukapheresis was performed 24 h after the last growth factor injection. $CD34^+$ cells were purified from leukapheresis products by immunomagnetic separation (Isolex-300; Baxter Immunotherapy, Irvine, Calif.) and cryopreserved by controlled-rate freezing in RPMI with 10% dimethyl sulfoxide. Purity of the $CD34^+$ cells was 94% by FACS analysis performed as described previously (31).

For PKH26-GL staining, 6×10^6 to 10×10^6 frozen $CD34^+$ cells were thawed at 37°C and washed twice with Iscove modified DMEM (Gibco-BRL). Cells were resuspended in serum-free medium and stained with PKH26-GL (Sigma, St. Louis, Mo.) according to the manufacturer's instructions. Equivalent aliquots of $CD34^+$ cells not treated with PKH26-GL were used for background control measurements in the subsequent FACS analyses. Four hours after PKH26-GL staining, 10^6 cells were transduced with either Mo-MLV or HIV-2 DNase I (Boehringer Mannheim)-treated (50 U/ml for 60 min at 37°C) *lacZ* vectors at a multiplicity of infection (MOI) of 1.0 in a total volume of 1.5 ml of Iscove modified with 10% FCS supplemented with Polybrene (4 μg/ml) and dNTPs (100 μM each), with or without cytokine stimulation (interleukin-3 [IL-3; 500 U/ml], IL-6 [500 U/ml], stem cell factor [40 ng/ml], GM-CSF [10 ng/ml], basic fibroblast growth factor [2.5 ng/ml], and erythropoietin [2.5 U/ml]). Cells were centrifuged in vector supernatants at 2,700 × *g* for 30 min and incubated at 37°C for 16 to 24 h. Following transduction, the cells were washed five times and then cultured in the dark for a further 48 h with or without the above cytokines. Two aliquots of PKH26-GL-treated or untreated $CD34^+$ cells were also cultured under the same conditions except for addition of the vectors.

CD34/38 labeling, three-color flow cytometry, and vector DNA detection in hematopoietic progenitor cells. At 48 h after transduction, PKH26-GL-stained or unstained $CD34^+$ cells were labeled with CD34-fluorescein isothiocyanate (BDIS) and/or CD38-Cy-chrome (Pharmigen, San Diego, Calif.) (31). Flow cytometric analysis and sorting of $CD34^+$ cells were performed on a FACStar^{plus} flow cytometer (BDIS) equipped with an argon-ion laser tuned at 488 nm. Data acquisition was performed with Lysis 2.0 (BDIS). Forward light scatter, orthogonal light scatter, and three-color fluorescent signals were determined for each cell, and the list mode data files were analyzed with Cut-a-Cluster software (BDIS). Cells were sorted into Falcon tubes containing Tris-EDTA (pH 8.0), 100 mM NaCl, proteinase K (200 μg/ml), and 1% sodium dodecyl sulfate. DNA was isolated by organic extraction and ethanol precipitation with glycogen as a carrier and amplified in parallel with a *lacZ* standard curve for 35 cycles (94, 60, and 72°C, 30 s each plateau) in 100-μl PCRs all prepared from the same mix containing 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM *lacZ* PCR primers (CCTTTG CGAATACGCCCACGCGATGGG and CGTACTGTGAGCCAGAGTTGCC CGGCGC), and 0.5 U of *Taq* polymerase. DNAs from each individual sort were adjusted to 200 cell equivalents per tube, and input DNA equivalence was assessed by PCR with human β-globin gene primers (53) in parallel with a human genomic DNA standard curve; in addition, 100-μl aliquots of each DNase-treated vector supernatant were simultaneously extracted and amplified to assess completeness of DNase treatment. A 5-μl aliquot of each PCR product was subjected to electrophoresis in 1% agarose followed by overnight alkaline Southern transfer and hybridization to a randomly primed, internal (^{32}P)-labeled *lacZ* or human β-globin probe under standard conditions.

RESULTS

Deletion in HIV-2 ψ abrogates replication. The three-plasmid system used in these studies (Fig. 1) was designed to express HIV-2 proteins except Env in *trans* from an mRNA that will not be encapsidated. Since the packaging determinants of HIV-2 are not well defined, the effect of a mutation in HIV-2 ψ on encapsidation and expression was first examined. HIV-2 ψ is 70% longer than HIV-1 ψ (75 versus 44 nt). To maximally disrupt the potential HIV-2 encapsidation signal while not interfering with splicing or *gag* translation, 61 nt were deleted from this region (Fig. 1); the deletion extends from 11 nt downstream of the major SD to 3 nt upstream of the *gag* start codon. As shown in Fig. 2, removal of the 61 nt blocked replication of HIV-2_{KR} but did not interfere with transient expression. Transient transfection of a proviral plasmid carrying the 61-bp ψ deletion alone (pE32Δψ) or the ψ deletion plus a truncated 3′ LTR (pE40) into T-cell lines highly permissive for the parental virus resulted in high but transient Gag protein (p26) expression and marked transient syncytium formation (HIV-2_{KR} is syncytium inducing in human T-cell lines). However, viral replication was abrogated (followed out to 6 months as illustrated). In addition, transfer of 30 ml of supernatant containing 210 ng/ml of p26 antigen from COS-1 cells electroporated with pE32Δψ to a culture of 10^7 CD4-LTR/β-gal indicator cells (28) was negative for any β-galactosidase-expressing cells when stained at 2, 7, and 33 days after transfer. In contrast, 0.005 ng of viral antigen per ml from pE32-transfected cells was detected in this assay. Potential transfer of coding sequences by pseudotyped vectors was later examined by a PCR-based assay (below).

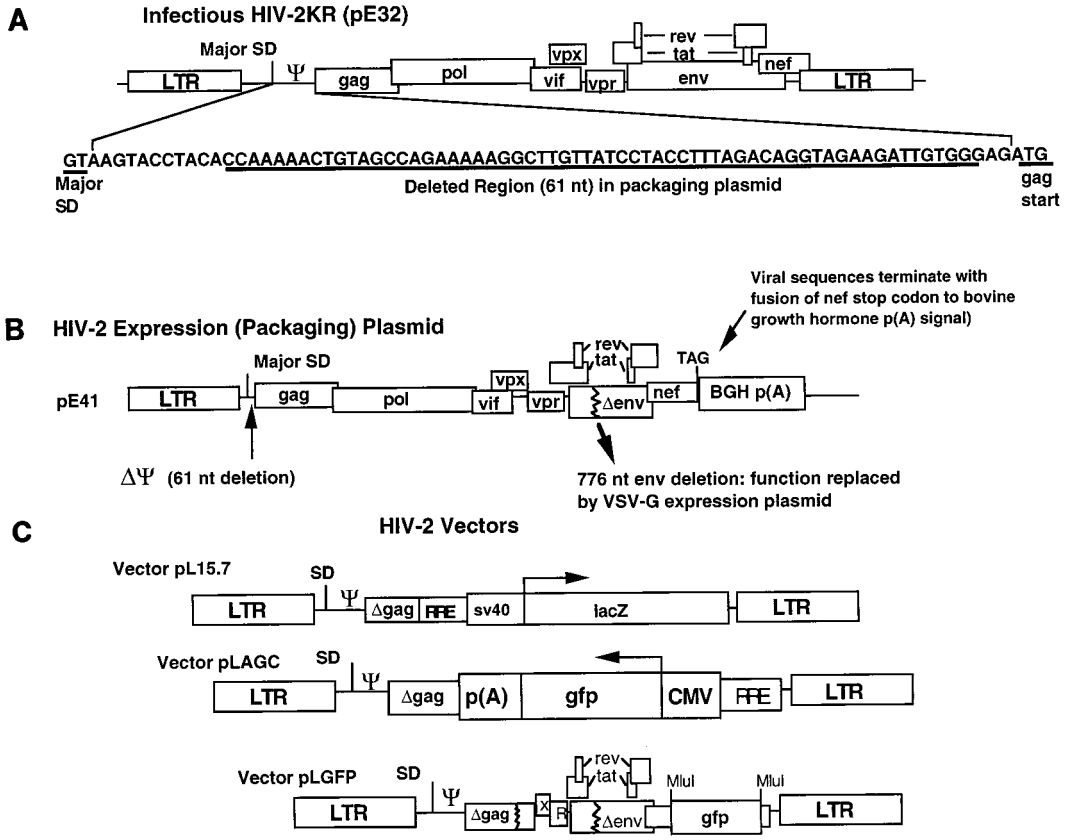

FIG. 1. Schematic representation of the HIV-2 lentivirus vector system. (A) Nucleotides deleted from the ψ region of HIV-2_{KR}.; (B) the protein expression plasmid used for *trans* packaging and the VSV-G expression plasmid supplying the deleted *env* function; (C) HIV-2-based vectors. Abbreviations: BGH, bovine growth hormone; CMV, cytomegalovirus.

Deletion in HIV-2 ψ prevents encapsidation. Although these experiments demonstrated that deletion of 61 of 75 bp in ψ permitted wild-type levels of viral gene expression while preventing both HIV-2 replication and transmission of coding sequences to target cells, they did not specifically measure the effect of the deletion on HIV-2 genomic encapsidation. An RNase protection assay was then used to directly compare levels of intracellular HIV-2 genomic RNA and of HIV-2 virion genomic RNA in supernatants of transfected cells. As shown in Fig. 3b, the ratio of virion to cellular genomic mRNA was markedly reduced by the ψ deletion alone (compare lanes A and B) or the ψ deletion in combination with the *env* and 3′ LTR deletions of pE41 (compare lanes E and F). These results identify the ψ region of HIV-2 as a determinant of genomic encapsidation.

HIV-2 expression by fully modified packaging plasmid. In addition to the ψ deletion, several other attenuating modifications were made in constructing the packaging plasmid (pE41) used for packaging VSV-G-pseudotyped vectors (Fig. 1). HIV-2 sequences were terminated precisely at the stop codon of the *nef* gene (pE40 retained a short stretch of the 3′ U3). The 3′ LTR was replaced with the bovine growth hormone polyadenylation signal, and a 776-bp span of *env* that encompasses the V3 loop was deleted. Figure 4 shows that high levels of p26 antigen were produced from transient transfection of these modified expression plasmids; in either COS-1 or human 293-T cells, 100 to 400 ng of p26 per ml was routinely generated. Figure 3 (lane E) shows the comparatively negligible levels of HIV-2 *gag* RNA present in pelleted virions from these cells.

Transduction of dividing and nondividing HeLa cells. Three HIV-2 vectors were used in this study (Fig. 1). HeLa cells were transduced with VSV-G-pseudotyped *lacZ*-encoding HIV-2 vector L15.7 prepared by triple cotransfection in 293-T cells (see Materials and Methods). *lacZ* titers scored 48 h after transduction for unconcentrated and ultracentrifuge-concen-

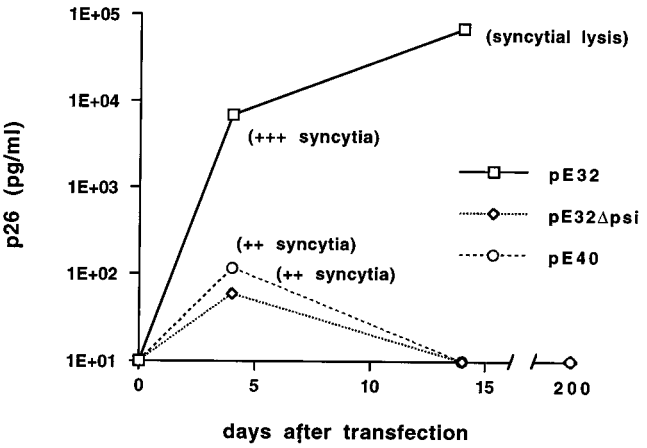

FIG. 2. ψ deletion alone abrogates replication but preserves transient HIV-2 protein expression. HIV-2_{KR} proviral plasmids pE32Δψ and pE40 were transfected into Molt4-8 T cells. The ordinate cutoff is 10 pg of p26/ml, the limit of sensitivity of the assay.

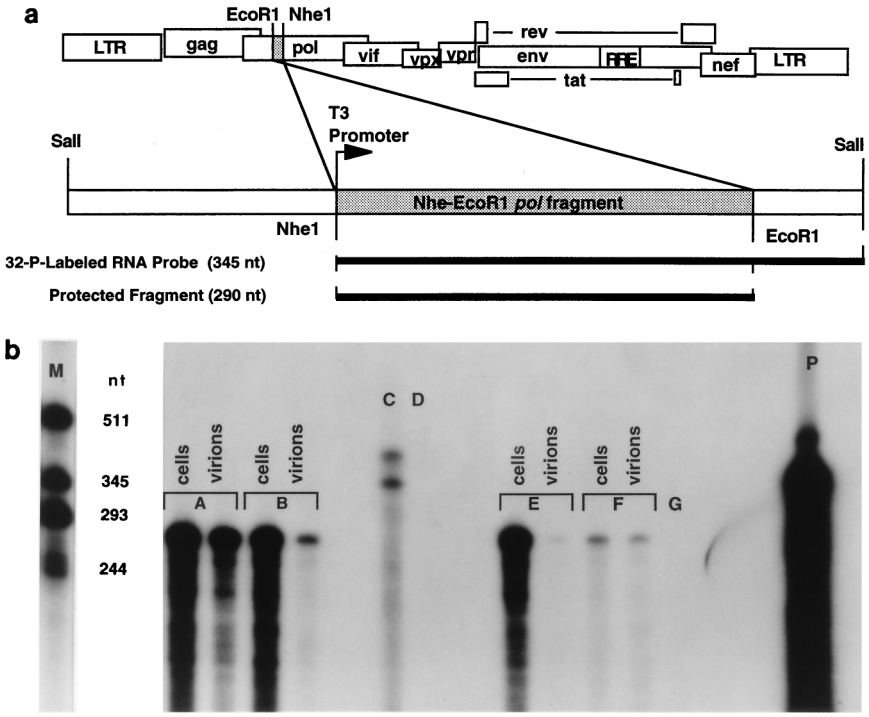

FIG. 3. RNase protection assay comparing amounts of intracellular HIV-2 genomic mRNA and of virion genomic mRNA. RNAs were harvested from 2×10^6 COS-1 cells, and from pelleted virions from the cell supernatants, 48 h after electroporation of 10 μg of plasmid DNA. RNAs were treated with 20 U of RNase-free DNase I for 4 h at 37°C and analyzed as described in Materials and Methods. (a) Probe design and expected fragments. (b) Lane M, ^{32}P-labeled RNA markers in vitro transcribed from templates of known size. Plasmids electroporated were pE32 (wild-type full-length HIV-2; lanes A), pE32Δψ (lanes B), and pE41 lanes E. Lanes F, separate transfection of pE32 (wild type). Results for cellular (lane C) and virion RNA (lane D) controls from COS-1 cells electroporated with a plasmid expressing only the probe sequence in sense orientation from the SV40 promoter are also shown. Lane P, free probe minus RNase (10% of the amount added to other samples to avoid overloading autoradiogram); unmarked lane just left of P, 100% of free probe added to other samples plus RNase; lane G, untransfected COS-1 cell RNA control. The sense transcript controls in lanes C and D indicate that substantial amounts of cellular RNA were not nonspecifically pelleted but the small amount of RNA measured in the Δψ (B, virions) and pE41 (E, virions) virion samples may in part represent cosedimented 0.2-μm-pore-size-filterable RNA-containing subcellular fragments in addition to encapsidated RNA.

trated supernatants are shown in Table 1. When HeLa cells were transduced as in the experiments reported in Table 1 but subsequently allowed to proliferate for 2 weeks, staining for β-galactosidase expression yielded uniformly blue-staining colonies of several hundred cells at titers 85 to 90% of those scored at 48 h, indicating stable, clonal maintenance of the transgene.

To examine the ability of vector L15.7 to transduce nondividing cells and compare this ability with that of a conventional retrovirus vector, HeLa cells were arrested in the G_1/S phase with the DNA polymerase α/δ inhibitor aphidicolin (24) (20 μg/ml), which was maintained during transduction and replenished daily until staining for β-galactosidase. FACS analysis of propidium iodide-stained cells at 24 h confirmed complete G_1/S arrest of the aphidicolin-treated cells (data not shown). Cell counts also showed that no cell proliferation occurred during the 4 days of aphidicolin exposure until X-Gal staining. By 24 h into aphidicolin treatment, cells were transduced with serial dilutions of Mo-MuLV *lacZ* retroviral vector LZRNL (VSV-G) or HIV-2 vector L15.7(VSV-G) for 4 h in the presence of Polybrene (4 μg/ml); 48 h after transduction, *lacZ* titers were scored. Figure 5 shows that aphidicolin markedly reduced the ability of the LZRNL vector to transduce HeLa cells whereas the HIV-2 vector was only minimally affected. That nondividing cells were transduced could be confirmed visually also: aphidicolin-arrested HIV-2 vector-transduced cells were uniformly present as clearly isolated single blue cells, whereas nonarrested transduced cells had proliferated into colonies of 4 to 16 blue-staining cells.

Lack of transfer of coding sequences by vector. To test for transfer of coding sequences by pE41-packaged vector, 5×10^4 log-phase HeLa cells were transduced at an MOI of 10 with DNase-treated L15.7 vector, yielding >98% transduction as assessed by X-Gal staining of 10% of the cells at 60 h. The remaining 90% were expanded for 15 days (four passages) and genomic DNA was prepared by proteinase K digestion, organic extraction, and ethanol precipitation. As shown in Fig. 6, 1 μg of this genomic DNA was negative by a sensitive, nested PCR/Southern blot assay for a 319-bp segment of *pol*, while simultaneous amplification in the same assay of the same amount of

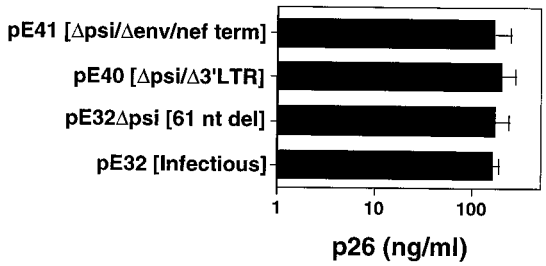

FIG. 4. p26 antigen production at 48 h in supernatants of COS-1 cells electroporated with HIV-2 expression plasmids. Bars indicate standard errors.

TABLE 1. HIV-2 vector transduction of dividing HeLa cells[a]

Assay	Transduction	
	Unconcentrated	Concentrated (10^7)
1	7.8×10^5	2.3
2	1.2×10^6	2.2
3	7.7×10^5	8.5
4	6.9×10^5	9.9
Mean	$(8.6 \pm 2.3) \times 10^5$	5.7 ± 4.1

[a] Values are mean transducing units per milliliter ± standard deviation. VSV-G-pseudotyped vector pL15.7 supernatants were cleared by two low-speed centrifugations and 0.45-μm-pore-size filtration and titered directly (unconcentrated) and after two rounds of ultracentrifugation at 50,000 × *g* (concentrated).

this DNA spiked with genomic DNA from as few as five cells from a chronically HIV-2-infected Molt4 T-cell line was positive. To this limit of sensitivity, therefore, VSV-G-pseudotyped vectors generated using pE41 did not transfer HIV-2 coding sequences to target cells. In addition, these transduced cells also produced no detectable p26 antigen when assayed at 1 and 3 weeks after transduction. Finally, after continued passage for 3 weeks, 50 ml of filtered supernatant from 10^7 log-phase cells was transferred to 5×10^6 CD4-LTR/beta-gal cells, which were negative by X-Gal staining at 96 h.

Transduction of T and monocytoid cell lines. GFP-expressing HIV-2 vectors were constructed to study transduction of T cells and monocytes. Vector LGFP employs internally encoded HIV-2 *tat* transactivation of the HIV-2 LTR to promote transcription of a Nef/GFP fusion protein. This fusion protein contains the 5′ *nef* myristoylation signal and localizes to cytoplasmic vesicles with the same distribution as the Nef protein (data not shown). VSV-G-pseudotyped LGFP previously titered on HeLa cells was used to transduce a T-cell line (Molt4) and a monocytoid cell line (U937). Flow cytometric analysis for GFP expression 48 h after transduction is shown in Fig. 7.

Transduction of human macrophages and NTN2 neurons. NTN2 neurons and monocyte-derived macrophages were plated as described in Materials and Methods and transduced with HIV-2 vectors. NTN2 neurons were plated on a Matrigel basement membrane matrix in the presence of mitotic inhibitors for 10 days and transduced with vector L15.7. As shown in Table 2, these postmitotic human neuronal cells (13, 14, 29, 48, 49) were efficiently transduced by the HIV-2 vector but not by the control Mo-MuLV *lacZ* vector.

Background β-galactosidase staining was seen in human monocyte-derived macrophages from some donors (data not shown). Therefore, vector pLACG was used to transduce macrophages. Because this vector contains an internally promoted *gfp* gene in reverse orientation to the HIV-2 LTR, the RRE is positioned downstream of the marker gene cassette and a separate polyadenylation signal is used for *gfp*. As shown in Table 2, titers on human macrophages exceeding 10^5/ml were achieved; in contrast, Mo-MuLV vector transduction of macrophages was negligible.

$CD34^+$ human hematopoietic progenitor cell transduction. Purified human $CD34^+$ hematopoietic progenitor cells isolated from growth factor-mobilized peripheral blood were transduced at an MOI of 1.0 for 16 to 24 h with pretitered Mo-MuLV LacZ vector (LZRNL) or HIV-2-based LacZ vector L15.7 in the presence or absence of stimulatory cytokines (see Materials and Methods); after washing and 48 h of subsequent culture, the cells were further stratified according to CD38 expression status and proliferation index. To distinguish dividing and nondividing cells, cells were stained with the lipophilic membrane-fluorescent tracking dye PKH26-GL prior to transduction. PKH26-GL has been used to accurately track the mitotic history of hematopoietic cells since partitioning between daughter cells reduces its fluorescence intensity by one-half with each cell division; the dye does not exchange spontaneously between labeled and unlabeled cells and does not identifiably alter hematopoietic cell physical properties or function in vivo (32, 59). The PKH26-GL staining profiles of $CD34^+$ cells were not affected by exposure to either vector as analyzed by flow cytometry (data not shown). Three-color FACS analysis showed that 2 to 5% of $CD34^+$ cells maintained a $CD38^-$ phenotype after 72 h in culture and 48 h posttransduction (data not shown). Among these, 12 to 17% had undergone zero to one cell division, while 83 to 88% of $CD34^+$ $CD38^-$ cells had undergone more extensive cell divisions and had diminished PKH26-GL content.

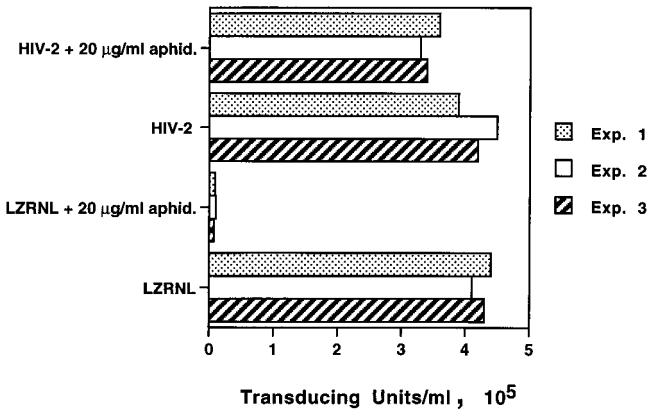

FIG. 5. Effect of mitotic arrest on an HIV-2 lentivirus vector compared to an Mo-MuLV retrovirus vector. HeLa cells were arrested in the G_1/S phase by treatment with 20 μg of aphidicolin (aphid.) per ml; cell cycle arrest (<0.1% G_2/M) was verified by flow cytometry after propidium iodide staining.

The two $CD34^+$ $CD38^-$ cell subsets with the highest and lowest PKH26-GL staining, corresponding to cells with low and high proliferative indices, were sorted and collected separately for DNA extraction. The samples were then analyzed by PCR and Southern blotting for the presence of the *lacZ* transgene to determine transduction by VSV-G-pseudotyped HIV-2 *lacZ* and LNL *lacZ* vectors. Results are shown in Fig. 8. In one experiment, the vector transduction and subsequent cell culture were carried out in the presence or absence of a stimulatory cytokine cocktail culture consisting of IL-3, IL-6, GM-CSF, basic fibroblast growth factor, stem cell factor, and erythropoietin. β-Globin sequences were amplified as internal controls to verify equivalent DNA input. As shown in Fig. 8A, Mo-MuLV vector DNA was observed in the $PKH26^{lo}$ (actively dividing) cells cultured in the presence of cytokines and to a

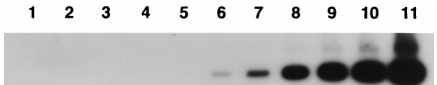

FIG. 6. Assay for transfer of HIV-2 coding sequences. Nested PCR amplifications followed by Southern blotting with an internal ^{32}P-labeled *pol* probe were performed with 1 μg of genomic DNA (present in all tubes except PCR blank in lane 1) from HeLa cells transduced at a high MOI, yielding an efficiency of >98% as described in the text. Lanes: 1, PCR without genomic DNA; 2 to 5, reactions containing 1 μg of genomic DNA from transduced cells; 6 to 12, reactions containing 1 μg of the same DNA from the L15.7-transduced cells coamplified with various cell equivalents of genomic DNA prepared from HIV-2-infected T cells: lane 6, 1 cell; lane 7, 5 cells; lane 8, 50 cells; lane 9, 100 cells; lane 10, 500 cells; lane 12, 1,000 cells.

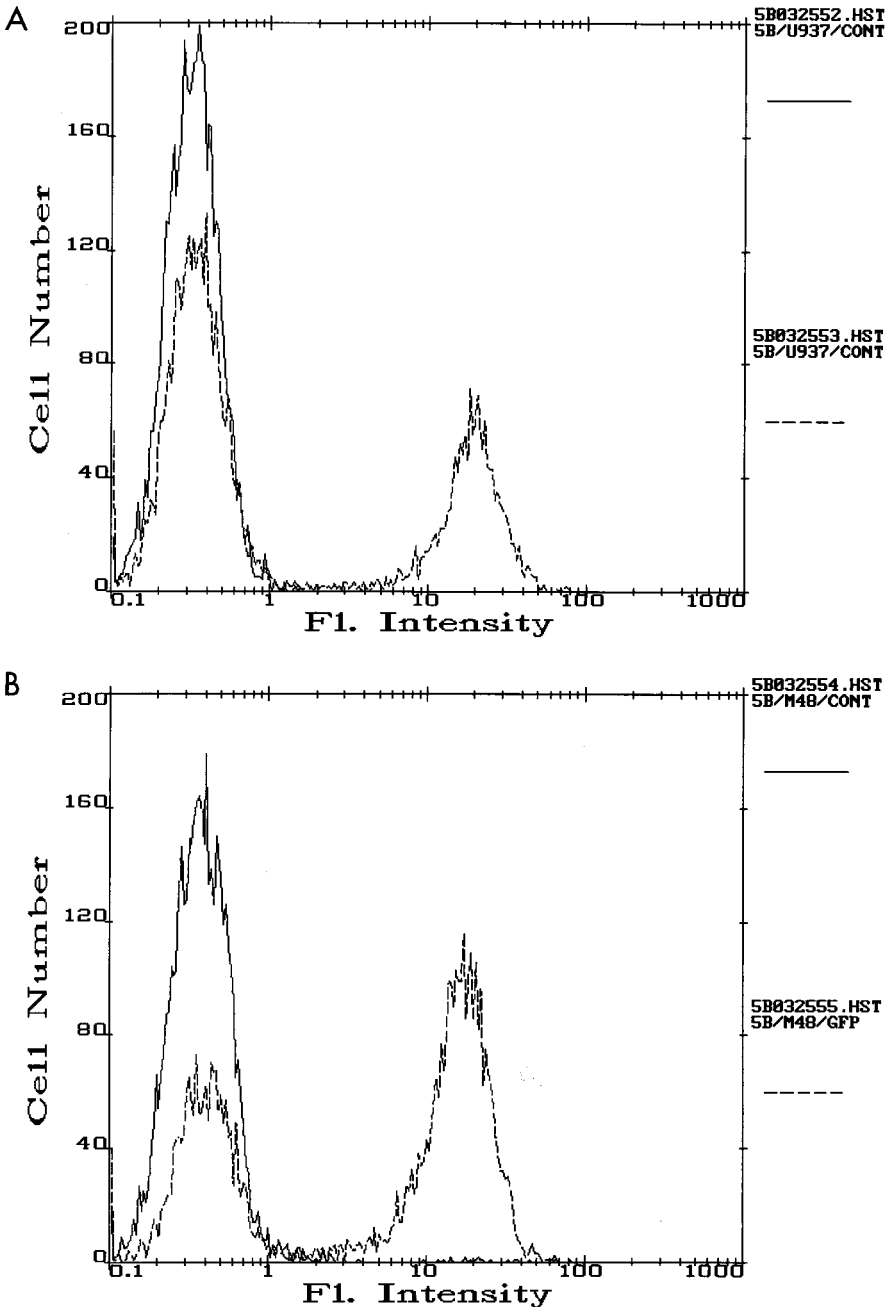

FIG. 7. Flow cytometric analysis for GFP expression in a T-cell line (Molt4; A) and a monocytoid cell line (U937; B) 48 h after transduction with an HIV-2 GFP vector (MOI = 1.0). Solid lines, untransduced control cells; dashed lines, transduced cells. Fl., fluorescence.

lesser extent in the PKH26^{lo} subset derived from nonstimulated cells. Most notably, no vector DNA was detected in cells from the PKH26^{hi} subsets (not actively dividing). In contrast, HIV-2 vector DNA could be detected in all four dividing and nondividing cell populations. Based on the LacZ standards and input total cell DNA, we estimate >1 copy of vector DNA per cell. Figure 8B presents results of an experiment in which cytokines were included in the in vitro cell culture. Again, the Mo-MLV vector showed restricted capacity to transduce the CD38$^-$ cells, while HIV-2 vectors transduced both PKH26^{hi} and PKH26^{lo} subsets efficiently (see the legend to Fig. 8). In both of these experiments, DNase-treated vector supernatants used for transduction were negative for the transgene sequences (Fig. 8A and B, lanes M and H), indicating that the detected DNA was not carryover DNA. We did not detect expression of the *lacZ* reported gene in these cells. It is not clear whether this was due to lack of promoter activity in these cells or lack of proviral DNA integration. Unfortunately, the number of transduced cells was too low to allow us to examine the latter issue.

DISCUSSION

We report a replication-defective, three-plasmid lentivirus vector system derived from a parental lentivirus with demonstrated apathogenicity in a standard animal model. (SIV-derived

TABLE 2. Comparative transduction of monocyte-derived macrophages and NTN2 neurons by Mo-MuLV and HIV-2 vectors[a]

Cell type	Transduction	
	Mo-MuLV vector LNL6-GFP	HIV-2 vector LACG
HeLa	$(1.0 \pm 0.6) \times 10^6$	$(1.0 \pm 0.8) \times 10^6$
MDM	$<1 \times 10^1$	$(5.4 \pm 1.1) \times 10^5$
	Mo-MuLV vector LZRNL	HIV-2 vector L15.7
HeLa	$(0.8 \pm 0.3) \times 10^6$	$(1.1 \pm 0.2) \times 10^6$
NTN2	$<1 \times 10^1$	$(2.3 \pm 1.7) \times 10^5$

[a] Values are mean transducing units per milliliter ± standard deviation from three experiments. HeLa cells and third-replate NTN2 neurons were transduced with pretitered HIV-2 vector pL15.7 and Mo-MuLV vector LZRNL; monocyte-derived macrophages (MDM) were transduced with HIV-2 vector pLAGC, and an Mo-MuLV GFP-encoding vector (LNL6-GFP) and scored by epifluorescence microscopy at 48 h. No background X-Gal staining of untransduced NTN2 cells occurred, even if cells were stained for 1 week. Heat treatment (56°C, 40 min), leaving the VSV-G expression plasmid pHCMV-G out of the producer cell transfection, or addition of 10 μM zidovudine reduced transduction of macrophages and NTN2 neurons to <1/ml.

vectors have been reported, but HIV-1 virions were used to package them [52]). Similar to HIV-1-based vectors, the HIV-2-based vectors can transduce both dividing and nondividing cells at high efficiency. Consistent with a large body of previous studies, transduction by Mo-MuLV vectors in this study was restricted to dividing cells. In addition to high efficiency on dividing cell lines, HIV-2 vectors efficiently transduced aphidicolin-arrested cells, primary macrophages, and postmitotic NTN2 neurons. Concentration of the VSV-G-pseudotyped vectors by ultracentrifugation was readily achieved.

HIV-1-based vectors in previous studies have included true vectors, in which viral structural proteins are supplied fully in *trans* (46), and simpler systems that employ modified HIV-1 proviruses in which the vector itself supplies one or more of the viral structural proteins in *cis*. The HIV-2-based vector system described here is of the former type and contains additional deletions of 5′ and 3′ *cis*-acting regions illustrated in Fig. 1.

Safety issues will require extensive investigation for gene therapy vectors derived from primate lentiviruses (16). A three-plasmid HIV-1 vector system deleted in multiple accessory genes has also been described and may lessen risk (66), although some cell types may require more than Gag/Pol and Rev. For example, hepatocytes in vivo were transduced efficiently in vivo only if Vpr and Vif were supplied (25). In contrast to HIV-1 vectors, safety of HIV-2 vectors can be addressed by studies in primates susceptible to pathogenicity. Although infection of macaques with HIV-2_{KR} can be achieved, the animals have remained free of symptoms or disease for more than 2 years; matched animals infected with HIV-2_{EHO} rapidly developed AIDS; interestingly, prior infection with HIV-2_{KR} either delayed or prevented disease induction by HIV-2_{EHO} (38). Deletion of HIV-2 accessory genes (e.g., *vpr*, *vpx*, *vif*, and *nef*) is under investigation. We have minimized risk for recombination (42) by distributing viral functions to separate DNAs, avoiding large regions of homologous sequence overlap, and showing that packaging of the mRNA coding for viral proteins is minimal. In addition, transfer of coding sequences to target cells was not detected.

The nature of the HIV-2 packaging signal has received limited study and produced conflicting results (19, 41, 52). Our results suggest that, as for HIV-1, deleting most of the region between the major SD and the *gag* start codon abrogates replication, prevents incorporation of HIV-2 genomes into viral particles, and, in combination with the *env* deletion and LTR modifications, prevents detectable transfer of coding sequences to heavily transduced target cells. These results are consistent with the results of McCann and Lever, who reported that deletions of up to 40 of the 75 nt (53%) in HIV-2 ψ reduced encapsidation by only 33 to 70% (41); a larger deletion, such as that of the present study (61 nt, 83%), would appear to be required to reduce HIV-2 genomic mRNA encapsidation to nonspecific levels. Additional *env* and LTR deletions make regeneration of wild type-HIV-2 impossible. Stable HIV-1 packaging lines using the native HIV-1 envelope have been described (8, 15, 54).

The $CD38^-$ subset of human hematopoietic progenitors contains cells capable of both multilineage differentiation and long-term repopulating ability. Up to a third of $CD38^-$ $CD34^+$ cells remain in G_0/G_1 phase after cytokine stimulation as used in our study (1). Numerous studies have shown that conventional murine leukemia virus-based vectors cannot transduce this subset of hematopoietic precursors in vitro or permit efficient chimeric reconstitution of NOD/SCID mice with transduced human $CD34^+$ cells (33). Our results with HIV-2 vectors suggest that the ability of lentiviruses to infect nondividing cells may extend to both cycling and noncycling $CD38^-$ $CD34^+$ human hematopoietic progenitor cells. A clear difference between the murine vector and the lentivirus vector in the infective phase up to the point of proviral DNA formation is indicated by the experiments in Fig. 8. Expression of either

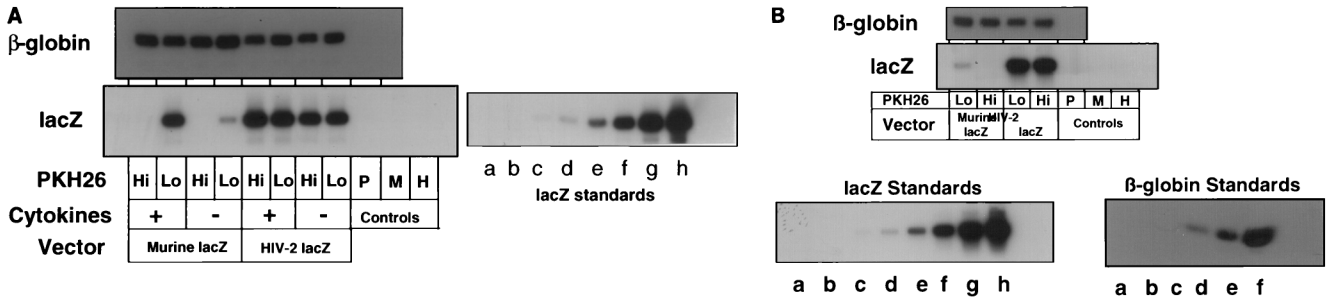

FIG. 8. PCR analyses of transduced $CD34^+$ cell subsets for *lacZ* and β-globin DNA (single-copy cellular gene DNA input control). Cells were transduced with DNase-treated vectors and sorted by flow cytometry into $CD34^+$ $CD38^-$ and $PKH26^{hi}$ or $PKH26^{lo}$ fractions before DNA extraction, PCR, and Southern blotting with an internal *lacZ* or β-globin probe. (A) Analysis of cells FACS sorted as $CD34^+$ $CD38^-$ and $PKH26^{hi}$ or $PKH26^{lo}$ after transduction in the presence (+) or absence (−) of cytokines as detailed in Materials and Methods. (B) Cells were transduced with each DNase-treated vector in the presence of cytokines and similarly sorted. LacZ standards (lanes a to h): 0, 1, 4, 16, 64, 256, 1,024, and 4,096 copies of the *lacZ* gene; β-globin standards (lanes a to f): genomic DNA equivalent to 0, 1, 5, 50, 500, and 5,000 U937 cells. β-globin PCRs for both experiments were amplified simultaneously; the standard curve is shown in the bottom panel of B. P, PCR blank; M, DNase-treated Mo-MuLV (LZRNL) vector supernatant; H, DNase-treated HIV-2 *lacZ* vector supernatant.

β-galactosidase or GFP could not be detected in these cells, and hence the completeness of the transduction process remains uncertain. It is not clear if these results represent transcriptional shutoff or lack of integration in these primitive hematopoietic progenitors, since unintegrated retroviral DNA, both that of lentiviruses and that of murine retroviruses, is transcriptionally silent (11, 46). In lieu of reporter gene expression, inverse PCR assays to detect integrated proviruses were performed but were insufficiently sensitive to verify integrated proviral DNA from the low number of cells remaining after the repeated sorting for CD38 negativity (data not shown). Methodological constraints thus limit the conclusions that can be drawn about the integration state from the human $CD34^+$ cell experiments because transgene expression could not be detected and because the low numbers of cells obtainable after two rounds of sorting prevented demonstration of viral-cellular DNA junctions. Although Fig. 8 shows a clear difference between the abilities of the Mo-MuLV vector and the HIV-2 vector to generate proviral DNA in the $CD38^-$ cells, it may be that hematopoietic stem cells with both pluripotent differentiation capacity and self-renewal capacity will harbor blocks to lentivirus vectors analogous to those seen for HIV-1 in resting G_0 T cells (55, 56, 58, 64). To prove stable gene transfer to functional, repopulating stem cells, hematopoietic reconstitution with vector-transduced cells in an in vivo model will be required.

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