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Production of human immunodeficiency virus type 1 (HIV-1) pseudoviruses using linear HIV-1 envelope expression cassettes

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Abstract

HIV-1 pseudoviruses constitute an important tool in HIV-1 vaccine and entry inhibitor research. Single-cycle pseudoviruses carrying functional envelopes are generated by co-transfecting HEK293T cells with pNL4-3.LucR^-E^- and *Env* expression plasmids. However, cloning of *Env* genes is time consuming and single *Env* clones are not representative of the diversity of HIV-1 in a patient's blood sample. A new method to construct *Env* expression cassettes is proposed which can be used for the rapid generation of heterogeneous HIV-1 pseudoviruses without a cloning step. The linear *Env* expression cassettes are constructed by ligating PCR amplified *Env* genes between a 5′ CMV promoter and 3′ SV40 polyadenylation element. The resulting cassettes generate pseudoviruses carrying heterogeneous *Env* variants of a primary HIV-1 isolate derived from viral RNA or proviral DNA. The influence of *cis*-acting sequences upstream of the *Env* gene on infectivity was compared between pseudoviruses generated from plasmids and linear expression cassettes. The results suggest that the presence of these upstream sequences tends to result in higher infectivity of pseudoviruses when present in heterogeneous *Env* expression cassettes, but they do not enhance infectivity of pseudoviruses generated with homogeneous *Env* expression constructs. Using linear expression cassettes allows for the rapid production of heterogeneous patient-derived functional *Env* genes.

Keywords: HIV-1 pseudovirus; Envelope; HIV-1

1. Introduction

Studying envelope-specific antibodies contributes to the Herculean challenge to design an HIV-1 vaccine (Haynes and Montefiori, 2006; Burton et al., 2005; Emini and Koff, 2004; Mascola, 2003; McMichael and Hanke, 2003). There is also a pressing need to develop new anti-HIV-1 drugs including entry inhibitors. The high variability of the HIV-1 Env protein accounts for immune escape from neutralizing antibodies and variable sensitivity to entry inhibitors (Sterjovski et al., 2006; Labrosse et al., 2003). Recently, several laboratories replaced infectious HIV viruses by so-called single-cycle or "pseudoviruses" to study neutralizing antibodies (Li et al., 2005, 2006; Beddows et al., 2005; Binley et al., 2004; Zhang et al., 2004; Richman et al., 2003; Quinnan et al., 1998) or drugs in entry inhibition assays (Siebert et al., 2006; Madani et al., 2004; Wei et al., 2002).

The current generation of pseudovirus-based assays has several advantages over traditional replicating virus systems in peripheral blood mononuclear cell (PBMC) cultures. First, pseudoviruses are replication-incompetent and can be safely handled outside of an expensive biosafety level 3 laboratory. Second, the generation of a new pseudovirus stock simply requires transfection of a suitable mammalian cell line such as HEK293T cells. Third, neutralization or entry inhibitor assays can be performed in a continuous cell line, thus alleviating the requirement of primary donor cells. Together, these elements ensure that pseudovirus-based assays are precise, reproducible and easy to standardize (Li et al., 2005, 2006).

Traditionally, HIV-1 pseudoviruses are produced using a two-plasmid system. The first plasmid expresses a functional

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Env gene while the second (e.g. pNL4-3.LucR$^-$E$^-$) carries the complementary HIV-1 information necessary for the production of single-cycle infectious pseudovirus. The *Env* fragments are (RT−) amplified from either proviral DNA or viral RNA and the amplicons are cloned into an expression vector.

According to the literature, sequences upstream of the *Env* gene are considered important for the production of infectious pseudovirus (Hammarskjöld et al., 1989; Lu et al., 1990). However, infectious pseudoviruses can also be produced using only *Env* gp160 expressing vectors (Leo Heyndrickx; unpublished data). Nevertheless, increased levels of *Env* expression may yield more infectious pseudovirus stocks. Therefore, the relevance of these upstream sequences in *Env* expression constructs still remains to be identified.

Pseudoviruses are typically more sensitive to antibody neutralization than their parental primary HIV viruses. One possible explanation is that a single clone can be relatively easily neutralized, compared to the viral quasispecies present in primary infected cells (Li et al., 2005; Louder et al., 2005). Indeed, a single clone may not be representative of the diversity of *Env* quasispecies. Moreover, cloning of individual envelopes into plasmids is laborious, as it generally requires amplification of *Env*, purification of the amplicon, ligation, transformation, plasmid DNA preparation and finally the selection of a functional *Env* clone.

The use of viruses that contain *Env* glycoproteins that are representative of the quasispecies circulating in a patient, as compared to "traditional" cloned pseudoviruses, is an obvious next step to monitor neutralizing antibody responses as well as entry inhibitor efficiency in a system that is more relevant to the in vivo situation. The present study proposes a rapid and efficient method to produce linear *Env* expression cassettes without cloning. The pseudoviruses constructed with these cassettes contain heterogeneous *Env* genes derived from viral RNA or proviral DNA of parental primary virus isolates. Furthermore, the influence of the first exon of *Rev*, present in *cis* as an upstream sequence in the envelope expressing constructs, was evaluated in terms of the single-cycle infectivity of the various types of pseudoviruses.

2. Materials and methods

2.1. HIV-1 isolates and biological clones

A panel of four primary HIV-1 viruses (VI 1090, VI 829, VI 824 and VI 2809) was previously isolated from patients attending the AIDS clinic at the Institute of Tropical Medicine in Antwerp, Belgium. Proviral DNA was isolated from patient's PBMCs after short-term co-cultivation with PHA (phytohemagglutinin; Murex Diagnostics, Kent, United Kingdom)-stimulated PBMCs from seronegative donors. Viral RNA was extracted from four plasma samples (PIC 3654, PIC 7901, PIC 561 and PIC 3226) from HIV-1 infected persons visiting the same clinic. This panel of HIV-1 samples was supplemented with two reference HIV-1 strains, SF162 (obtained through the AIDS Research and Reference Reagent Program from Dr. Jay Levy; Cheng-Mayer and Levy, 1988) and MN (kindly provided by Dr. Suzan Zolla-Pazner). Four biological clones, VI 829-1 (derived from the primary isolate VI 829), CA10-3 (derived from the primary isolate CA10), VI 525-1 and VI 525-5 (both derived from the primary isolate VI 525) (Beirnaert et al., 2001) and two molecular clones, pNL4-3 (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Malcolm Martin; Adachi et al., 1986) and pMJ4 (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Drs. Thumbi Ndung'u, Boris Renjifo and Max Essex; Ndung'u et al., 2001) were also used to generate HIV-1 pseudoviruses (Table 1).

Table 1
Characteristics of HIV-1 virus strains used to generate HIV-1 envelope expression constructs

Virus strain	Type of virus isolate	Env subtype[a]	CXCR4/CCR5
VI 1090	Primary isolate	CRF02_AG	CCR5
VI 829	Primary isolate	C	CCR5
VI 824	Primary isolate	D	CCR5
VI 2809	Primary isolate	A	CCR5
PIC 3654	Plasma RNA	B	CCR5
PIC 7901	Plasma RNA	G	CCR5
PIC 561	Plasma RNA	D	CCR5
PIC 3226	Plasma RNA	B	CCR5
VI 525-1	Biological clone	A	CCR5
VI 525-5	Biological clone	G	CXCR4
VI 829-1	Biological clone	C	CCR5
CA10-3	Biological clone	CRF01_AE	CXCR4
pNL4-3	Molecular clone	B	CXCR4
pMJ4	Molecular clone	C	CCR5
SF162	Reference strain	B	CCR5
MN	Reference strain	B	CXCR4

[a] The subtype of the virus isolates were determined by *Env* HMA (V3–V5) or by partial sequence analysis.

2.2. Cell lines and HIV-1 plasmids

The human kidney cell line HEK293T (obtained from ATCC) was maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, Bornem, Belgium) supplemented with 10% fetal bovine serum (FBS; Lonza, Verviers, Belgium), 2 mM L-glutamine and gentamycin (Lonza). The green fluorescence protein-transduced human osteosarcoma cells (GHOST), expressing either the CXCR4 or CCR5 co-receptor (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Vineet N. Kawal Ramani and Dr. Dan R. Littman. Mörner et al., 1999) were cultured in DMEM (Lonza) supplemented with 10% FBS, 0.03% L-glutamine (Lonza), geneticin (100 μg/ml) (Invitrogen, N.V., Merelbeke, Belgium), hygromycin B (25 μg/ml) (Roche Diagnostics, Mannheim, Germany), puromycin (1 μg/ml) (Sigma–Aldrich) and penicillin/streptomycin (Invitrogen). Both cell lines were grown at 37 °C with 5% CO_2 in a humidified incubator and were split twice a week.

The pNL4-3.LucR$^-$E$^-$ (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Nathaniel Landau. He et al., 1995; Connor et al., 1995) has frame shift mutations in *Env* (E$^-$) and *Vpr* (R$^-$) and a luciferase reporter gene inserted into the *Nef* gene. This plasmid requires

co-transfection with an *Env* expression construct to produce infectious pseudovirus.

2.3. Amplification of Rev/Env and Env from proviral DNA sequences

For DNA extraction, $5–10 \times 10^6$ PBMCs from HIV-1 seropositive blood donors were resuspended in 200 μl lysis buffer (Merck, Darmstadt, Germany) and treated overnight at 56 °C with 100 μg/ml proteinase K (Qiagen, Chatsworth, CA), followed by phenol chloroform extraction to purify the DNA. Proviral *Env* DNA sequences were amplified by nested polymerase chain reaction (PCR) using the GeneAmp XL PCR kit (Applera, Lennik, Belgium). The first round forward primer was 'Wou26' (5′-GCATCTCCTATGGCAGGAAGAAG-3′) and the reverse primer was 'Wou29' (5′-TGTAAGTCATTGGTCTTAAAGGTACCTG-3′). The cycle conditions for the first round PCR were 1 cycle at 94 °C for 60 s, 16 cycles at 94 °C for 15 s, 50 °C for 30 s, 68 °C for 240 s, followed by 12 cycles at 94 °C for 15 s, 68 °C for 240 s, with an increment of 5 s per cycle and a final extension step at 68 °C for 10 min. For the second-round PCR, primers were designed with an additional 11 nucleotide sequence (underlined and italicized), as required for ligation of the 5′ CMV promoter and 3′ SV40 polyadenylation element of the TOPO® tools technology (see Section 2.6). To amplify the *Rev/Env* fragment, the forward primer 5RevEnv_TOPO (5′-*CGGAACAAGGG*CATYTGCTATGGCAGGAAGAAGCGGA-3′) and the reverse primer 3OUT1B_TOPO (5′-*TGAGTCAAGGG*CTTAAAGGTACCTGAGGTCTGACTGG-3′) were used. To amplify the *Env* fragment, the forward and reverse primers were 'JFES_TOPO' (5′-*CGGAACAAGGG*AATTCAGAGCAGAAGACAGTGGCAATG-3′) and 'Wou28 Not_TOPO' (5′-*TGAGTCAAGGG*CGCCGGCGTTTGACCACTTGCCACCCAT-3′), respectively, and the cycle conditions were the same as in the first round. Amplified DNA products were visualized on a 0.8% agarose gel and purified using the QiaQuick® PCR purification kit (Qiagen). For the amplification of *Rev/Env* or *Env* from plasmid DNA, only the second round primers were used.

2.4. Amplification of Rev/Env from viral RNA

Viral RNA was extracted from the plasma of HIV-1 seropositive patients using the QiaAmp® Viral RNA Mini kit (Qiagen), followed by reverse transcription with expand RT (Roche Molecular Biochemicals, Mannheim, Germany) using primer 3INN2B (5′-GGGACTAACCGTTTTGATGTGTG-3′), as described by the manufacturer's instructions. The obtained cDNA was used for a nested PCR (expand high fidelity PCR system, Roche Molecular Biochemicals), as described in the previous section.

2.5. HIV-1 (Rev) Env expression plasmid clones

The *Env* or *Rev/Env* containing PCR amplicons were ligated into the pcDNA4/TO (Invitrogen) expression vector after appropriate restriction enzyme digestion. The ligation product was then transformed into One Shot® Top10 cells (Invitrogen) (Fig. 1). From clones carrying the correct insert, plasmid DNA was isolated using the Wizard® Plus DNA purification system (Promega Benelux BV, Leiden, The Netherlands).

2.6. Linear envelope expression cassettes (LEC)

For the production of LEC, we used two methods. The plasmid-derived LEC (LEC-PD) contain the Env or Rev/Env

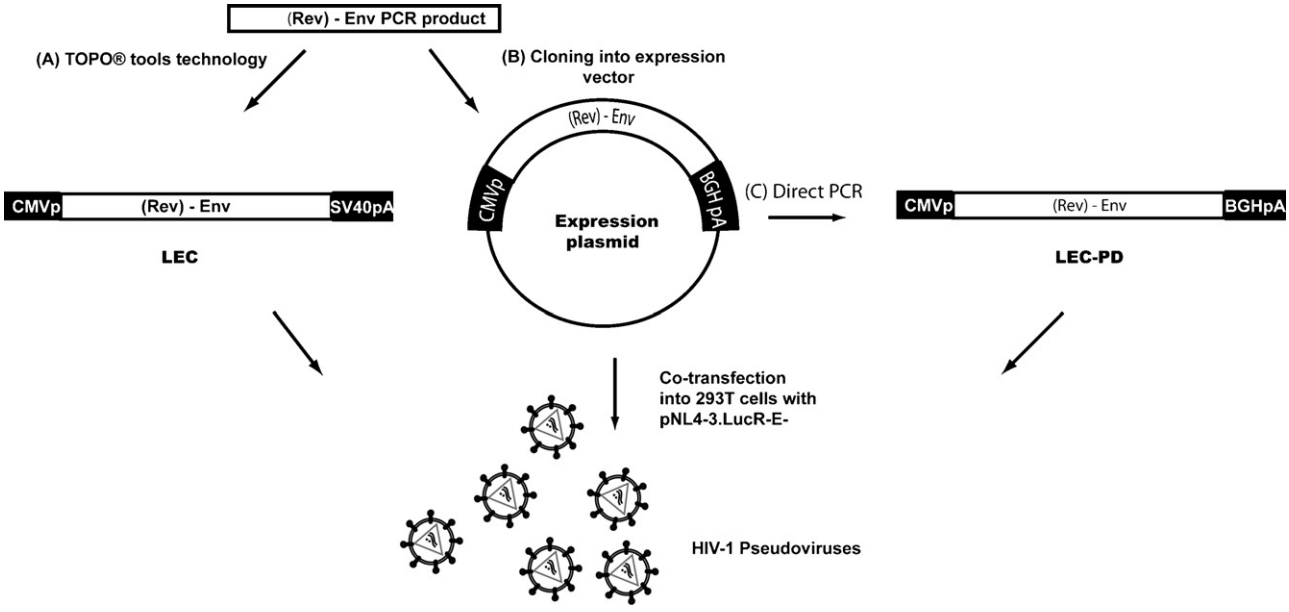

Fig. 1. Generation of HIV-1 (*Rev*) *Env* expression constructs starting from PCR-amplified (*Rev*) *Env* fragments. (A) Construction of LECs by ligation of a 5′ promoter and 3′ SV40 polyadenylation element to the (*Rev*) *Env* PCR product using the TOPO® tools Technology (Invitrogen). (B) Cloning of the (*Rev*) *Env* PCR product resulting in (*Rev*) *Env* expression plasmids. (C) Generation of LEC-PD by amplification of the corresponding (*Rev*) *Env* plasmid. HIV-1 pseudoviruses were generated by transfection of HEK293T cells with the (*Rev*) *Env* expressing constructs and pNL4-3.LucR$^-$E$^-$.

gene with a CMV promoter and BGH polyadenylation sequence. They were PCR amplified from the corresponding plasmids. The utilized primers were specific for the CMV promoter (CMV_FP#3; 5′-CGCGTTGACATTGATTATTGACTAGTTA-3′) and the BGH polyadenylation sequence (BGH_RP#2; 5′-AAGCCATAGAGCCCACCGCA-3′). The cycle protocol was 1 cycle at 94 °C for 2 min, 16 cycles at 94 °C for 15 s, 55 °C for 30 s, 68 °C for 240 s, 12 cycles at 94 °C for 15 s, 240 s at 68 °C and with an increment of 5 s per cycle and a final extension step at 68 °C for 10 min.

Secondly, LEC were made using TOPO® tools technology (Invitrogen) as described in the manufacturer's instructions (Fig. 1). This technique was originally described by Cheng and Shuman and utilizes the properties of vaccinia DNA topoisomerase I to join the envelope-containing PCR products to a functional CMV promoter (5′ end) and an SV40 polyadenylation (3′ end) element (Cheng and Shuman, 2000). In this study, the PCR primers used to amplify the *Rev*/*Env* or *Env* gene were adapted by adding specific sequences on the 5′ and 3′ ends, including a six base pair sequence needed for cleavage by DNA topoisomerase I and a 5-nucleotide overhang complementary to the overhang sequences of the 5′ and 3′ elements (see Section 2.3 for details about the PCR). After ligation of the PCR template with the 5′ and 3′ elements, the ligation product was kept on ice until the final PCR amplification of the LECs. For this PCR, the GeneAmp XL PCR kit (Applera) was used with primers specific for the 5′ and 3′ elements provided in the TOPO® tools technology kit (Invitrogen). These primers are specific for either the CMV promoter or the SV40 polyadenylation element of the ligation product. The cycle protocol was 1 cycle at 94 °C for 2 min, 10 cycles at 94 °C for 30 s, 55 °C for 30 s, 68 °C for 120 s, 25 cycles at 94 °C for 30 s, 55 °C for 30 s and 120 s at 68 °C with an increment of 5 s per cycle and a final extension step at 68 °C for 10 min.

All PCR products were visualized on a 0.8% agarose gel and purified using the QiaQuick® PCR purification kit (Qiagen).

2.7. Heterogeneity of LEC by the heteroduplex mobility assay (HMA)

The heterogeneity of LEC was analyzed by HMA. First, a region including the V3–V5 regions of gp120 (primers ES7 and ES8, described by Delwart et al., 1993) was PCR amplified from the LEC. These PCR fragments were subcloned into the pCR2.1-TOPO® vector by TA cloning, as described by the manufacturer's instructions (Invitrogen). The ligation mix was transformed into One Shot® Top10 cells (Invitrogen). Individual transformants were picked and used in a PCR reaction to amplify the V3–V5 fragment (primers ES7 and ES8). The resulting PCR fragments were further processed by analyzes of heterogeneity using the heteroduplex mobility assay (HMA).

The HMA was performed as described before (Delwart et al., 1993; Heyndrickx et al., 2000). Briefly, the same region was amplified from *Env* reference plasmids (Heteroduplex Mobility Analysis HIV-1 *env* subtyping kit (cat no. 2751) obtained from the NIH AIDS Research and Reference Reagent program, Division of AIDS, NIAID, NIH from Dr. James Mullins). Heteroduplex molecules were obtained by mixing 5 μl (containing 50–250 ng) of the PCR fragment with 5 μl of the reference fragment and adding 1.1 μl of 10× annealing buffer (1 M NaCl, 100 mM Tris–HCl, 20 mM EDTA). The DNA fragments were denatured at 94 °C for 3 min and re-annealed by rapid cooling on ice prior electrophoresis on a 5% polyacrylamide gel, containing 5% urea, in 1× TBE buffer (88 mM Tris–borate, 89 mM boric acid, 2 mM EDTA) at 250 V for 3 h. Detection of the heteroduplexes was achieved by staining with ethidium bromide and visualization under UV light.

2.8. Pseudovirus production

Pseudoviruses were produced in a 24-well plate by co-transfection of 70–80% confluent HEK293T cells with 400 ng pNL4-3.LucR$^-$E$^-$ and 1000 ng *Env* expressing plasmid or 400 ng LEC (unless otherwise indicated) using the calcium phosphate technique (ProFection® Mammalian Transfection Systems, Promega Benelux BV). After 24 h, the medium was replaced with medium containing 1 mM sodium butyrate (Sigma–Aldrich). Two days after transfection, the pseudoviruses were harvested, passed through 45 μm pore sterile filters (Millex® HV, Millipore NV, Brussels, Belgium). FBS was added (final concentration 10%) prior to storage at −80 °C until use.

2.9. Luciferase activity assay

A fixed volume of undiluted or twofold diluted HIV-1 pseudovirus (50 μl) was used to infect 4×10^3 GHOST cells (150 μl) in white 96-well plates (Corning-Costar, Elscolab, Kruibeke, Belgium). After incubating the cultures for 72 h at 37 °C in 5% CO_2, the cells were washed once with phosphate-buffered saline (PBS) (Lonza). The cells were lysed with 5 μl of cell culture lysing reagent (Promega) for 15 min and 50 μl luciferase substrate (Promega) was then added. The infectivity was measured using a Topcount™ (Canberra-Packard, Zellik, Belgium) as relative light units (RLU). In each experiment, mock controls were included, that consisted of medium harvested from HEK293T cells transfected with the empty expression vector (pcDNA4/TO) and pNL4-3.LucR$^-$E$^-$. All infection experiments were done in triplicate.

3. Results

3.1. Production of infectious HIV-1 pseudoviruses with LEC: proof of principle

Env expression constructs from three different primary HIV-1 viruses (VI 824, VI 829 and VI 1090) were used to compare the infectivity of pseudovirus obtained using LEC-PD and the corresponding *Env* expression plasmids (Fig. 2). Pseudoviruses generated using LEC-PD resulted in comparable or even higher luciferase signals after infection of GHOST cells than those made using the corresponding *Env* expression plasmids. This indicates that the concept of using linear LECs for the production of infectious HIV-1 pseudoviruses is good. Infectivity of

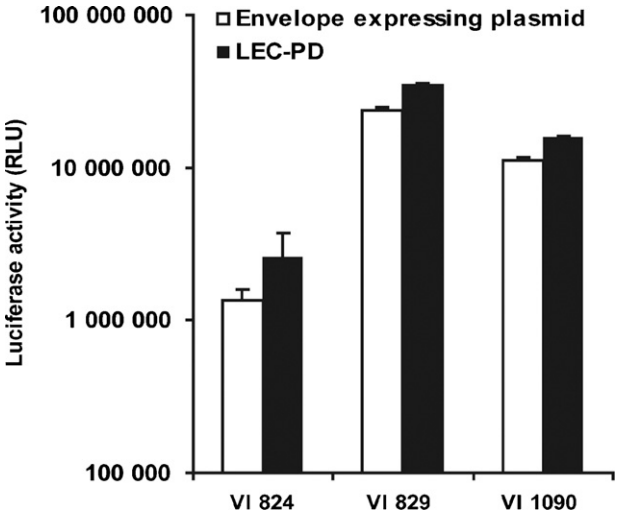

Fig. 2. Proof of principle demonstrating infectious pseudoviruses can be generated with LECs. LEC-PD were generated by direct PCR amplification of *Env* plasmids. To generate pseudoviruses, the *Env* plasmid and corresponding LEC-PD were used side-by-side for co-transfection into HEK293T cells together with the pNL4-3.LucR$^-$E$^-$. Pseudoviruses were harvested 72 h after transfection and used to infect GHOST cells. The luciferase activity was measured 72 h after infection. The data are shown ± S.D. (data originating from one experiment in triplicate; background <1000 RLUs).

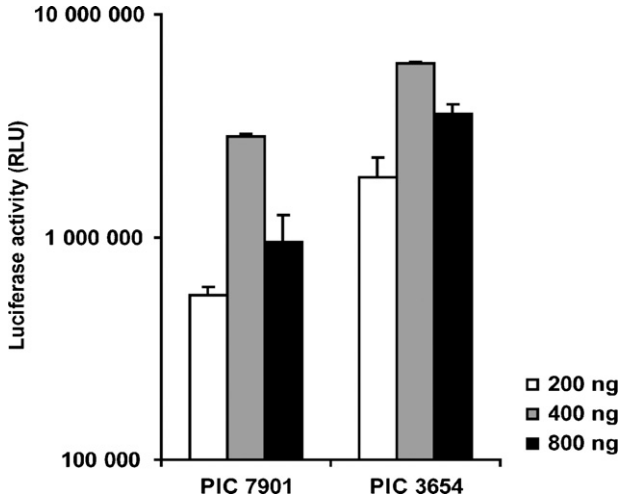

Fig. 3. Optimisation of LEC concentration to construct pseudoviruses. Three concentrations (200, 400 and 800 ng) of Rev Env LECs derived from viral RNA of plasma PIC 7901 and PIC 3654 were co-transfected into HEK293T cells with pNL4-3.LucR$^-$E$^-$ to generate HIV-1 pseudoviruses. Pseudoviruses were harvested 72 h after transfection and used to infect GHOST cells. The luciferase activity was measured 72 h after infection. The data are shown ± S.D. (data originating from one experiment in triplicate; background <1000 RLUs).

mock-derived pseudovirus typically resulted in <1000 RLU for all experiments.

3.2. Influence of DNA ratio on infectivity of pseudoviruses

In previous experiment using *Env* plasmids, the ratio of pNL4-3.LucR$^-$E$^-$ to Env in (co)transfection was 1:2.5 (400 ng:1000 ng, respectively). To optimize the production of pseudovirus with LEC, different amounts of the *Rev*/*Env* LEC (200, 400 and 800 ng) were co-transfected with 400 ng pNL4-3.LucR$^-$E$^-$, resulting in a pNL4-3.LucR$^-$E$^-$ to *Env* ratio of 1:2, 1:1, and 2:1, respectively. The resulting pseudoviruses were used to infect GHOST cells and, 72 h post-infection, the RLU were monitored. For these experiments, LEC were constructed from two plasma samples (PIC 7901 and PIC 3654). The results (Fig. 3) show that the highest RLU value was obtained using a 1:1 ratio. Therefore, in further transfection experiments, equal amounts (400 ng) of the LEC construct and pNL4-3.LucR$^-$E$^-$ vector were used.

3.3. Infectivity of pseudoviruses constructed with Rev/Env and Env expression constructs

According to the literature, the first exon of *Rev*, which acts as a *cis*-acting element of the *Env* sequence, may be essential for the production of infectious pseudoviruses (Hammarskjöld et al., 1989; Lu et al., 1990). In order to analyze the influence of *Rev* in the *Env* expression constructs, LEC expressing only *Env* (further referred to as *Env* LEC) as well as LEC expressing the first exon of *Rev* upstream of the *Env* sequence (further referred to as *Rev*/*Env* LEC) were constructed starting from both proviral DNA and viral RNA. Using the TOPO tools technology, the CMV promoter and SV40 polyadenylation elements were ligated to the (*Rev*) *Env* PCR product, immediately followed by a final amplification step resulting in a yield of ∼1.5 μg DNA per reaction. The LECs were purified and co-transfected with pNL4-3.LucR$^-$E$^-$ into HEK293T cells. All LEC constructs resulted in infectious pseudoviruses with luciferase activities ranging from 5×10^4 to 1×10^7 RLUs. The infectivity of the *Rev*/*Env* LEC pseudovirus was consistently higher as compared to the *Env* LEC pseudoviruses for six out of the seven constructs tested. The increase in infectivity rose up to 240 times when using equal volumes of pseudovirus to infect the GHOST cells (Fig. 4A). This result clearly suggests that the presence of the *Rev* gene in the LEC supports more efficient expression of the functional *Env* polyprotein, resulting in a more infectious pseudovirus.

In Fig. 4B, the influence of the first exon of *Rev* was evaluated when using (*Rev*) *Env* expressing plasmids to construct the pseudoviruses. Hereto, amplicons containing *Rev*/*Env* and *Env* genes were generated and cloned into the pcDNA4/TO vector. From each cloning experiment, several clones (∼10), all containing the desired *Env* gp160 fragment, were used for pseudovirus production and screened for infectivity. The RLUs resulting from the *Rev*/*Env* and *Env* plasmids are shown in Fig. 4B. In contrast to the observations made with the LEC, the presence of the first exon of *Rev* in *cis* with *Env* in the *Env* expressing plasmids seems to produce less infectious pseudovirus. For VI 2809 and VI 525-5, infectivity of the *Rev*/*Env*-derived pseudovirus was very low ($\leq 10^4$ RLU).

3.4. Side-by-side comparison of pseudovirus derived from molecularly cloned HIV-1 constructs

In order to fairly assess the infectivity of pseudoviruses generated with the different approaches mentioned above, two full-length molecular clones (pNL4-3 and pMJ4) were used.

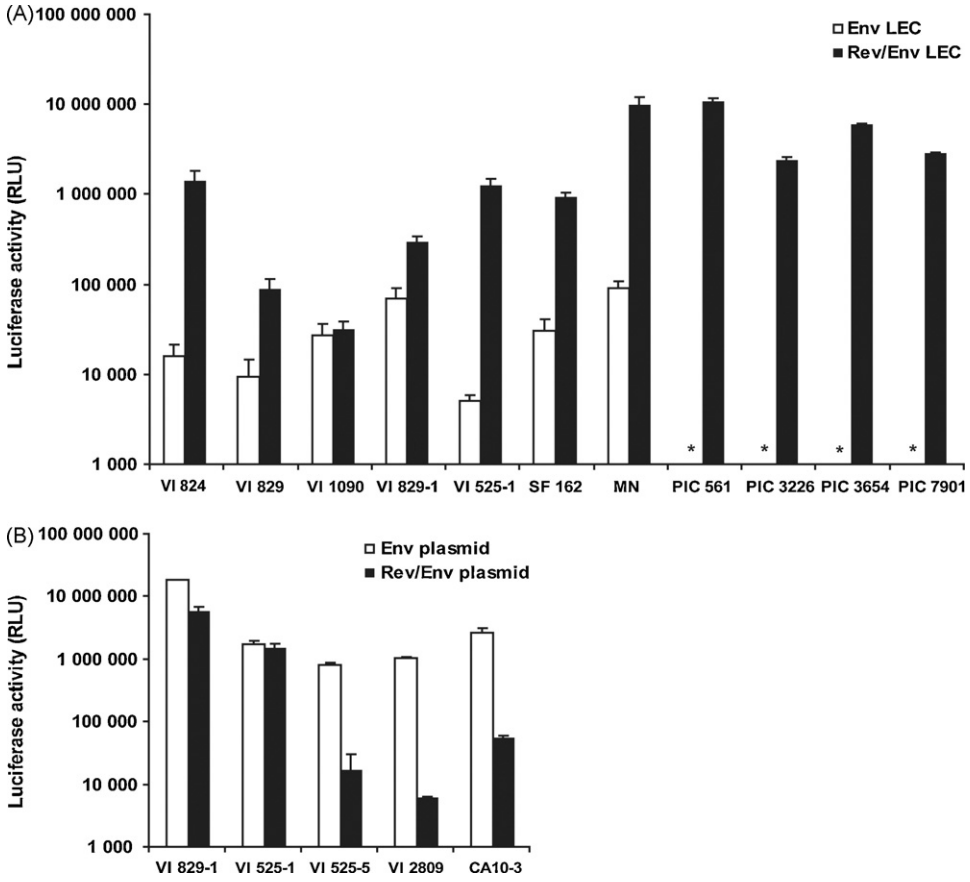

Fig. 4. Infectivities of the pseudoviruses generated with (*Rev*) *Env* LECs (A) and plasmids (B) after co-transfection of HEK293T cells with the pNL4-3.LucR^-E^-. Pseudoviruses were harvested 72 h after transfection and used to infect GHOST cells. The luciferase activity was measured 72 h after infection. The data are shown ± S.D. (data originating from one experiment in triplicate; background < 1000 RLUs). No *Env* LECs were constructed for the strains marked with (*).

Both *Rev*/*Env* and *Env* were amplified and further processed using the three different approaches, resulting in (*Rev*) *Env* LEC-PD, (*Rev*) *Env* LEC, and (*Rev*) *Env* plasmids. Side-by-side transfections of these constructs allowed us to compare identical *Env* genes and evaluate the impact of the first exon of *Rev* on pseudovirus infectivity (Fig. 5). The results show that for the plasmids and LEC-PD, the presence of *Rev* did not have an important impact on infectivity. For the LECs, however, the infectivity was clone-dependent, *Rev* did not have an effect on pMJ4 (Fig. 5B) and it enhanced pNL4-3 (Fig. 5A). The generation of LECs was repeated twice for pNL4-3 and pMJ4 starting from separate PCR products of (*Rev*) *Env* and the results were concordant in both cases. Overall, more experiments are needed to define the exact role of *Rev* in pseudovirus production. Furthermore, luciferase activities (RLU) were directly proportional to the pseudovirus dilutions used over at least a 2-log range (data not shown).

3.5. Heterogeneity of the Env protein in the obtained HIV-1 pseudoviruses

From the previous results it is evident that *Rev*/*Env* LEC can be used for the production of infectious pseudoviruses. The purpose of amplifying *Rev*/*Env* directly from viral RNA or proviral DNA is to produce pseudoviruses that reflect the diversity present within a patient's blood sample. In order to verify the heterogeneity, HMA was applied on the primary isolate VI 1090 and the plasma sample PIC 7901. Hereto, the ES7–ES8 (covering V3–V5) PCR fragment derived from the *Rev*/*Env* LEC was TA-cloned into the pCR2.1-TOPO vector. From individual clones, the ES7–ES8 fragments were amplified and used for the formation of DNA heteroduplexes with the corresponding PCR fragment of a reference strain. As shown in Fig. 6, variation in the heteroduplex mobilities was found for both tested isolates which confirmed the heterogeneity present in the *Rev*/*Env* LECs.

4. Discussion

HIV-1 pseudoviruses constitute a powerful tool to characterize functional *Env* genes, identify potential *Env* immunogens for vaccine design, and to evaluate neutralizing antibodies in vaccine trials. Furthermore, they can also be used to evaluate new entry inhibitors or for the follow-up of phenotypic drug resistance in patients receiving entry inhibitors (e.g. Fuzeon). In the present study, a novel alternative method for the production of pseudoviruses expressing heterogeneous *Env* polyproteins was evaluated.

This TOPO tools methodology can employ either viral RNA or proviral DNA as starting material and encompasses the construction of PCR-generated linear *Rev*/*Env* expression cassettes

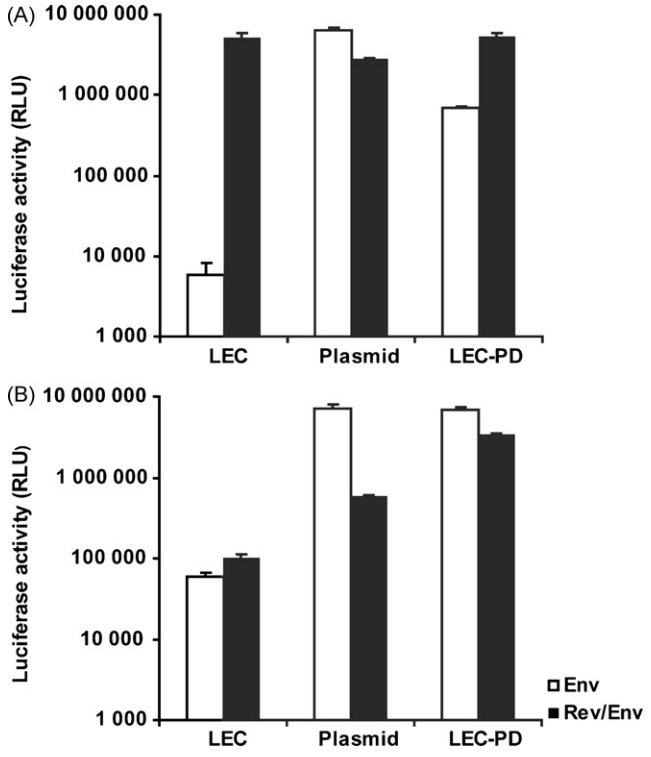

Fig. 5. Side-by-side comparison of molecularly cloned (*Rev*) *Env* expression constructs to evaluate the impact of *Rev* as a *cis*-acting upstream sequence on pseudovirus infectivity. Starting from molecular clones pNL4-3 (A) and pMJ4 (B), *Rev*/*Env* and *Env* constructs were made using three different approaches. HIV-1 pseudoviruses were generated by co-transfection of HEK293T cells with pNL4-3.LucR$^-$E$^-$. Pseudoviruses were harvested 72 h after transfection and used to infect GHOST cells. Luciferase activity was measured 72 h after infection. The data are shown ± S.D. (data are representative of two independent experiments performed in triplicate; background <1000 RLUs).

(LEC) without any requirement of cloning, transformation, plasmid DNA preparation, and last but not least, the selection of functional *Env* clones. As a consequence, pseudovirus is generated much faster (at least 2–3 days less), allowing for high throughput applications. In addition, the infectivities of all ($n = 13$) tested *Rev*/*Env* LECs were high enough (range: 3×10^5 to 10×10^6) to perform several experiments with the same batch of pseudovirus. One drawback of the use of LECs may be that repetition of an experiment involving pseudovirus constructed with LEC requires reproduction of the LEC as well. Therefore, plasmids expressing heterogeneous populations of *Env* genes, as used in a neutralization assay by Richman et al. (2003), might be advantageous for some applications needing repeated production of pseudoviruses due to the fact that the plasmids can be stored in glycerol.

Some investigators state that the upstream *cis*-acting *Rev* sequence in the *Env* expression plasmids is essential for the production of infectious pseudoviruses (Hammarskjöld et al., 1989; Lu et al., 1990). Others construct *Env* expression plasmids containing upstream *Env* sequences without further explanation (Cham et al., 2006; Beddows et al., 2005; Binley et al., 2004; Zhang et al., 2004; Quinnan et al., 1998). In this study, both *Env* and *Rev*/*Env* LECs from several isolates were compared in order to analyze the influence of the upstream *cis*-acting elements on pseudovirus infectivity. When starting from primary and biologically cloned isolates or plasma, the heterogeneous *Rev*/*Env* LECs generated more infectious pseudoviruses than the corresponding *Env* LECs. This suggests that the first exon of *Rev* may play an enhancing role as a *cis*-acting element on the *Env* sequence. However, when LECs were produced starting from a plasmid (LEC-PD), no differences in infectivity were found between the *Rev*/*Env* and *Env* LEC. Moreover, the effect of *Rev* on the infectivity of pseudovirus produced with LEC starting from molecular clones was dependent on the strain, resulting in an enhancement effect (pNL4-3) or no effect (pMJ4).

Rev is known to regulate nuclear export of the *Env* mRNA via binding to the RRE (*Rev*-responsive element), a secondary structure located in the *Env* coding sequence. Absence of *Rev* might affect post-transcriptional utilization of *Gag* and *Env* mRNA, because *Rev* may counteract the *cis*-acting negative regulatory sequences in the *Gag* and *Env* genes (Sodroski et al., 1986). Since *Rev* is provided in *trans* by the pNL4-3.LucR$^-$E$^-$ construct in this system, the production of infectious pseudoviruses might not be expected to be reduced by the absence of upstream *cis*-acting *Rev* sequences. Indeed, we observed that the upstream sequences

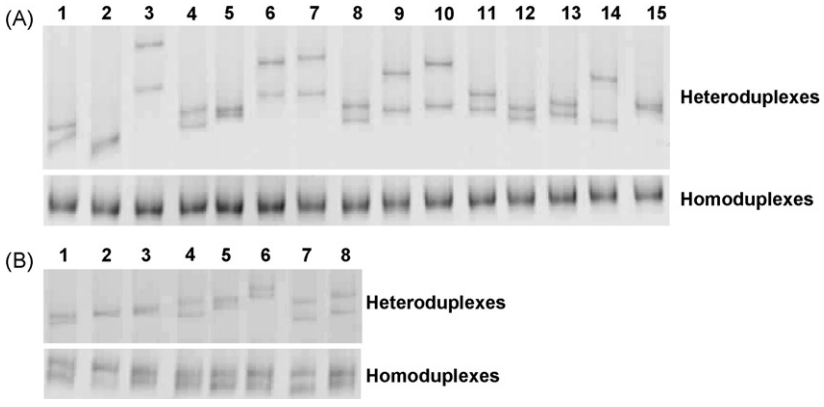

Fig. 6. Heteroduplex mobility assay analysis of the LECs. V3–V5 PCR fragments of the VI 1090 and PIC 7901 LECs were reannealed with the corresponding PCR fragment of a reference strain (*Env* HMA HIV-1 *Env* subtyping kit). Heteroduplexes were resolved on a 5% polyacrylamide gel containing 5% urea and stained with ethidium bromide prior visualization with UV light. (A) Heteroduplexes of 15 clones of virus strain VI 1090. (B) Heteroduplexes of 8 clones of plasma PIC 7901. The difference in the heteroduplex mobility of the clones demonstrates the heterogeneity of the LEC constructs.

in the *Env* expression plasmids were not mandatory for efficient production of infectious pseudoviruses. In addition, it has been suggested that the U1 snRNA sequence and the *Rev*/*Tat* 5′ splice donor site are essential for mRNA stabilization and binding of *Rev* to RRE (Lu et al., 1990). Since these non-coding sequences are absent in the *Env* expression plasmids, which generate more infectious pseudoviruses than the *Rev*/*Env* plasmids, our results do not confirm the requirement of *Rev* for the production of infectious pseudoviruses. Overall, both *Env* expression plasmids and *Rev*/*Env* LECs generate highly infectious pseudoviruses. Further studies are needed to clarify the complex role of upstream sequences on the expression of *Env*.

Recently, Kirchherr and colleagues reported a similar method, called the promoter PCR method, for the generation of HIV-1 pseudoviruses from homogeneous *Rev*/*Env* LECs to be used in neutralization assays. Results of this study indicated that the pseudoviruses derived from the *Rev*/*Env* LECs and the traditional *Rev*/*Env* plasmids have similar neutralization characteristics (Kirchherr et al., 2007).

In summary, the proposed LEC method has several advantages. It is straightforward, time and cost effective and ideal for high throughput production of pseudoviruses of many HIV-1 strains without cloning of the *Env* gene.

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