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Lv2, a Novel Postentry Restriction, Is Mediated by both Capsid and Envelope

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The characterization of restrictions to lentivirus replication in cells identifies critical steps in the viral life cycle and potential therapeutic targets. We previously reported that a human immunodeficiency virus type 2 (HIV-2) isolate was restricted to infection in some human cells, which led us to identify a step in the life cycle of HIV-2 detected after reverse transcription but prior to nuclear entry. The block is bypassed with a vesicular stomatitis virus glycoprotein G (VSV-G) envelope (A. McKnight et al., J. Virol. 75:6914–6922, 2001). We hypothesized that, although the restriction is apparent at a post-reverse transcription step, the lack of progress results from a failure of the virus to reach a cellular compartment with access to the nucleus. Here we analyzed molecular clones of the restricted virus, MCR, and an unrestricted virus, MCN. Using sequence analysis and gene swapping, we mapped the viral determinants to *gag* and *env*. Site-directed mutagenesis identified a single amino acid at position 207 in CA to be responsible for the *gag* restriction. Pseudotype experiments indicate that this step is also important for the infection of cells by HIV-1. The HIV-1 NL4.3 core is restricted if supplied with a restricted MCR envelope but not with VSV-G. Also the NL4.3 envelope rescues the restricted core of HIV-2 MCR. Abrogation experiments with MLV demonstrate that the restriction is distinct from Fv1/Ref1/Lv1. We propose that this represents a new lentiviral restriction, Lv2. Thus, the envelope and capsid of HIV act to ensure that the virus is delivered into an appropriate cellular compartment that allows postentry events in viral replication to proceed efficiently.

Retroviral particles must contain all the necessary components to interact with cellular processes so that integration into the host genome is achieved. Here we show for human immunodeficiency virus type 2 (HIV-2) that the viral *gag* and *env* genes act in concert to enable postentry steps.

For HIV and simian immunodeficiency virus (SIV), fusion at the cell surface is mediated by the interaction of viral envelope glycoprotein with cell surface receptor CD4 and a coreceptor molecule, usually either CXCR4 or CCR5 (9). Fusion leads to delivery of the viral core inside the cell; once inside, reverse transcription takes place. HIV cores are likely to interact with components of the cytoplasm (4, 5). Eventually the preintegration complex, which contains viral DNA and proteins, integrase, matrix, Vpr, and reverse transcriptase (5, 11, 12, 17, 22, 33), arrives at the nucleus and traverses the nuclear membrane by interaction with cellular proteins involved in this transport.

Apart from the reverse transcription process itself, the steps immediately following fusion and surrounding reverse transcription are largely undefined. One approach to mapping early events is to study cells and/or viruses with postentry restrictions to replication. For example, the *Fv1* gene in mice confers resistance to murine leukemia viruses (MLVs) preventing a post-reverse transcription pre-nuclear entry step (3, 14, 26). The same restriction in human cells, termed Ref1, also prevents the replication of MLV-N. A similar restriction for the prevention of infection of monkey cells by HIV-1 and -2 and SIVmac, termed Lv1, occurs at or after reverse transcription but prior to nuclear entry (2, 8, 10, 16, 19, 20, 32, 34, 39). Escaping such cross-species barriers is likely to be required for successful zoonosis to occur.

Mapping the viral genes responsible for overcoming such postentry restrictions can give insight into intracellular steps in the viral life cycle. The MLV gene responsible for overcoming the Fv1 restriction was mapped to CA within *gag*. The cellular gene for Fv1 was mapped to a murine endogenous retroviral *gag* gene. This led to the present model that incoming virus must avoid an interaction with endogenously expressed Gag for infection to proceed (42).

The viral envelope has been implicated in postentry events for HIV-1 (7, 31, 36). Schmidtmayerova et al. (36) reported that T-cell line-adapted viruses of HIV-1 are restricted at a post-reverse transcription step in primary human macrophages. Postentry restrictions to infection by HIV in proliferating human cells are uncommon. A postentry restriction element for HIV-2 in actively dividing human cells has been reported (29). An HIV-2 virus (prCBL-23) can fuse, enter, and reverse transcribe in these restrictive cell types, but preintegration complexes do not locate to the nucleus. The preintegration complexes, however, have all the requirements for successful transport into the nucleus because prCBL-23 pseudotypes bearing the vesicular stomatitis virus glycoprotein G (VSV-G) envelope overcome the restriction element. It was concluded that prCBL-23 virus is delivered into a cellular compartment that does not have direct access to the nucleus (29).

Here we have determined that both *env* and *gag* together are responsible for the restriction and that the same proteins in

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HIV-1 are similarly tailored to use this cellular route. We further demonstrate that the step in the viral life cycle is distinct from Ref1 and Lv1.

MATERIALS AND METHODS

Cell lines. GHOST/CXCR4 (6) and HeLa/CD4 (human squamous epithelial carcinoma) (27) cells, human glioma cell lines U87/CD4/CXCR4 (18) and NP2/CD4/CXCR4 (40), and *Mus dunni* tail fibroblasts (MDTF) (25) were maintained in Dulbecco's minimal Eagle's medium (DMEM) supplemented with 4% fetal calf serum (FCS), 60 μg of penicillin/ml, 100 μg of streptomycin/ml, and 1 mg of G418/ml. DMEM for U87/CD4/CXCR4, NP2/CD4/CXCR4, and GHOST/CXCR4 additionally contained 1 μg of puromycin/ml.

Generation of full-length HIV-2 molecular clones by long-distance PCR. Genomic DNA from peripheral blood mononuclear cells (PBMC) (prCBL23) or H9 cells (CBL23) was isolated following standard molecular biology techniques 7 days after infection with viral supernatant of prCBL23 or CBL23 virus (29, 37), respectively. Full-length molecular clones were generated by a long-distance PCR. First, a primer pair for the 5′ half of the HIV-2 genome was used to amplify a 2.2-kb fragment (long terminal repeat [LTR]-*gag*) with primers AF (5′-GGACTGCGGCCGCCCCAGAGGAATTTGGGCACAAGTCAGG-3′) and AR (5′-AGTCGACCCCTGCTCGAGGTGCAGTAAGTCC-3′). The underlined sequence in AF encodes a *Not*I restriction site, and the underlined sequences in AR encode *Sal*I and *Xho*I sites. The *Xho*I site is a natural site occurring in this position in several HIV-2 isolates; however, the *Not*I and *Sal*I sites were added to the primer sequences for cloning purposes. The 3′ *pol*-*env*-3′LTR 8.5-kb fragment was generated with primers BF (5′-GGACTTACTGCACCTCGAGCAGGG-3′, *Xho*I site underlined) and BR (5′-TTTAACATATGGACCAGGCGGCGACTAGGAGAGATGGG-3′, *Nde*I site underlined). The 2.2-kb amplicon was digested with *Not*I and *Sal*I and cloned into a previously *Not*I/*Xho*I-digested pGEM-TEasy vector (Promega). The 8.5-kb 3′ amplicon was cloned into the T-tailed pGEM-TEasy vector and excised from positive recombinants with a *Xho*I/*Nde*I digest. The full-length molecular clone was obtained by digesting the 2.2-kb clones with *Xho*I/*Nde*I and cloning the 8.5-kb *Xho*I/*Nde*I 3′ ends into place (Fig. 1a).

For long-distance PCR, 3.75 U of Expand Long template polymerase mix was used along with 3 pmol of each primer, 500 μM concentrations of each deoxynucleoside triphosphate, 0.5 to 1 μg of genomic DNA, and 2.25 mM $MgCl_2$ supplied with buffer 3 of the Expand Long template kit. PCRs amplifying the 8.5-kb fragments were performed with an Applied Biosystems GeneAmp 9700 thermal cycler with the following settings for the pre-PCR: 94°C for 2 min followed by 10 cycles of 92°C for 10 s, 60°C for 30 s, and 68°C for 6 min. The PCR settings were 30 cycles of 94°C for 10 s, 60°C for 30 s, and 68°C for 8 min, with a 15-s extension per cycle. A final elongation cycle at 68°C for 7 min was added to complete PCR products. The 1.8-kb amplification products were generated with the following settings for pre-PCR: 94°C for 2 min followed by 15 cycles of 92°C for 10 s, 60°C for 30 s, and 68°C for 1 min. The PCR settings were 30 cycles of 94°C for 10 s, 60°C for 30 s, and 68°C for 1.5 min, with a 5-s extension per cycle, followed by a 7-min final elongation at 68°C.

Construction of recombinant proviruses. (i) MCR and MCN. The 5′ and 3′ HIV-2 proviral genomes were generated by long-distance PCR as described above. The full-length genomes were sequenced with ABI Prism BigDye sequencing kits and a Perkin Elmer 3100 sequencer. Sequencing data were analyzed with Sequencher, version 3.1.1, software, and subsequent full-length molecular constructs were designed with the aid of Gene Construction kit software.

(ii) MCRmcn*gag* and MCNmcr*gag*. A 2.2-kb *Not*I/*Xho*I fragment encompassing the 5′ LTR (R/U5) and the *gag* gene were cloned from MCR into MCN to generate MCNmcr*gag* (and vice versa).

(iii) MCRmcn*nef* and MCNmcr*nef*. A unique *Pml*I restriction site is located 92 bp downstream of the *nef* gene in both MCR and MCN constructs (position 8625). A 1,088-bp *Pml*I/*Nde*I (963 bp for MCR) fragment was excised from MCN and subcloned into the *Pml*I/*Nde*I site of MCR. The resulting clone MCRmcn*nef* maintains a stop codon within the cytoplasmic tail (CPT) of MCR; *env*, however, encodes a full-length *nef* gene. As the nucleotide sequences of both molecular clones are identical from the start of the *nef* open reading frame (ORF) to the *Pml*I site, no other nucleotide changes were transferred between the clones in this region.

(iv) MCRΔenv. While cloning MCRΔstop, a clone was isolated lacking a single nucleotide within the *Tth*111L restriction site. Sequencing demonstrated that the change at position 7795 from wild-type GACGTGGTC to GACGGGTC. The frameshift causes early termination of gp160.

(v) MCRmcn*env* and MCNmcr*env*. Two *Bsa*BI restriction sites in the *env* ORF (positions 6328 and 8286) were used to excise and exchange the ORF between MCR and MCN to generate *env* swaps.

(vi) MCRmcn*gagenv* and MCNmcr*gagenv*. LTR-*gag* sequences of clones MCRmcn*env* and MCNmcr*env* were swapped by *Not*I/*Xho*I digestion.

(vii) pMP11-MCRenv, pMP11-MCNenv, and pMP11-sMCNenv. Envelope coding regions were PCR amplified from molecular clone MCR or MCN with primers 5′-GGATAAGTCGCGGCCGCATGATGGGTGGTAGAAATCAGCTGC-3′ and 5′-GCCAGGGAAGCTTTCATAGGAGGGCGATCTCTGCTCCC-3′. The underlined sequences mark a *Not*I and a *Hin*dIII restriction site, respectively. The PCR products were cloned into expression vector pMP11 (35) to generate plasmids pMP11-MCRenv and pMP11-MCNenv. Construct pMP11-sMCN was generated by subcloning a *Bst*EII/*Pml*I fragment from MCR *env* containing the CPT into pMP11-MCN. The resulting clone contains the MCR CPT, which codes for a stop codon shortly after the transmembrane region, leading to the early termination of the CPT. DNA sequences have been confirmed by sequencing.

Mutation of MCR *gag*. Nucleotide point mutations in the *gag* gene of MCR were generated by site-directed mutagenesis changing the MCR nucleotide to the corresponding nucleotide MCN. The MCR LTR-*gag* sequence was excised (*Not*I/*Xho*I) and subcloned into vector pGEM-TEasy. Primers MAfwd (5′-GAATTCTTACAGTTTTAGGTCCATTAGTACCGACAG-3′), CA1fwd (5′-CAGGGAGATCTTAATGAAGAAGCAGCAGATTG-3′), and CA2fwd (5′-CAAACAGATCCAGCAGTGAAGAATGGATGACC-3′) were used in combination with the reverse complementary primer to generate single mutations, MCR-MA (D→G), MCR-CA1 (I→V), and MCR-CA2 (T→A). Double (MCR-MA/CA2) or triple (MCR-MA/CA1/CA2) mutants were generated with the single mutant (MCR-MA for generating MCR-MA/CA2) or the double mutant (MCR-MA/CA2 for generating MCR-MA/CA1/CA2).

Site-directed mutagenesis. PCR-based site-directed mutagenesis was performed according to the QuikChange XL site-directed mutagenesis kit (Stratagene, Inc.). Briefly, an anticomplementary primer pair was designed that contained the desired nucleotide changes. Primers were added to the template DNA, and the mutation reaction was carried out in a thermal cycler. The reaction mixture contained between 50 and 500 ng of template DNA, 200 μM concentrations of each deoxynucleoside triphosphate, 0.2 μM concentrations of each high-performance liquid chromatography-purified primer, 1.25 to 2.5 U of *Pfu* Turbo polymerase, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 0.1% (vol/vol) Triton X-100, 0.1 mg of bovine serum albumin/ml, and 2 to 8% (vol/vol) dimethyl sulfoxide. Thermal cycling conditions were 95°C for 30 s, 16 cycles of 95°C for 30 s, 55°C for 1 min, and 68°C for 2 min/kb. Ten microliters of the product was digested with 10 U of *Dpn*I, and 1 μl was transformed into *Escherichia coli* XL1-Blue bacteria.

Quantitative strong-stop PCR. U87/CD4/CXCR4 and HeLa/CD4 cells were seeded overnight in a 24-well plate at a density of 10^5 in DMEM and 4% FCS. The following day, the medium was replaced with 500 μl of fresh medium containing 500 focus-forming units (FFU) of either MCN or MCR pretreated with DNaseI (Roche) (50 U of DNase/ml plus 5 mM $MgCl_2$) for 1 h at 37°C (29). Virus titers were determined on U87/CD4/CXCR4 cells (data not shown). At 1, 2, 6, and 18 h postinfection, the inocula were removed and the cells were washed twice with 1 ml of phosphate-buffered saline (PBS). Total DNA was prepared with a QIAamp blood DNA mini kit according to the manufacturer's protocol.

The isolated DNA was subjected to quantitative PCR (Q-PCR) to determine the number of strong-stop transcripts present. Each 25-μl reaction mixture contained the following components: 1× Quantitech Probe PCR buffer (Qiagen), 900 nM forward primer (5′-AGCTGCCAGTTAGAAGCAAGTTAAGT-3′), 300 nM reverse primer (5′-TGTTATTCAGATGAACACCGAATGA-3′), 150 nM probe (5′-6-carboxyfluorescein-TTCCCATCTCTCCTAGTCGCCGCCT-6-carboxy-tetrafluorescein-3′), and 100 ng of total DNA. A standard curve was prepared with the MCR molecular clone in the background of 200 ng of genomic DNA (data not shown). PCR conditions consisted of one cycle of denaturation (95°C for 10 min) followed by 45 cycles of amplification (95°C for 15 s, 60°C for 1 min). All real-time PCR amplifications, data acquisition, and analysis were performed with the ABI PRISM 7000 sequence detection system.

Production of molecular clone-derived virus, mixed core-*env* particles and VSV-G pseudotypes. 293T cells were passaged 48 h prior to transfection. In a 10-cm^2 tissue culture dish, a semiconfluent (40 to 60%) monolayer was seeded 24 h later. The transfection reagent FuGENE6 was used according to the manufacturer's instructions. Briefly, a total of 2 μg of DNA was combined with 46 μl of FuGENE6 in a total volume of 80 μl of serum-free Optimem (Gibco, Invitrogen Corp.). The suspension was incubated for 15 min at room temperature. Eight milliliters of DMEM, containing 4% (vol/vol) FCS, 60 μg of penicillin/ml, and 100 μg of streptomycin/ml, was added to the transfection suspension. The trans-

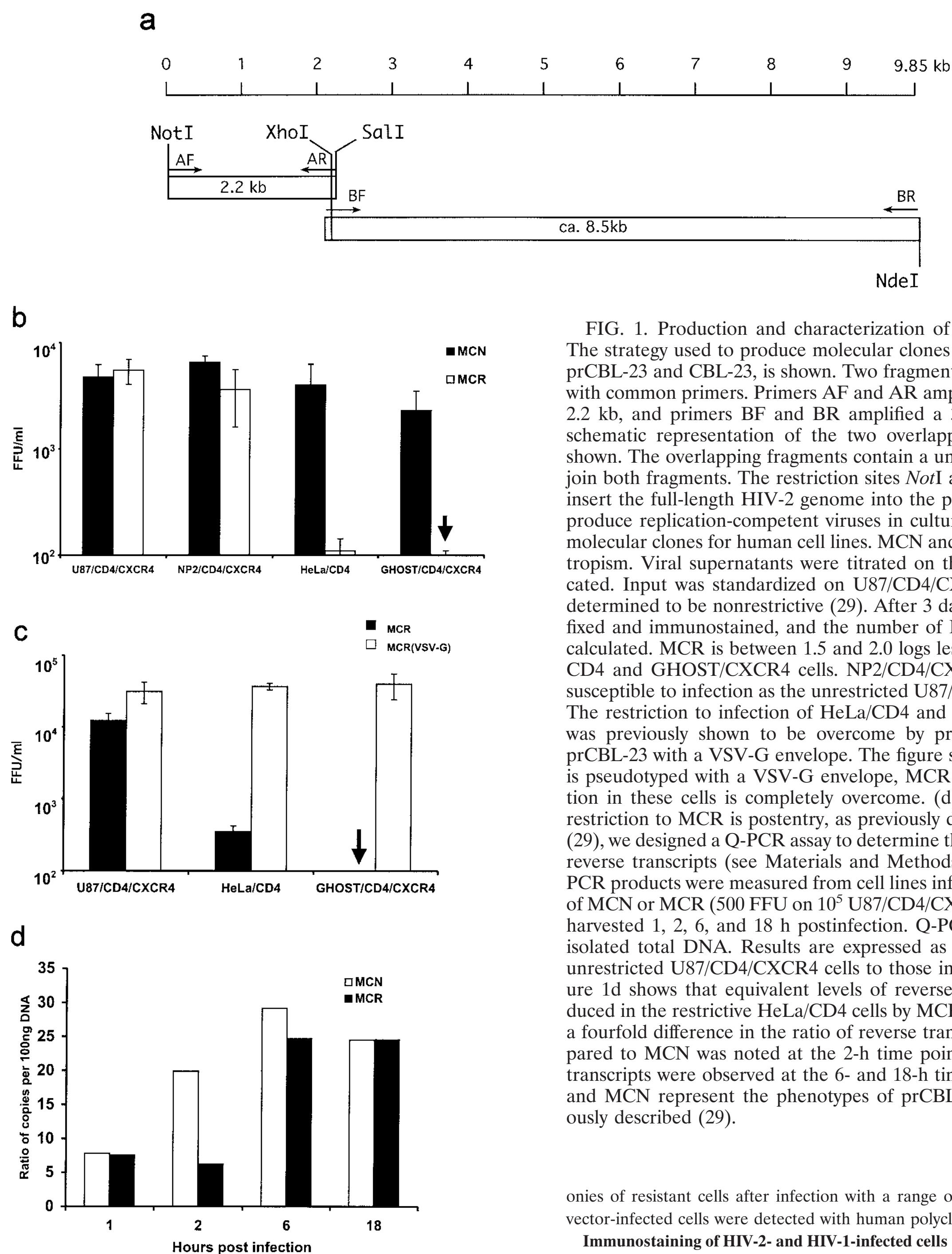

FIG. 1. Production and characterization of molecular clones. (a) The strategy used to produce molecular clones of the HIV-2 isolates, prCBL-23 and CBL-23, is shown. Two fragments were PCR amplified with common primers. Primers AF and AR amplified a 5′ fragment of 2.2 kb, and primers BF and BR amplified a 3′ 8.5-kb fragment. A schematic representation of the two overlapping PCR products is shown. The overlapping fragments contain a unique *Xho*I site used to join both fragments. The restriction sites *Not*I and *Nde*I were used to insert the full-length HIV-2 genome into the pGEM-TEasy vector to produce replication-competent viruses in culture. (b) Tropism of the molecular clones for human cell lines. MCN and MCR were tested for tropism. Viral supernatants were titrated on the four cell lines indicated. Input was standardized on U87/CD4/CXCR4 cells previously determined to be nonrestrictive (29). After 3 days, infected cells were fixed and immunostained, and the number of FFU per milliliter was calculated. MCR is between 1.5 and 2.0 logs less infectious on HeLa/CD4 and GHOST/CXCR4 cells. NP2/CD4/CXCR4 cells are just as susceptible to infection as the unrestricted U87/CD4/CXCR4 cells. (c) The restriction to infection of HeLa/CD4 and GHOST/CXCR4 cells was previously shown to be overcome by providing the restricted prCBL-23 with a VSV-G envelope. The figure shows that when MCR is pseudotyped with a VSV-G envelope, MCR (VSV-G), the restriction in these cells is completely overcome. (d) To confirm that the restriction to MCR is postentry, as previously described for prCBL23 (29), we designed a Q-PCR assay to determine the levels of strong-stop reverse transcripts (see Materials and Methods). Briefly, strong-stop PCR products were measured from cell lines infected with equal doses of MCN or MCR (500 FFU on 10^5 U87/CD4/CXCR4 cells). Cells were harvested 1, 2, 6, and 18 h postinfection. Q-PCR was performed on isolated total DNA. Results are expressed as the ratio of copies in unrestricted U87/CD4/CXCR4 cells to those in HeLa/CD4 cells. Figure 1d shows that equivalent levels of reverse transcripts were produced in the restrictive HeLa/CD4 cells by MCR and MCN. Although a fourfold difference in the ratio of reverse transcripts for MCR compared to MCN was noted at the 2-h time point, equivalent levels of transcripts were observed at the 6- and 18-h time points. Thus, MCR and MCN represent the phenotypes of prCBL23 and CBL23 previously described (29).

fection mixture was added to the cells and incubated at 37°C (5% [vol/vol] CO_2) in a humidified incubator. Supernatants were harvested after 48 to 72 h.

The molecular clones were transfected as described above. More specifically, VSV-G pseudotype particles were generated by cotransfection of 1 μg of MCRΔenv and 1 μg of VSV-G envelope expression vector pMDG. HIV-1 pseudotyped particles were generated with an HIV-1 core vector (p8.91) and pMP11-MCR or pMP11-sMCN. HIV-2 pseudotype particles were generated from MCRΔenv in the presence of HIV-1 *env* expression vector pSVIII-NL4.3env. U87/CD4/CXCR4, HeLa/CD4, and GHOST/CXCR4 cells (10^5) were infected overnight, and the proportion of green fluorescent protein (GFP)-positive cells was determined by flow cytometry with a FACScan (Becton Dickinson). Titers of puromycin-encoding vectors were determined by counting colonies of resistant cells after infection with a range of virus doses, and HIV-2 vector-infected cells were detected with human polyclonal HIV-2 serum.

Immunostaining of HIV-2- and HIV-1-infected cells and calculation of restriction. The immunostaining method has been described previously (41). Briefly, methanol-acetone (1:1)-fixed cells infected with HIV-2 or HIV-1 were immunostained with HIV-2 human serum diluted 1/4,000 or anti-HIV-1 p24 monoclonal antibody diluted 1/40 (1:1 mix of EVA 365 and 366 from the Medical Research Council AIDS Reagent Program, Potters Bar, United Kingdom). Second-layer β-galactosidase conjugates of goat anti-human immunoglobulin G (HIV-2) or goat anti-mouse immunoglobulin G (HIV-1) were used to detect first-layer antibodies at a dilution of 1:400 (Southern Biotechnology Associates, Inc., Birmingham, Ala.). Infected cells were stained blue with X-Gal (5-bromo-4-chloro-3-indoyl-β-galactopyranoside) in PBS containing 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, and 1 mM magnesium chloride. Foci of infection, which stained blue, were counted, and virus infectivity was estimated as FFU per milliliter. The restriction (*n*-fold) is calculated as the ratio of infectivity of unrestricted to restricted cells.

TABLE 1. Amino acid comparison of MCR and MCN

Protein	Length[a]	No. of changes	Domain[b]	Position no.[c]	Amino acid encoded by:	
					MCR	MCN
Gag	517	5	MA	65	D	G
			CA	207	I	V
			CA	314	T	A
			P6	470	K	N
			P6	503	Y	C
Pol	1,035	5		43	I	L
				73	S	G
				75	I	M
				507	G	E
				563	V	I
Vif	215	1		187	D	G
Vpx	112	1		29	I	V
Vpr	105	0				
Tat	130	2		126	C	R
				127	S	G
Rev	107	1		70	D	G
Env	858	15	Leader	−9	A	T
				40	E	D
			V1	**120**	T	S
				132	K	E
				154	N	S
				156	A	T
			V2	**171**	S	D
			V4	**394**	K	R
			Near CD4 bs	**433**	E	G
			Near CD4 bs	**437**	D	N
				636	S	G
			CPT	718	L	S
			CPT	719	Stop	W
			CPT	738	T	A
			CPT	758	A	V
Nef (MCN)	256					
Nef (MCR)	69	0		70	Stop[d]	

[a] Number of nucleotides from position 1 of the initiation codon (ATG) to the last base before the termination codon (TAA, TGA, or TAG). The Pol polyprotein does not start with an ATG codon.
[b] The domains in Gag and Env are based on the HIV-2 prototype molecular clone ROD. bs, binding sequence.
[c] Boldface type indicates amino acid differences in the SU protein.
[d] Stop, stop codon.

MLV-N and MLV-B virion VSV-G pseudotype production. VSV-G-enveloped MLV-N/B *gag* particles were produced from a combination of three plasmids as described previously (30, 42). MLV-N and MLV-B *gag* cores were expressed from pCIG3-N and pCIG3-B, respectively, and pseudotyped with VSV-G envelope expressed from pMDG. A proviral reporter genome, pCNCG, encoding enhanced GFP (EGFP) was packaged into the pseudovirions. One microgram of pCNCG, 1 μg of pCIG3-N or -B, and 1 μg of pMDG were mixed, and a confluent layer of 293T cells was transfected in the presence of 6 μl of FuGENE6. Pseudotype particles were harvested 48 and 72 h posttransfection, and virus titers were determined on MDTF.

Abrogation assay. HeLa/CD4, U87/CD4/CXCR4 and HOS/CD4/CXCR4 cells were plated in 48-well trays at 5×10^3 cells per well 24 h prior to infection. Then, MVL-N and -B pseudotype viruses were added to the cells at a multiplicity of infection (MOI) of approximately 16. After 3 h the cells were washed three times with PBS and challenged with MCN or MCR. At 48 h postinfection, the cells were screened for MLV expression by visualization of EGFP expression under fluorescence, and at 72 h posttransduction, cells were immunostained for MCN and MCR infection with human polyclonal HIV-2 serum.

To show that MLV-N particles could inhibit Lv1-mediated retroviral and lentiviral restriction in HeLa/CD4, an inhibition assay was performed, 10^5 HeLa-CD4 cells were plated and grown overnight. The cells were then exposed to a dose of MLV-Npuro(VSV-G) vector at an MOI of 10. Two hours later, the cells were washed and exposed to an MOI of 0.1 of HIV, equine infectious anemia virus (EIAV), MLV-N. or MLV-B VSV-G pseudotypes. The increase in GFP-positive cells compared to nohabrogated controls was determined by flow cytometry.

Nucleotide sequence accession numbers. The GenBank accession numbers for MCN and MCR are AY509259 and AY509260, respectively.

RESULTS

Production of molecular clones of restricted HIV-2 prCBL-23 and nonrestricted CBL-23 viruses. The restricted primary isolate prCBL-23 and the nonrestricted CBL-23 were used to infect PBMC and H9 cells, respectively, and DNA was prepared. Figure 1a shows the strategy used for PCR cloning. A two-step PCR was designed to amplify a 2.2-kb 5′ LTR-*gag* fragment and an overlapping 8.5-kb 3′ *pol*-LTR from each viral species. Fragments were subcloned and joined via a common internal *Xho*I site to generate full-length molecular clones (Fig. 1a).

As previously described (29), prCBL-23, but not CBL-23, is restricted in HeLa/CD4 and GHOST/CXCR4 cells, whereas both viruses can infect U87/CD4/CXCR4 with equal efficiency. Thus, molecular clones (16 of CBL-23 and 22 of prCBL-23) were produced and tested for their infectivity on HeLa/CD4 cells compared with U87/CD4/CXCR4 cells, the unrestricted cell type (data not shown). Two clones representing prCBL-23 and CBL-23 (MCR and MCN, respectively) were selected for further gene mapping and analysis.

Figure 1b shows that the nonrestricted virus MCN plates to equivalent titers on all four cell lines tested, U87/CD4/CXCR4, NP2/CD4/CXCR4, HeLa/CD4, and GHOST/CXCR4 (4.7×10^3, 6.5×10^3, 4.0×10^3, and 2.3×10^3 FFU/ml, respectively). By comparison, the restricted clone, MCR, could infect U87/CD4/CXCR4 and NP2/CD4/CXCR4 cells equally well (5.5×10^3 and 3.6×10^3 FFU/ml), whereas infectivity on HeLa/CD4 cells was reduced by at least 15-fold on both HeLa/CD4 and GHOST/CXCR4 cells (1.1×10^2 and $<10^2$ FFU/ml, respectively). Thus, the molecular clones MCN and MCR display the same tropism phenotype for infection of these human cells as has been previously described for prCBL-23 and CBL-23 isolates (29).

We tested whether VSV-G pseudotyping could overcome the block to infection by MCR of HeLa/CD4 and GHOST/CXCR4 as previously observed for the noncloned prCBL-23 (29). We cotransfected MCR with a VSV-G envelope expression construct and used pseudotyped virus to infect permissive and nonpermissive cells. Figure 1c shows that MCR plated to a titer of 1.2×10^4 FFU/ml on U87/CD4/CXCR4 cells, whereas the same virus input resulted in only 3.5×10^2 FFU/ml on HeLa/CD4 cells and <100 FFU/ml on GHOST/CXCR4 cells. The VSV-G pseudotyped virus, however, plated equivalently well on all three cell lines (3.1×10^4, 3.6×10^4, and 4.0×10^4 FFU/ml). Thus, a VSV-G envelope can rescue the restricted infection of MCR on HeLa/CD4 and GHOST/CXCR4 cells.

To confirm that the restriction to MCR is postentry, as previously described for prCBL23 (29), we designed a Q-PCR assay to determine the levels of strong-stop reverse transcripts (see Materials and Methods). Figure 1d shows that equivalent levels of reverse transcripts were produced in the restrictive HeLa/CD4 cells by MCR and MCN. Although a fourfold difference in the ratio of reverse transcripts for MCR compared

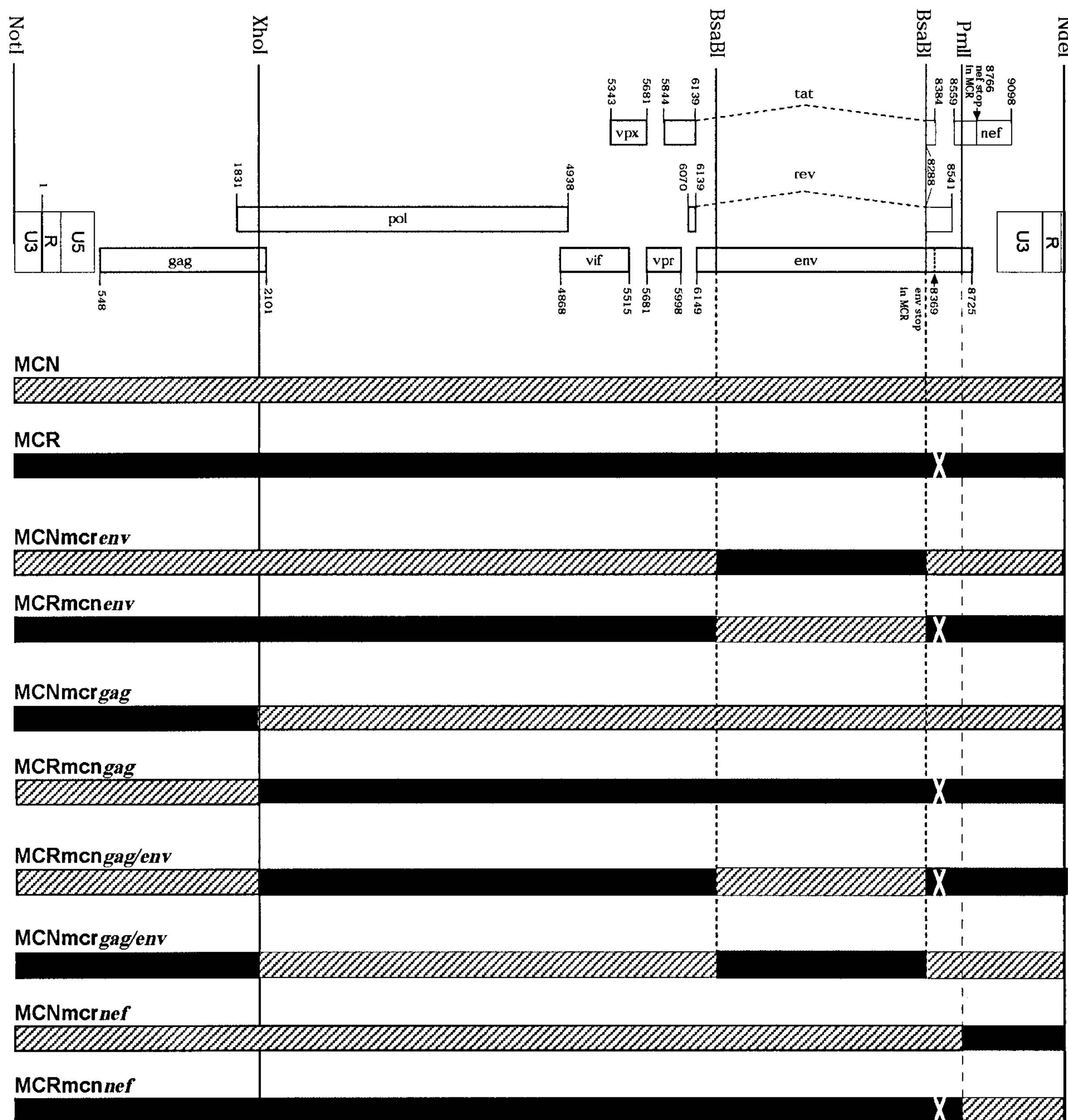

FIG. 2. Schematic genome representation of MCN and MCR and recombinants thereof. A representation of the HIV-2 genome is shown. The nucleic acid numbering system is in accordance with the convention of the first base of the 5′ R region being number 1. The terminal cloning sites of the molecular clones are indicated (*Not*I and *Nde*I). Additional unique sites (*Xho*I and *Pml*I) used for cloning are also shown (*Bst*EII is not shown). The *Bsa*BI site allows the transfer of the gp120 ectodomain of *env* between the molecular clones. The spliced donor/acceptor of *tat* and *rev* were adopted from the ROD molecular clone (accession no. M15390). A premature stop codon within the envelope of MCR is indicated with an arrow (position 8369). Also, there is a premature stop codon in the *nef* reading frame at position 8764 in MCR. The full-length molecular clone and swapped fragments of MCN (molecular clone nonrestricted) and MCR (molecular clone restricted) are represented by grey and solid fills, respectively. The white cross within MCR represents the premature stop codon in the CPT of the *env* gene. Gene swaps are indicated in italics preceded by the clone of origin in lowercase letters. For example, the MCN clone containing the envelope gene for MCR is MCNmcr*env*.

to MCN was noted at the 2-h time point, equivalent levels of transcripts were observed at the 6- and 18-h time points. Thus, MCR and MCN represent the phenotypes of prCBL23 and CBL23 previously described (29).

Sequence comparison of MCN and MCR and production of recombinant molecular clones. The genomes of MCN and MCR were sequenced, and comparative sequence analysis showed amino acid differences throughout the genomes. As

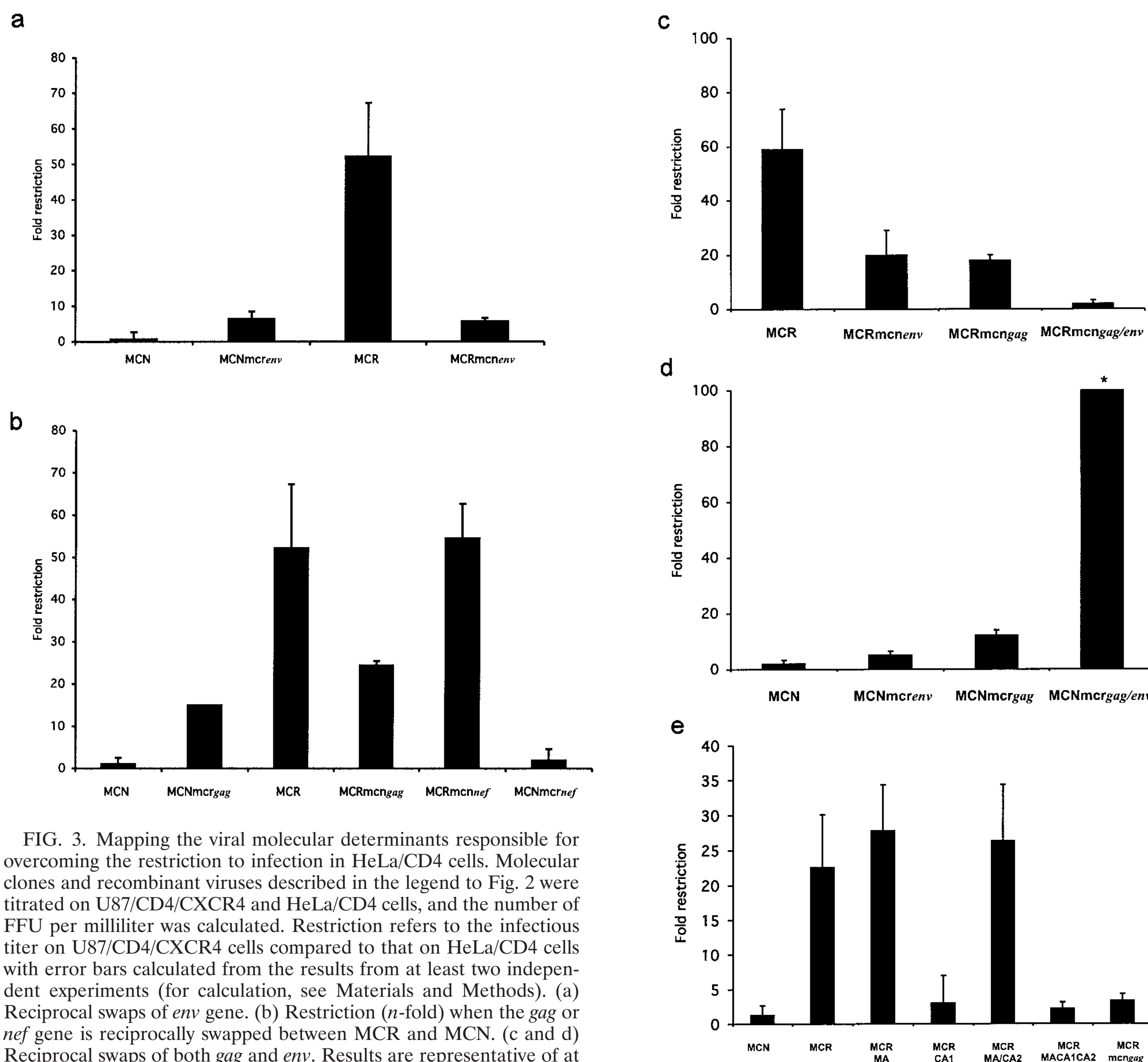

FIG. 3. Mapping the viral molecular determinants responsible for overcoming the restriction to infection in HeLa/CD4 cells. Molecular clones and recombinant viruses described in the legend to Fig. 2 were titrated on U87/CD4/CXCR4 and HeLa/CD4 cells, and the number of FFU per milliliter was calculated. Restriction refers to the infectious titer on U87/CD4/CXCR4 cells compared to that on HeLa/CD4 cells with error bars calculated from the results from at least two independent experiments (for calculation, see Materials and Methods). (a) Reciprocal swaps of *env* gene. (b) Restriction (*n*-fold) when the *gag* or *nef* gene is reciprocally swapped between MCR and MCN. (c and d) Reciprocal swaps of both *gag* and *env*. Results are representative of at least three experiments. (e) A single point mutation in *gag* rescues the *gag* restriction. Site-directed mutagenesis of MCR was used to introduce amino acid changes in Gag at positions 65 (MA), 209 (CA1), and 314 (CA2) or combinations of these. Viruses were titrated, and the restriction was calculated and compared to those of MCR and MCN. The results are representative of at least three experiments. Molecular clones MCR-MA (containing the MA mutation at position 65) and MCR-MA/CA2 (containing mutations at positions 65 and 314) showed no rescue. MCR-CA1 and the triple mutant MCR-MA/CA1/CA2 rescued the restriction to levels seen for MCRmcn*gag*.

shown in Table 1, amino acid differences were noted in Gag (5), Pol (5), Vif (1), Vpx (1), Tat (2), Rev (1), and Env (15), including a premature stop codon at position 719 in the *env* gene, resulting in a truncated CPT shortened by 140 amino acids. This early termination codon was found in six of six independently generated MCR clones. The remaining amino acids of the CPT were conserved between both clones. In Nef there was an early truncation after 70 amino acids in MCR that was not present in MCN (256 amino acids long). The Vpr was identical between both viruses.

Within the LTR region of MCR, there were nine changes and a nucleotide deletion at position 9082 which resulted in a 125-bp deletion in U3 which was not observed in MCN. Figure 2 also shows the restriction sites used to swap genes between MCR and MCN. Swaps were made between *env* (*Bsa*BI to *Bsa*BI), LTR-*gag* (*Not*I to *Xho*I), and *nef* (*Pml*I to *Nde*I). The stop codon for the MCR *env* was repaired by site-directed mutagenesis.

The molecular clones resulting from the gene swaps and mutagenesis were used to determine the genes relevant to the restriction. U87/CD4/CXCR4 cells were used as the controls for unrestricted infection and compared to infection of HeLa/CD4 cells as well as GHOST/CXCR4 cells for restricted infection. For these mapping studies and because the rate of infection can vary from experiment to experiment, the ratio of

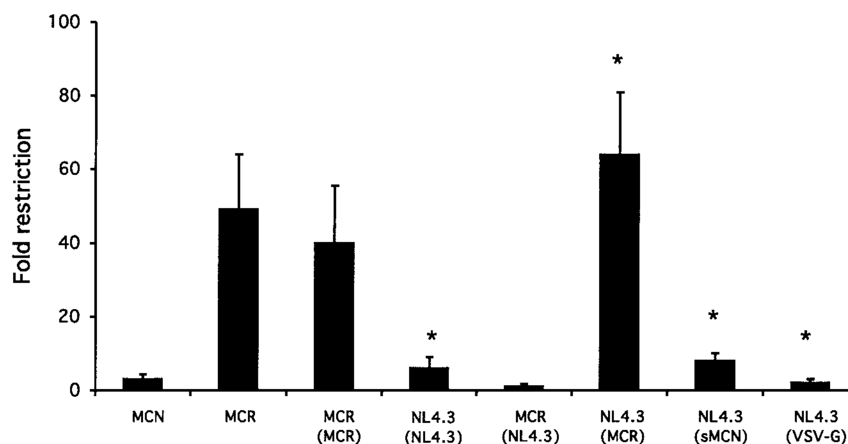

FIG. 4. The HIV-1 (NL4.3) core is restricted, but the NL4.3 envelope rescues the restriction in HeLa/CD4 cells. The restriction refers to the infectious titer on U87/CD4/CXCR4 cells compared to that on HeLa/CD4 cells. Error bars were calculated from the results from at least two independent experiments (see Materials and Methods). MCRΔenv or HIV-1 NL4.3Δenv was pseudotyped with NL4.3, MCR, sMCN, or VSV-G envelopes called MCR (NL4.3), NL4.3 (MCR), NL4.3 (sMCN), and NL4.3 (VSV-G). *, the HIV-1 NL4.3 core-based vectors encoding GFP were pseudotyped with MCR, sMCN, and VSV-G in a similar way (Materials and Methods). The titers of these viruses were determined by flow cytometry, and the restriction (*n*-fold) was plotted as the reduction of the HeLa/CD4 cell titer compared to that of U87/CD4/CXCR4 cells, as described before.

infectivity and absolute titer were measured. FFU per milliliter were calculated, and the restriction (*n*-fold) was derived (the FFU per milliliter ratio of HeLa/CD4 to U87/CD4/CXCR4 cells) (see Materials and Methods).

Role of envelope in restriction. To determine the role of envelope in the tropism of MCR, we repaired the premature stop codon in the envelope gene, resulting in a full-length CPT of TM (gp41). This repair resulted in a small rescue of the restrictive phenotype. A detailed site-direction mutagenesis study of the envelope-mediated restriction suggests that the major determinant of the envelope restriction is SU (S. Reuter, submitted for publication). The envelopes were swapped from amino acid 40 to amino acid 636, which encompasses all of the amino acid differences in the SU protein (Table 1) between MCR and MCN. Figure 3a shows the restriction of these viruses. The nonrestricted envelope rescued MCR by almost 90% (MCRmcn*env*). In contrast, the MCR envelope conferred only a partial restricted phenotype to MCN (MCNmcr*env*). This result suggests that MCN contains other genes which may rescue the restricted phenotype.

***gag* but not *nef* is partially responsible for the block to infection of HeLa/CD4 cells.** We further investigated whether other genes might rescue MCR from the block to infection of HeLa/CD4 cells. To determine whether Nef was involved in the restriction, we swapped a *nef*-LTR fragment between MCR and MCN by using *Pml*I/*Nde*I (MCRmcn*nef* and MCNmcr*nef*). Figure 3b shows that when the *nef* genes and LTR were exchanged between MCR and MCN there was no change in restriction. Thus, it is unlikely that Nef or the LTR region is involved in the restriction.

Convenient restriction sites allowed the exchange of *gag* between clones, and we next determined whether these genes could be involved in the restricted phenotype of MCR. When the restricted *gag* was substituted into MCN, the resulting recombinant, MCNmcr*gag*, was partially restricted in HeLa/CD4 cells (Fig. 3b). Also, the nonrestricted core of MCN was able to partially rescue the restricted phenotype of MCR (MCRmcn*gag*). Thus, in addition to *env*, the *gag* gene is involved in the restricted phenotype of MCR.

Both Env and Gag are required to fully rescue or confer the restricted phenotype of MCR. Since neither Gag nor envelope alone could fully rescue the restriction for MCR, we swapped both the *env* and *gag* genes from the unrestricted MCN into restricted MCR to produce the recombinant MCRmcn*gag*/*env*. Figures 3c and d show that MCRmcn*gag*/*env* completely recovered efficiency of infection of HeLa/CD4 cells. Also, *env* and *gag* from the restricted virus conferred the full restricted phenotype to MCN (MCNmcr*gag*/*env*) (Fig. 3d). Thus, both *env* and *gag* are responsible for the restricted tropism of MCR for HeLa/CD4 cells.

The restriction due to *gag* can be rescued by a V to I amino acid change at position 207. It was observed that a single amino acid in the envelope gene (SU) is responsible for the part of the restriction conferred by the envelope gene with pseudotypes of PrCBL-23 and CBL-23 envelopes and a ROD core (Reuter, submitted). We sought to determine the genetic location of the restriction contributed by *gag*. From the sequence analysis, we identified three changes in Gag, one in MA (position 65), and two in CA (positions 207 and 314). We also noted two differences in the P6 domain, but because the restriction is early in the viral life cycle and the role of P6 is seen at budding (13, 15, 21), we initially investigated the MA and CA differences. To do this, we generated a series of point mutations in MCR *gag* to the equivalent nucleotides in MCN *gag*. Site-directed mutagenesis of MCR was used to introduce amino acid changes in Gag at positions 65, 209, and 314 or combinations of these. Molecular clones MCR-MA (containing the MA mutation at 65) and MCR-MA/CA2 (containing mutations 65 and 314) showed no rescue of restriction compared to MCR. In contrast, MCR-CA1 and the triple mutant MCR-MA/CA1/CA2 rescued the restriction almost to MCN levels. In addition, both MCR-CA1 and MCR-MA/CA1/CA2

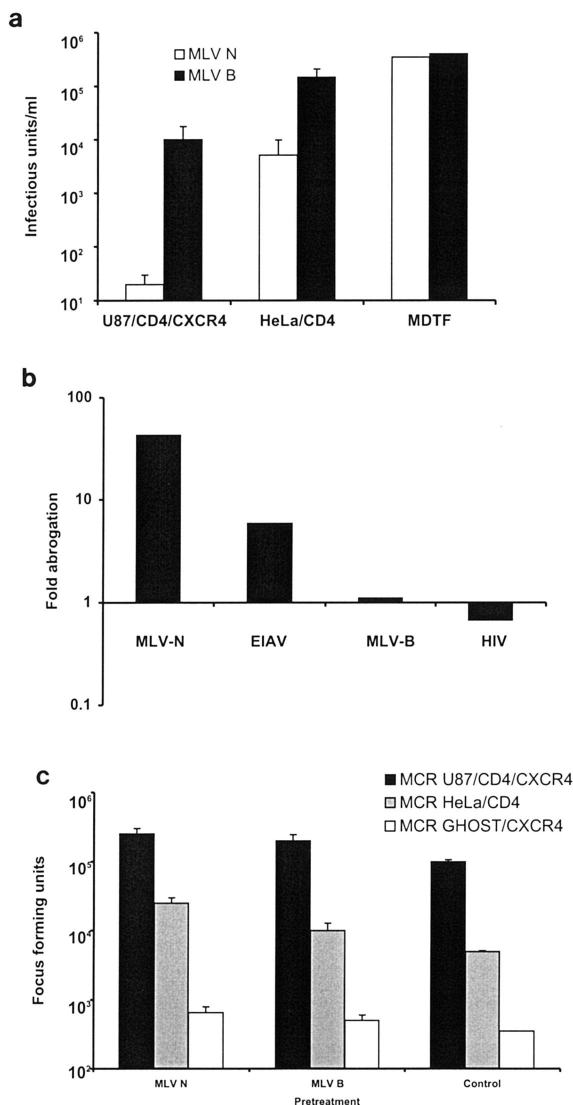

FIG. 5. The restriction to infection of HeLa/CD4 and GHOST/CD4/CXCR4 cells is distinct from that of Fv1/Ref1/Lv1. (a) The Ref1-restricted MLV-N (VSV-G) and unrestricted MLV-B (VSV-G) were titrated on U87/CD4/CXCR4 and HeLa/CD4 cells and on the Fv1-null murine cell line MDTF. (b) Abrogation of Ref1 in HeLa/CD4 cells by MLV-N. Cells (10^5) were pretreated either with control medium or with an MOI of 10 of MLV-Npuro (VSV-G) particles. Two hours later, the cells were exposed to an MOI of 0.1 of various VSV pseudotyped retroviral and lentiviral vectors encoding GFP. Inhibition of infection was plotted as the increase (*n*-fold) in the proportion of GFP-positive cells compared to the untreated cells and was measured by flow cytometry. (c) Pretreatment of cells with an MOI of 16 of MLV-N (VSV-G) or MLV-B (VSV-G) did not abrogate the restriction to infection of MCR on HeLa/CD4 or GHOST/CXCR4 cells.

showed the same phenotype as MCRmcn*gag*. From these results, we conclude that the *gag* mutation 207 I→V in the MCR capsid is entirely responsible for the *gag* restriction.

HIV-1 Env, but not core, can rescue the block to infection of MCR. We determined whether this crucial step in the life cycle of HIV-2 might also be used by HIV-1. We first determined whether HIV-1 envelope could rescue MCR virus. From MCR we generated an envelope deletion construct, MCRΔ*env*. We also generated envelope expression constructs of the MCR and MCN with a truncated CPT (sMCN) (see Materials and Methods). MCRΔ*env* and p8.91, encoding the capsid of NL4.3, were pseudotyped with envelopes derived from MCR, sMCN, VSV-G, and NL4.3. Pseudotyped viruses were plated on HeLa/CD4 and U87/CD4/CXCR4, and their restrictions (*n*-fold) were determined. As shown in Fig. 4, the MCR envelope conferred restriction on the MCR core as expected. Interestingly, the envelope of HIV-1, NL4.3 could rescue restricted MCR core. NL4.3 itself with its homologous envelope is unrestricted. Furthermore, the MCR envelope conferred restriction on the NL4.3 core, which was rescued by the unrestricted sMCN envelope. Pseudotyping with VSV-G confirmed that this envelope, similar to HIV-2, bypasses the restriction. Thus, we have shown that the envelope of HIV-1 can rescue the restriction and that the core of NL4.3 is also susceptible to this restriction if delivered to an unsuitable compartment.

Restriction conferred by either *gag* or *env* is distinct from Ref1/Lv1. Saturable factors that restrict MLV in mice (Fv1) (3, 14) and humans (Ref1) (43) have been described and mapped to the viral CA (14). There are two alleles of Fv1. Cells expressing $Fv1^N$ restrict MLV-B, those expressing $Fv1^B$ restrict MLV-N, and null cells do not restrict. Abrogation of Ref1 by MLV-N CA also inhibits HIV-1 and HIV-2 in monkey cells and is termed Lv1 (lentiviral restriction element 1) (2, 10, 20, 32, 34). Although not yet proven, it is likely that Ref1 and Lv1 are similar. The block to infection described in this paper appeared unlikely to be the same as Ref1/Lv1 because it is overcome by VSV-G (29). Since our restriction partially mapped to *gag*, which encodes the gene for CA, we determined whether the block to infection is Ref1/Lv1. We first compared the levels of Ref1 expression in our unrestricted U87/CD4/CXCR4 cells with our restricted HeLa/CD4 cells. MLV-N and MLV-B *gag*/*pol* EGFP vectors pseudotyped with VSV-G envelope were titrated on both cell types. We used the Fv1 null cell line MDTF as a positive control for viral infection (Fig. 5a). If Ref1 played a role in the restriction described in this paper, we would expect its activity to be higher in restrictive HeLa/CD4 cells. Figure 5a shows that, on the contrary, Ref1 was active to higher levels in the unrestrictive U87/CD4/CXCR4 cells. Although MLV-N and MLV-B had the same titer on MDTF, infection of MLV-N was reduced in both human cells types but was far more restricted in U87/CD4/CXCR4 cells.

The Ref1 restriction element can be saturated by preinoculation with MLV-N but not MLV-B *gag*-containing particles (43). Also shown in Fig. 5b, MLV-N treatment enhanced MLV-N and EIAV vector infection 60-fold and 5-fold, respectively, but did not enhance unrestricted HIV-1 and MLV-B vectors. These results are in agreement with previous levels of Ref1 inhibition seen in human cells (16). In contrast, pretreatment of restricted HeLa/CD4 and GHOST/CXCR4 cells had no effect on the restricted phenotype (compared to U87/CD4/CXCR4 cells) of HIV-2 MCR (Fig. 5c). Thus, we conclude that the block to MCR infection in HeLa/CD4 and GHOST/CXCR4 cells is distinct from that of Ref1/Lv1.

DISCUSSION

We show, for the first time, that both Gag and Env of HIV-2 act to facilitate an early fusion or entry step. This study arose

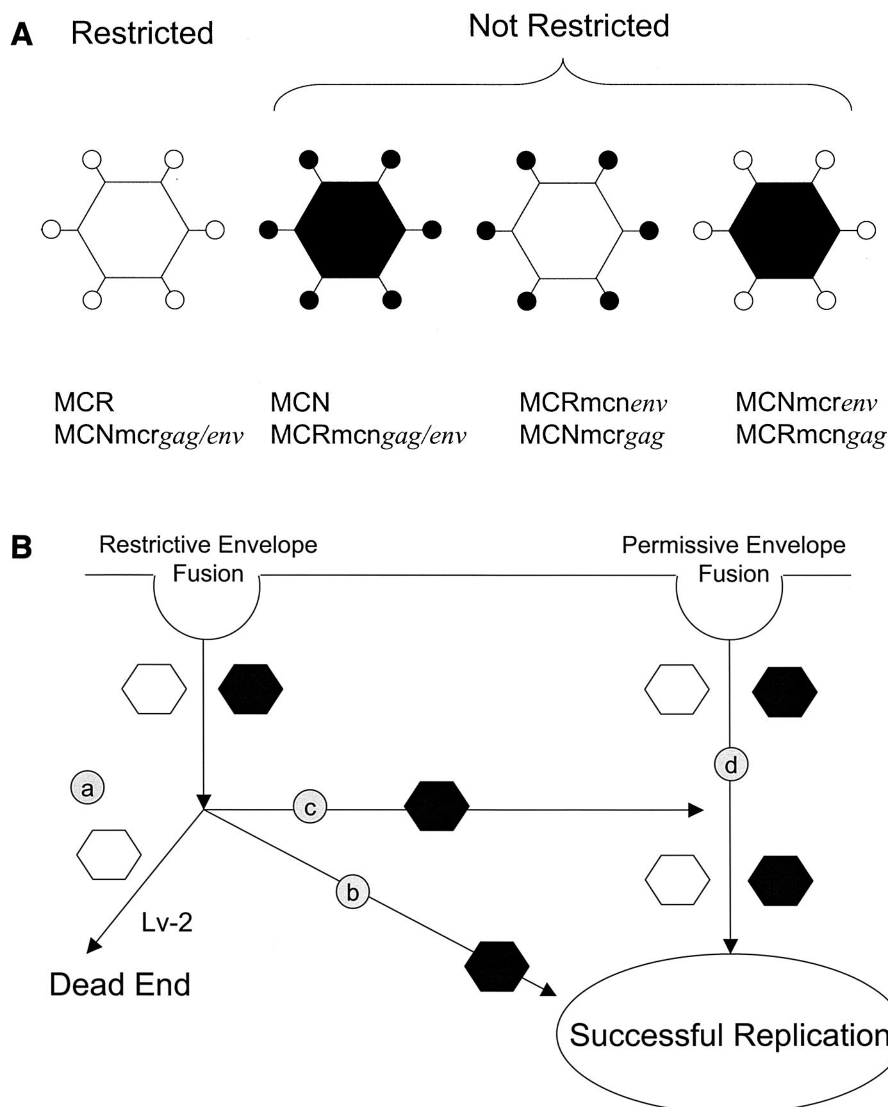

FIG. 6. (A) A schematic diagram of MCR, MCN, and chimeras shows their phenotypes on HeLa/CD4 cells. (B) A working model for the results of the tropism studies are presented here. Restrictive cells have at least two routes of entry. One, which is mediated by a restrictive MCR envelope, can result in a route where Lv2 is operative, if the virus also has a restrictive core (a). This entry mechanism will result in a dead end to infection. If the virus has an unrestrictive core, such as MCN, then the Lv2 is bypassed (b) or the virus is rerouted to a permissive pathway (c and d). Nonrestrictive envelopes deliver the core to a permissive pathway (d) and bypass Lv2.

from a previous report on the relative tropism of a primary isolate of HIV-2 (prCBL-23) and a laboratory-adapted isolate (CBL-23) cultured from the same individual (29). Both isolates were equally efficient at infection of CD4-expressing human T-cell lines as well as primary PBMC. PrCBL-23, however, was less able to infect other human cells such as HeLa/CD4 and HOS/CD4/CXCR4 cells. The restriction to infection of prCBL-23 was mapped to a postentry event because fully reverse transcribed viral DNA could be detected in restrictive cells. Although the restricted step in infection is detected post-reverse transcription, we hypothesize that it results from a failure during or after plasma membrane fusion because it can be bypassed by pseudotyping the restricted virus with a VSV-G envelope. Since the VSV-G envelope will redirect virion entry via an endocytic route, we further hypothesized that the restriction occurred because the virus was delivered to an inappropriate cellular compartment during or after fusion at the plasma membrane.

To map the viral genes responsible for this restriction, we produced molecular clones of the restricted (MCR) and nonrestricted (MCN) viruses. By a combination of sequence comparison, gene swapping, and site-directed mutagenesis we have located the genes responsible for the restricted phenotype to both *gag* and *env*. In gene swapping experiments, if either *gag* or *env* alone was swapped between molecular clones, the restricted or unrestricted phenotype was only partially conferred; the combination of both *gag* and *env*, however, resulted in a complete exchange of phenotypes. There is a truncation of the

CPT of the *env* gene in MCR but not in MCN. No significant effect was seen if this tail was repaired. A detailed analysis of the envelope gene of this restriction is described elsewhere (Reuter, submitted).

Thus, besides its role in engaging receptors at the cell surface to induce cell fusion, the envelope also ensures that the virion is placed inside the cell correctly to undergo postentry events. Schmidtmayerova et al. (36) have suggested that early fusion events may influence the cellular compartment in which postentry events occur in macrophages. An envelope-dependent block of SIV in macrophages (31) was shown to occur after nuclear entry (23).

As for Ref1/Lv1, where CA was identified as responsible for a postentry block, we also identified the CA protein to be partially responsible for the restriction described here. However, we show that the restrictive step to replication in this study is distinct from that of Ref1/Lv1. Unlike Ref1/Lv1, it is rescued by VSV-G, and we show that it is not abrogated by N-MLV (or B-MLV), which was previously shown to rescue the Ref1 restriction (42). Indeed, we find that the unrestricted reference cell U87/CD4/CXCR4 has far more Ref1/Lv1 activity (at least 18-fold) than the restricted HeLa/CD4 and GHOST/CXCR4 cells. Furthermore, Ref1/Lv1 is envelope independent (1). Taken together, these results strongly suggest that the restriction described here is different from that of Ref1/Lv1. We propose to call it Lv2.

Heterokaryon experiments of restrictive and nonrestrictive cells fused with the human T-cell leukemia virus type 1 envelope show that the Lv1 restriction is a dominant-negative factor (10). Results from our heterokaryon experiments with either polyethylene glycol or human T-cell leukemia virus type 1 envelope-mediated fusion, however, cannot be interpreted. Homokaryon fusion results in rescue of MCR restriction. We believe that this may be due to general perturbation of the plasma membrane. Thus, it may not be possible to characterize the dominance of all restriction factors at a cellular level.

A producer cell-dependent restriction has been reported for MLV, which phenotypically resembles the restriction described here, as it is manifested post-reverse transcription. Nuclear localization was only observed if MLV was pseudotyped by VSV-G (38). We are currently investigating the possibility that the restriction described here is also producer cell dependent. So far, no effect on relative tropism has been seen when the restricted virus is produced from at least four different cell types (data not shown).

Because we determined both the capsid and envelope to be responsible for overcoming this cellular restriction, we conclude that it most closely resembles that described by Chackerian et al. for HIV-1 infection of macaque cells (7). In this case, isolates of HIV-1 are restricted either before or after reverse transcription but prior to nuclear entry. The block to infection is overcome by expression of the appropriate coreceptors (implying a role for envelope) or by supplying the HIV-1 envelope with SIV genes including the CA (7). It was also observed that restriction can occur prior to reverse transcription if, for example, an HIV-2 ROD core is used together with a restricted envelope in pseudotype experiments (Reuter, submitted).

Our results thus far do not resolve whether Gag and Env act simultaneously (together or in parallel) or sequentially in this restrictive step. However, it is unlikely that a physical interaction (at least not prior to fusion) is required because the restricted virus has a truncated CPT with only 35 amino acids remaining that are conserved between the restricted and unrestricted virus and exchange of the CPTs had little effect (Reuter, submitted). Mapping of an amino acid responsible for the Env defect to the N terminus of SU (Reuter, submitted) strongly suggests that Gag and Env act independently in this step. It is interesting, however, that while a nonrestrictive envelope almost completely rescues an MCR core, the MCR restrictive envelope only partially conferred restriction to the MCN core. Our present model explaining this result is shown in Fig. 6. Fusion into the restrictive cell can occur by two routes. A restrictive route results in delivery of the viral core to a compartment where Lv2 is operative and results in a dead end. However, an unrestrictive core, delivered by this route, can either avoid Lv2 or reroute to a permissive pathway. The permissive envelope of MCN may also deliver directly to this nonrestricted pathway.

This specific event is also likely to be needed for HIV-1. In our experiments with the NL4.3 core, there was no rescue of the restriction. This suggests that it is not adapted to this step, implying that HIV-1 does not use this pathway. Alternatively, the NL4.3 core itself may be restricted and thus unable to rescue the block. Nevertheless, the NL4.3 envelope is very potent at rescuing the restriction, strongly suggesting that HIV-1 may need to avoid this pathway and has indeed adapted its envelope to bypass the block. We are currently examining these possibilities.

It has been suggested that a permissive compartment is one in which the virus can access the nucleus (29). Studies have suggested that nuclear targeting of HIV-1 is dependent on an intact cytoskeleton (24). The microtubule network and dynein aid movement towards the nucleus (28). Access to these cellular components may be crucial for efficient replication of any virus.

Collectively, these observations indicate that, in addition to Ref1/Lv1, other cellular factors modulate HIV and SIV at a postentry preintegration step (29). The identification of such host factors and the corresponding viral determinants may present novel therapeutic targets to the replication of HIV.

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