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A Nonproducer, Interfering Human Immunodeficiency Virus (HIV) Type 1 Provirus Can Be Transduced through a Murine Leukemia Virus-Based Retroviral Vector: Recovery of an Anti-HIV Mouse/Human Pseudotype Retrovirus

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The expression of a human immunodeficiency virus (HIV) type 1 provirus (F12-HIV) cloned from a nonproducer, chronically infected CD4 down-regulated Hut-78 cell clone (F12) does not lead to the formation of viral particles and, upon transfection in HeLa CD4$^+$ cells, confers resistance to HIV superinfection without affecting the CD4 receptor exposure. In an attempt to transfer the anti-HIV properties of F12-HIV into human primary cells, we constructed a Moloney murine leukemia virus-based retroviral vector containing an F12-HIV genome lacking the 3′ long terminal repeat and part of the *nef* gene, which was expressed under the control of its 5′ long terminal repeat. The F12-HIV genome was inserted in the orientation opposite to that of the murine leukemia virus transcriptional unit and was designated the N2/F12-HIV *nef*− antisense vector. Lymphoblastoid CEMss cells, as well as human peripheral blood lymphocytes, were successfully transduced by the recombinant retrovirus emerging from the producer PA317 clones. CEMss clones expressing the F12-HIV *nef*− antisense vector became resistant to HIV superinfection even at the highest utilized multiplicity of infection (10^5 50% tissue culture infective doses per 10^6 cells). In transduced CEMss cells the viral interference induced by the F12-HIV expression is not due to CD4 HIV receptor down-regulation. Nonproducer, interfering HIV proviruses transduced into retroviral vectors may, therefore, provide an alternative strategy for the protection of CD4$^+$ human primary cells from HIV infection, which strategy may be used in designating a safe and efficient gene therapy protocol for patients with AIDS.

The physically nondefective human immunodeficiency virus type 1 (HIV-1) genome cloned from the nonproducer HIV-1 chronically infected Hut-78/F12 cell clone (10) shows two peculiarities: (i) it is a nonproductive HIV-1 variant and (ii) its expression protects the host cell from HIV superinfection. In spite of the ability to transcribe its genome and to produce viral proteins, the F12-HIV genome is totally unable to generate even aberrant HIV viral particles, possibly as a result of the many amino acidic mutations scattered along the nondefective genome (3).

We have previously shown that HeLa CD4$^+$ cells expressing a transfected F12-HIV provirus become resistant to HIV superinfection without demonstrating any evidence of CD4 down-regulation (7). This anti-HIV viral interference was also demonstrated by transfecting the pNL4-3 HIV-1 infectious molecular clone, indicating that a blockage may occur in F12-HIV-expressing cells after the full retrotranscription of the superinfecting HIV (7). Thus, even if experiments are in progress to better define the mechanism of action of F12-HIV-induced interference, the negative transdominant effect(s) of an F12 product(s) on the HIV wild-type replication could be hypothesized.

The possibility that mutated forms of a viral protein inhibit the replication of their cognate virus was first reported by Friedman and colleagues (11), who demonstrated that a truncated form of the herpes simplex virus transactivator protein VP16 impedes the life cycle of the herpes simplex virus. Similar data were subsequently reported for HIV as well. In fact, a negative transdominant effect on the replication of HIV was demonstrated in cells expressing a mutated form of either *rev* (21), *tat* (12, 26), or *gag* (41) HIV-1 genes, and a clinical protocol based on the anti-HIV action of the M10-mutated form of the *rev* gene has recently been approved. Thus, the hypothesis of a gene therapy strategy based on intracellular immunization induced by viral negative *trans*-dominant proteins has been widely accepted.

The importance of viral interference induced by the expression of the F12-HIV genome could remain theoretical unless an efficient method of transducing this provirus in human blood-derived cell cultures is found. Among the different methodologies developed so far (i.e., viral and nonviral delivery), the murine retroviral vector-based gene transfer has been a mainstay of clinically applicable gene therapy protocols because of its safety, efficiency, and stable integration of the transduced genomic material (24).

The aim of the present study was the construction of a retroviral particle able to transduce the functionally defective and interfering F12-HIV genome in human cells. Previous attempts to transfer an F12-HIV genome lacking both long terminal repeats (LTRs) under the control of the Moloney murine leukemia virus (MLV) LTRs in the pLj retroviral vector (8) were met with two major difficulties: (i) the low level of expression of the F12-HIV genome, with a consequent low level of HIV interference; and (ii) the inability of this retroviral construct to integrate into the host genome. We tried to circumvent these limitations by constructing retroviral vectors in

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which the F12-HIV genome was expressed under the control of the HIV-1 LTR. We demonstrate that one of these molecular constructs, in which an F12-HIV provirus deprived of most of the *nef* gene (*nef*−) was expressed in an antisense (AS) orientation with respect to the Moloney MLV transcription unit, (i) was packaged in amphotropic retroviral particles by the PA317 cell line; (ii) was stably integrated in the host cell genome; (iii) showed strong expression of the HIV LTR-driven F12-HIV genome; (iv) induced the F12-HIV phenotype, i.e., nonproductivity and the ability to protect the host cell from HIV superinfection; and (v) showed no cell toxicity.

PA317-producing cell clones able to constitutively release amphotropic retroviral particles carrying the F12-HIV genome may therefore be used to protect primary human cells, i.e., T lymphocytes, monocytes-macrophages, and hematopoietic precursors of HIV-1 infection.

MATERIALS AND METHODS

Construction of the retroviral vector. The full-length F12-HIV proviral DNA was previously cloned from a Hut-78/F12 cell genomic library (3). The N2 retroviral vector was provided by E. Gilboa (14).

To obtain the N2/F12-HIV *nef*− AS construct (Fig. 1, top), the whole F12-HIV provirus (3) was digested with *Tha*I (which recognizes a single site in the leader sequences in F12-HIV) and *Sma*I (which cleaves the U3 region of both LTRs). The *Tha*I-*Sma*I F12-HIV genome was then ligated with the pUc19/F12-HIV 5′ LTR (which was previously cut at the pUc *Kpn*I site), blunted with the T4 DNA polymerase (Boehringer GmbH, Mannheim, Germany), and dephosphorylated. To insert the F12-HIV genome into the *Xho*I cloning site of N2, an *Xho*I site was added at the unique *Xba*I site of F12-HIV (nucleotide [nt] 1). Then, after digestion with *Xho*I (which in F12-HIV recognizes a single site in the *nef* gene), the DNA fragment encompassing the F12-HIV genome from nt 1 to 8930 was finally inserted at the *Xho*I site of the N2 vector and the AS construct was recovered.

All modifying enzymes were used by following the manufacturer's recommendations. The *Escherichia coli* XL-1 blue strain was used for the amplifications of pUc-based plasmids, whereas in the amplifications of pBR-based plasmids, the *E. coli* JM 109 strain was utilized. Bacterial transformations were performed by following the Bio-Rad protocols of the electroporation method.

Cell culture and transfection. CEMss, Hut-78, H9/HTLV-IIIB, and C8166 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). HeLa, NIH 3T3, GP+E86 (23), and PA317 (25) cells were grown in Dulbecco's modified minimal essential medium supplemented with 10% FCS. For the G418 (GIBCO; 50% of activity) selection, CEMss and Hut-78 cells were grown in the presence of 1 mg of the antibiotic per ml, whereas both the packaging and the NIH 3T3 cells were selected with 0.5 mg of G418 per ml.

Cell cloning was performed by seeding 96-well plates with 0.5 cell per well (7). Human peripheral blood lymphocytes (PBLs), obtained as previously described (30), were stimulated with phytohemagglutinin for 48 h and then cultivated in RPMI 1640 medium supplemented with 20% FCS and 50 U of recombinant human interleukin 2 (Roche, Nutley, N.J.) per ml.

Transfections of packaging-cell monolayers were performed by the calcium-phosphate precipitation method (43). Briefly, 10 μg of plasmid DNA was precipitated and added to subconfluent cultures in 10-cm-diameter dishes. Four hours later, the precipitate was removed and the cells were washed and supplied with fresh complete medium. Two days later, supernatants were removed and used as a retrovirus source.

Virus infections and HIV superinfections. Supernatants from both transfected GP+E86 and PA317 cells were used to infect, respectively, subconfluent monolayers of PA317 (after a single cycle of infection) or CEMss cells (after four cycles of infection). Cocultivations were performed in order to infect either CEMss cells or human PBLs with the amphotropic retrovirus released by PA317 producer cell clones. Producer cells (10^5) were seeded together with either 10^5 CEMss cells or 10^6 PBLs, both of which were in 1 ml of culture. After 24 h, PA317 cells were removed. In order to completely eliminate the producer cells, the culture plates were changed every 24 (CEMss) or 12 (PBLs) h.

All infected cells were pretreated with 8 μg of Polybrene per ml. Forty-eight hours after the infection cycles, the G418 selection was started.

Titers of the amphotropic retrovirus preparations were assayed as CFU per milliliter on NIH 3T3 cells as previously described (8).

Supernatants from acutely infected CEMss cells were used as the source of HIV (HTLV-IIIB and NL4-3 strains). HIV titers (ranging from 1×10^6 to 3×10^6 50% tissue culture infective doses [$TCID_{50}$] per ml) were measured as previously described (8) by scoring the syncytium number on C8166 cells 5 days after the infection with serially diluted virus preparations.

HIV release after the superinfection experiments was monitored by reverse transcriptase (RT) assay (30).

DNA, RNA, and protein analyses. Genomic DNA was prepared by standard methods (31). Total RNA was extracted by the guanidine-isothiocyanate method (5), and the poly(A)$^+$ fraction was obtained through separation with oligo(dT)-coupled dynabeads (Dynal, Oslo, Norway). Southern and Northern (RNA) blots were performed as previously described (31).

The probes were ^{32}P radioactively labelled by the random-primed method at a specific activity of 0.5×10^9 to 2×10^9 cpm/μg of DNA. The F12-HIV genomic probe was obtained by excising the provirus from the N2/F12-HIV *nef*− constructs. The *neo* probe was the full-length Tn*5* bacterial transposon. F12-HIV *env* and *nef* DNAs were obtained after the cloning, into the pUc19 *Eco*RI site, of the products of the DNA PCR amplification performed with *Eco*RI-tailed oligonucleotide primers overlapping the start and stop codons of each gene. The F12-HIV U3-LTR probe was recovered by *Xba*I-*Sma*I digestion of the F12-HIV 5′ LTR cloned in pUc19. The N2-specific probes were recovered after N2 digestion with *Nhe*I and *Sac*I for the U3-LTR probe, *Sma*I and *Spe*I for the LTR-R/U5 probe, and *Eco*RI and *Spe*I for the N2-ψ site-specific probe.

Intracellular p24 antigen in retrovirus-infected cells was determined by lysing 10^5 cells in 200μl of a solution containing TNE buffer (31) and 0.1% Triton X-100. After 5 min of incubation on ice, cell lysates were briefly spun and supernatants were tested by enzyme-linked immunosorbent assay (ELISA) by the antigen capture assay (Abbott, North Chicago, Ill.). HIV-related proteins were detected by a radioimmunoprecipitation assay performed as described in reference 7.

Fluorescence-activated cell sorter (FACS) analyses. CD4 receptors on the plasma membrane were detected by indirect immunofluorescence and analyzed with a cytofluorometer as described in reference 38. Briefly, 10^6 cells were incubated with the appropriate concentration of the anti-CD4 monoclonal antibody (Ortho Diagnostics, Raritan, N.J.) in 100 μl of phosphate-buffered saline (PBS) containing 2.5% FCS. After 60 min of incubation on ice, samples were washed twice in PBS and further incubated for 60 min in ice with 100 μl of 1:20 goat anti-mouse immunoglobulin G conjugated with fluorescein isothiocyanate (Ortho Diagnostics). After three washes with PBS, samples were fixed with a 2% (vol/vol) solution of formaldehyde and analyzed with a cytofluorometer (FACscan; Becton Dickinson, Mountain View, Calif.).

Intracytoplasmic HIV *gag*-related proteins were detected by direct immunofluorescence. Cells (10^6) were washed three times with PBS buffer complemented with 2.5% FCS and resuspended in 200 μl of PBS-EDTA (10 mM). A solution of PBS–2% (vol/vol) formaldehyde was then added, and the cells were incubated for 10 min at room temperature. After being washed twice with the PBS-FCS buffer, the cells were resuspended in 40 μl of cold PBS-EDTA and 400 μl of cold methanol was added drop by drop, after which the cells were incubated on ice for an additional 10 min. Cells were then washed three times and incubated for 1 h at room temperature with a 1:50 dilution of KC57-RD1 (Coulter Corp., Hialeah, Fla.) anti-*gag* HIV monoclonal antibody coupled with phycoerythrin. Finally, cells were washed three times with PBS-FCS buffer and analyzed with a cytofluorometer. Both uninfected cells and phycoerythrin-conjugated nonspecific mouse immunoglobulin G1 antibodies (Coulter Corp.) were used as negative controls.

RESULTS

Functional analysis of the retroviral construct. The N2/F12-HIV *nef*− AS construct was obtained by inserting into N2 the F12-HIV molecular clone, deprived of most of the *nef* gene and of the whole 3′ LTR, in the orientation opposite to that of the N2 retroviral vector (Fig. 1). This retroviral construct was designed to reduce, as much as possible, the theoretical possibility of rearrangement between homologous regions of MLV and HIV LTRs.

Transient transfection experiments were performed with HeLa, NIH 3T3, and PA317 cells, and the intracellular presence of the F12-HIV *gag* proteins on 10^5 cells was assessed 2 days later. Cell transfection with the N2 vector alone was used as a negative control. Our results (Table 1) show that the deletion of the 3′ LTR and most of the *nef* gene did not negatively affect the expression of the F12-HIV genome. Conversely, a slight increase in the production of *gag* proteins was observed compared with that of the full-length F12-HIV genome inserted in the pUc19 plasmid (3). We observed significantly higher amounts of *gag* proteins in human cells than in mouse cells, both 48 h posttransfection and after G418 selection, probably because of the stronger activity of the HIV promoter in human versus mouse cells (40).

To determine if the retroviral construct was able to generate recombinant retroviral particles, both ecotropic (GP+E86) and amphotropic (PA317) packaging cells were transfected.

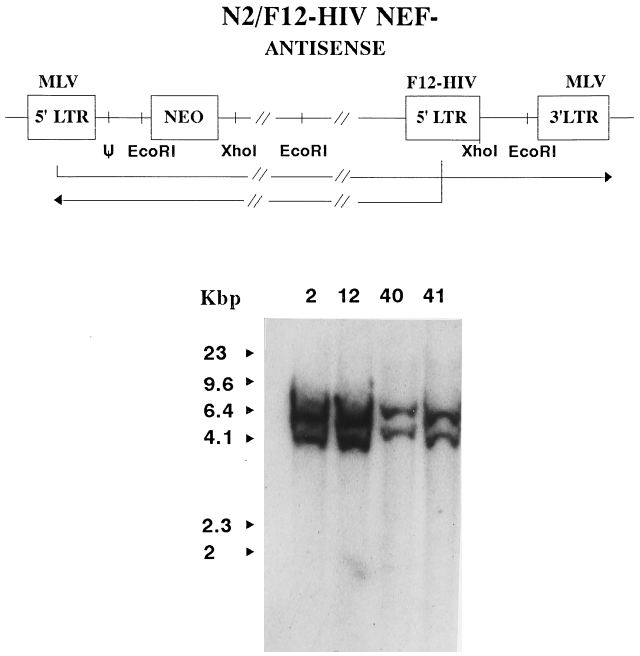

FIG. 1. (Top) Schematic map of the N2/F12-HIV *nef*− AS construct. Arrows indicate the transcriptional orientations of, respectively, the MLV and F12-HIV LTR promoters. (Bottom) F12-HIV-probed Southern blot of *Eco*RI-cut genomic DNAs from transduced PA317 (lanes 2 and 12) and CEMss (lanes 40 and 41) cell clones. *Hin*dIII-digested lambda phage DNA (far left lane) was used as a molecular size marker.

After infection with the supernatant from ecotropic cells, G418-resistant (Neo^r) PA317 cells were recovered; in addition, the supernatant from transfected amphotropic PA317 cells was utilized to obtain F12-HIV-expressing Neo^r human CEMss cells. In order to assess the functionality of the N2/F12-HIV construct transduced into the cells by retrovirus infection, all the recovered Neo^r cell populations were then tested for intracytoplasmic accumulation of the F12-HIV-encoded *gag* proteins.

High levels of F12-HIV *gag* proteins were detectable in cells integrating the F12-HIV *nef*− AS genome, i.e., 23.3 ± 2.4 and 106.3 ± 7 pg of *gag* protein in, respectively, PA317 and CEMss cells. These positive values remained stable over a long period of time (>1 year; data not shown).

In order to evaluate the percentage of cells stably expressing

TABLE 1. Amount of intracytoplasmic F12-HIV *gag* protein in N2/F12-HIV-transfected cells

Construct	Amt[a] of intracytoplasmic F12-HIV *gag* protein detected				
	48 h p.t.[b] in cell type:			After G418 selection in cell type:	
	HeLa[c]	NIH 3T3	PA317	HeLa	NIH 3T3
N2	<20	<10	<10	<10	<10
N2/F12-HIV *nef*− AS vector	239.2 ± 20	25.7	121.1	>246	130.6
pUc/F12-HIV	134.5 ± 10	88.4	67.4	>246	ND[d]

[a] Values are expressed in picograms of p24 HIV protein in 10^5 cells.
[b] p.t., posttransfection.
[c] Average values of four independent experiments ± standard deviations are reported.
[d] ND, not determined.

the F12-HIV genome, the Neo^r PA317 and CEMss cells integrating the *nef*− genome were cloned by the limiting-dilution method. As detected by the intracellular antigen capture assay, 12 and 23% of, respectively, PA317 and CEMss cell clones were able to synthetize the *gag* proteins.

Isolation and characterization of producer PA317 cell clones. Supernatants from 11 PA317 clones expressing the F12-HIV *nef*− AS genome were titrated as CFU per milliliter on NIH 3T3 cells. As expected, considering the large size of the retroviral construct (about 11 kb), retroviral titers were relatively low, ranging between 4.6×10^2 to 4×10^4 CFU/ml.

DNA analysis of the two PA317 clones (Fig. 1, lanes 2 and 12) producing the highest retroviral titers indicates that the retroviral construct was fully integrated in the host genome (Fig. 1) and was efficiently transcribed, as demonstrated by the Northern blot analysis (not shown). Accordingly, intracytoplasmic direct immunofluorescence of the F12-HIV-specific *gag* proteins (Fig. 2) revealed that both clones are able to homogeneously express the F12-HIV genome.

Molecular characterization of transduced CEMss cells and human PBLs. In order to transduce the F12-HIV genome in HIV-susceptible cells, cocultivations of PA317 clones with both CEMss cells and phytohemagglutinin-stimulated fresh human PBLs were set up. Twenty-four hours after cocultivation, G418 was added to CEMss cells and the Neo^r cell populations were obtained 20 days later. The percentage of F12-HIV-expressing cells was assayed by FACS analysis for the

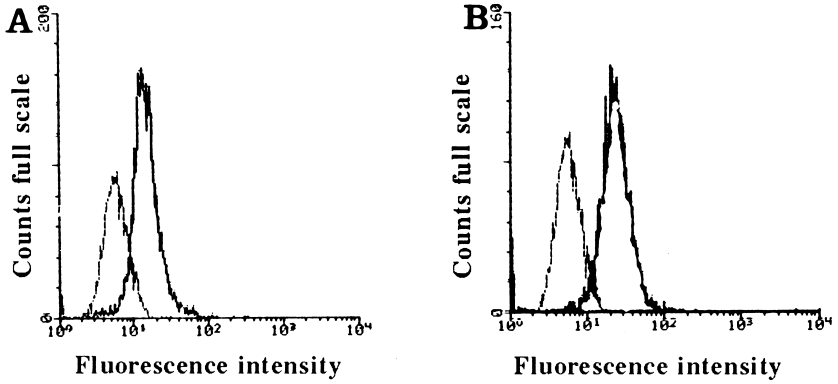

FIG. 2. FACS analysis for the presence of intracytoplasmic F12-HIV *gag* proteins in clone 2 (A) and clone 12 (B), selected PA317 cell clones transduced with the N2/F12-HIV *nef*− AS construct. In each panel, the slope of anti-*gag*-treated Neo^r N2-transduced PA317 cells is reported as a negative control (left curves).

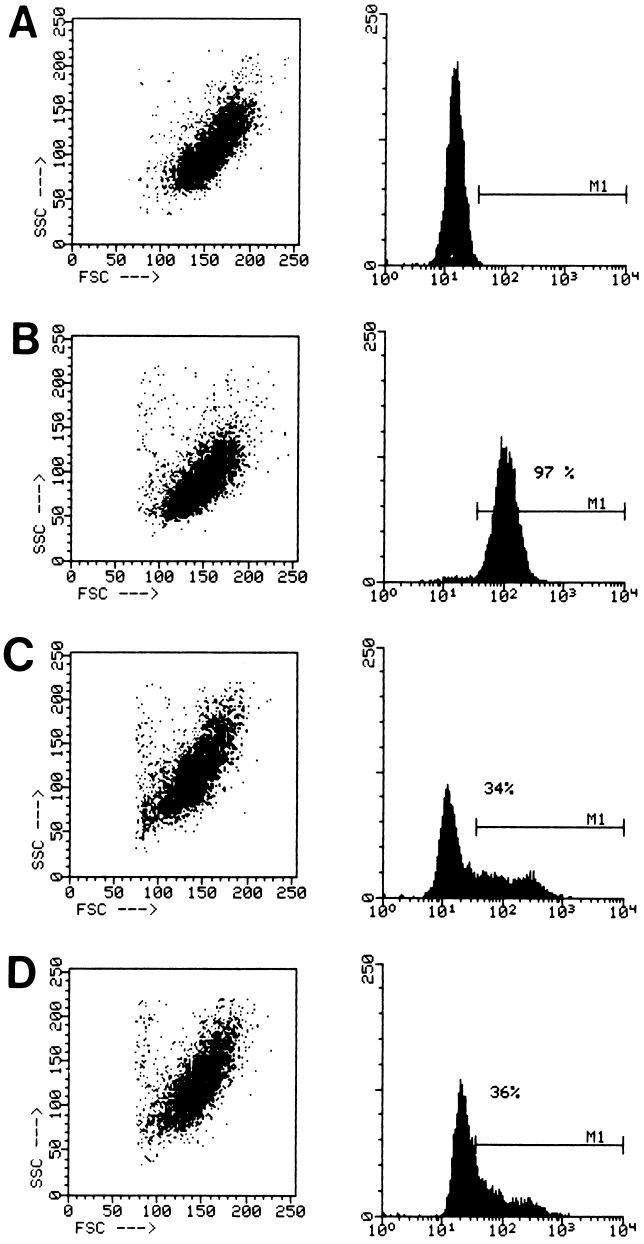

FIG. 3. FACS analysis for the presence of the intracytoplasmic F12-HIV *gag* protein in Neo^r CEMss cell populations after cocultivation with either PA317 clone 2 (C) or PA317 clone 12 (D). The same assay was performed on uninfected CEMss cells (negative control [A]) and on Hut-78/F12 cells (positive control [B]). Percentages of positive cells are also reported. M1, marker 1; SSC, side scatter; FSC, forward scatter.

HIV *gag* proteins. As shown in Fig. 3, about 35% of Neo^r cells expressed the F12-HIV genome. These data were replicated with Hut-78 cells (not shown). Moreover, 36 of the 60 CEMss cell clones scored (55%) expressed the F12-HIV genome, as assessed by the HIV-*gag*-specific ELISA.

Hybridization with either F12-HIV or *neo*-specific probes (Fig. 4) of *Xho*I-, *Eco*RI-, or *Xba*I-digested genomic DNA from Neo^r-transduced CEMss cells indicates that the F12-HIV genome fully integrated without apparent genomic rearrangements. This strongly suggests that major rearrangements of the transduced retroviral construct are not responsible for the in-

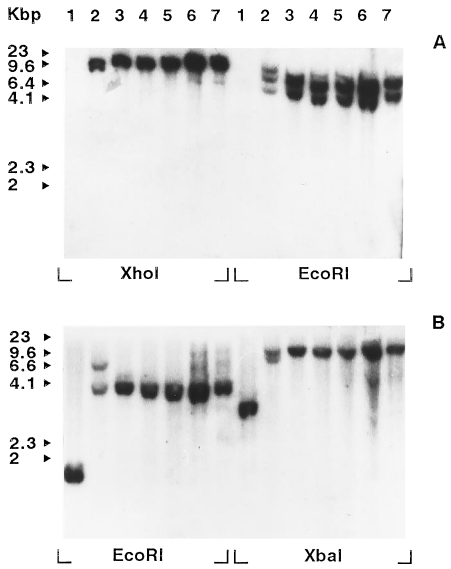

FIG. 4. Southern blot analysis of genomic DNA from Neo^r cell populations transduced by cocultivation with producer PA317 clone 2 or 12. The DNA was digested with the indicated restriction enzymes (*Xba*I cuts once in each of the N2 LTRs and once at nt 1 of the F12-HIV genome) and hybridized with the F12-HIV (A) or the *neo* (B) probe. DNAs from Neo^r N2-transduced CEMss cells (lanes 1); Neo^r CEMss cell populations transduced by uncloned N2/F12-HIV-expressing PA317 cells (lanes 2); Neo^r CEMss cells after cocultivation with either PA317 clone 2 (lanes 3) or clone 12 (lanes 4); Neo^r Hut-78 cells after cocultivation with either PA317 clone 2 (lanes 5) or clone 12 (lanes 6); and PA317 clone 12 (lanes 7) are shown. The same DNA molecular size marker as that in Fig. 1 was utilized.

ability of a fraction of transduced CEMss cells to express the F12-HIV genome.

In order to estimate the frequency of the infection events in human T cells, we tested the clonality of the Neo^r cells. The genomic DNA from transduced cells (CEMss and Hut-78) was analyzed by Southern blotting after *Hin*dIII digestion. The smear detectable after the hybridization with the *neo* probe (Fig. 5) clearly demonstrates that the T-cell-transduced cul-

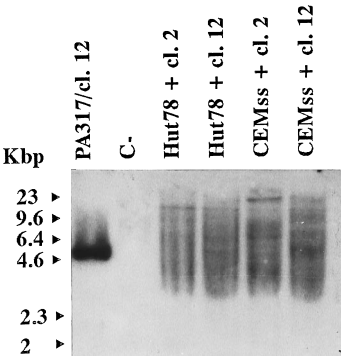

FIG. 5. Southern blot analysis of genomic DNA from Neo^r cell populations (either Hut-78 or CEMss cells) transduced by cocultivation with producer PA317 clone 2 (cl. 2) or 12 (cl. 12). DNAs were *Hin*dIII digested and hybridized with the *neo* probe. Starting from the N2 5′ LTR side of the N2/F12-HIV *nef*− AS construct, the first site recognized by the *Hin*dIII enzyme resides in the F12 *env* gene, about 3 kbp from the N2 5′ LTR end. As controls, DNAs from PA317 clone 12 (positive) and from parental CEMss (negative [C−]) cells were utilized. The molecular sizes of the same marker as that used for Fig. 1 are reported on the left.

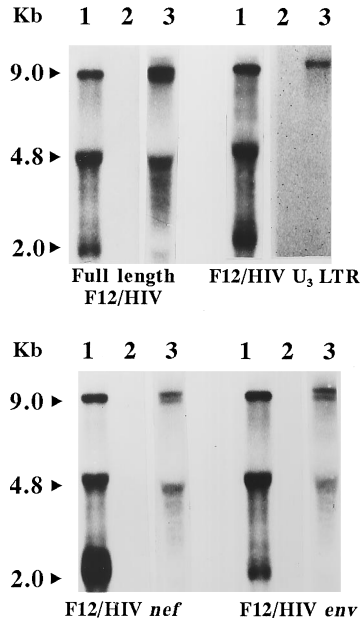

FIG. 6. Northern blot of poly(A)$^+$ RNA from F12 cells (lanes 1), parental CEMss cells (lanes 2), and CEMss clone 40 transduced with the N2/F12-HIV *nef*− AS vector (lanes 3). Five micrograms of RNA was hybridized with the indicated F12-HIV-specific probes. Molecular sizes of the major F12-HIV RNA species are indicated on the left.

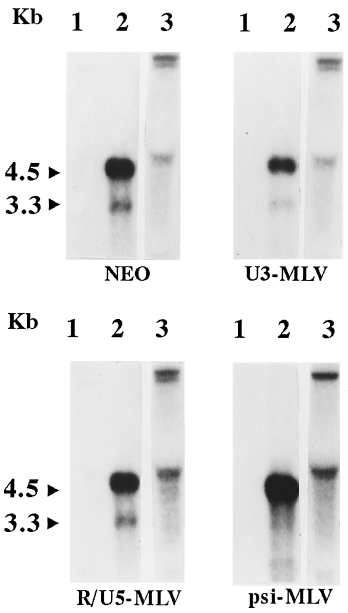

FIG. 7. Northern blot of the same poly(A)$^+$ RNAs as those used for Fig. 6 (except that in lanes 2, RNA from Neo^r CEMss cells transduced with a double-promoted retroviral vector, pLj [16], was loaded). The RNAs were hybridized with the indicated N2-specific probes. Molecular sizes of the major RNA species transcribed in CEMss cells by the pLj vector are reported on the left.

tures were largely polyclonal, thus excluding the possibility that the Neo^r cell populations emerge from rare infection events.

Thirty CEMss clones integrating and expressing the F12-HIV *nef*− AS genome were isolated and cultured in order to perform a comparative molecular characterization. In Fig. 1, the *Eco*RI pattern of two representative clones (lanes 40 and 41) is shown. No significant differences in HIV RNA or protein patterns were detected among different (as assessed by the variation in the integration sites) CEMss clones (data not shown).

Poly(A)$^+$ RNAs were hybridized with either F12-HIV-specific probes (i.e., the full-length, U3-LTR, *nef*, and *env* probes [Fig. 6]) or with four probes (see Materials and Methods) recognizing different regions of the N2 vector (Fig. 7). As demonstrated in F12-HIV-expressing HeLa CD4$^+$ clones (7), CEMss clones expressing F12-HIV *nef*− also express low levels of the double-spliced HIV RNAs (i.e., messengers specific for the regulatory proteins). In contrast, full-length and single-spliced F12-HIV RNAs are transcribed quite efficiently. The high-molecular-weight RNA doublet corresponds to the two full-length transcripts promoted, in opposite orientations, by the F12-HIV and the MLV 5′ LTRs. This observation was supported by the single (instead of the double) band at about 11 kb detectable by hybridizing the same poly(A)$^+$ RNAs with a probe overlapping the U3 region of the F12-HIV LTR, which could only be transcribed from the DNA chain codifying the N2 retroviral vector (Fig. 6).

The hybridization of the poly(A)$^+$ RNA with probes specific for the *neo* gene, the U3 and R-U5 regions of the N2 LTR, and the N2 ψ site (Fig. 7) showed the following. (i) Single- and double-spliced transcripts promoted by the F12-HIV 5′ LTR were recognized by each probe, suggesting that these RNA species were transcribed up to the U3 region of the MLV 5′ LTR. (ii) The single high-molecular-weight band obtained after hybridization with the N2-ψ-site-specific probe (which necessarily recognizes the N2-5′-LTR-promoted transcript) indicates that unspliced F12-HIV RNA did not include such sequences, suggesting a splicing event that did not occur in the single- and double-spliced F12-HIV RNAs. (iii) No spliced RNAs were transcribed by the MLV 5′ LTR; in fact, no additional RNA bands are detectable beside the doublet at 10 to 11 kb, the position of the single- and double-spliced F12-HIV mRNAs, the latter band being clear only after film overexposure. Accordingly, as already shown (Fig. 6), a single high-molecular-weight signal was detectable by hybridizing CEMss poly(A)$^+$ RNA with the F12-HIV U3-LTR probe that recognizes only the N2-5′-LTR-promoted transcripts.

The results obtained after the analysis of the viral proteins produced by the CEMss clones transduced with the N2/F12-HIV *nef*− AS vector (Fig. 8) were similar to those obtained

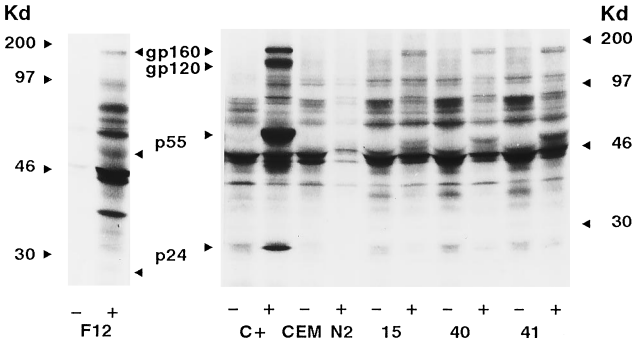

FIG. 8. Radioimmunoprecipitation assay of representative CEMss clones (clones 15, 40, and 41) transduced with the N2/F12-HIV *nef*− AS vector. The assay was performed with a mixture of either HIV-positive patients' sera (+) or HIV-negative donors' sera (−). Lysates from F12 and H9/HTLV-IIIB cells (C+) were used as positive controls, whereas lysates from N2-transduced CEMss cells (CEM N2) were utilized as a negative control. ^{14}C-labelled molecular size markers and HIV proteins detectable by radioimmunoprecipitation assay are also marked.

TABLE 2. CD4 FACS analysis of CEMss clones transduced with the N2/F12-HIV *nef*− AS vector

Cell clone	% of positive cells	Mean fluorescence intensity
CEM N2	99.55	491
F12	1.99	<10
5	89.55	501
15	95.81	431
17	69.17	384
21	29.21	138
22	93.25	488
28	97.03	530
31	78.89	436
35	99.31	528
40	85.63	509
41	92.23	465

with F12-HIV-transfected HeLa $CD4^+$ clones (7); in fact, no cleavage of the gp160 *env* glycoprotein was detectable, whereas reduced amounts (with respect to cells infected with a replication-competent HIV) of p55 and p24 *gag* proteins were observed. Finally, as already reported (7), no significant reduction of the CD4 exposure was detectable in any CEMss clone expressing the F12-HIV *nef*− genome (except for clone 21) (Table 2).

We also transduced the N2/F12-HIV *nef*− AS construct in fresh human PBLs. After 24 h of cocultivation, the transduction efficiency was assessed by testing the intracytoplasmic extracts by ELISA. Values clearly above the background values were obtained, i.e., 15 and 39 pg of *gag* protein from 10^5 PBLs 5 days after the end of the cocultivation with, respectively, PA317 clones 2 and 12. Accordingly, we estimate, by FACS analysis, that about 5% of PBLs were successfully transduced by the cocultures (Fig. 9). These data were consistent with the Southern analysis of the high-molecular-weight DNA extracted from transduced PBLs that shows the integration of the unarranged N2/F12-HIV genome in less than 10% of infected PBLs (data not shown).

HIV superinfection of transduced CEMss clones. Twenty-five CEMss clones were superinfected by HIV-1 (either the HTLV-IIIB or NL4-3 strain) at two different multiplicities of infection (MOI): 5×10^3 and 10^5 $TCID_{50}/10^6$ cells. The cytopathic effect of the HIV-1 superinfection was scored in terms of syncytium formation and cell viability. In addition, the RT activity of the supernatants was measured.

The formation of syncytia was not detected in any HIV-superinfected CEMss clone, whereas both wild-type CEMss cells and CEMss cells integrating the N2 vector rapidly formed large syncytia and died a few days after the HIV superinfection (Fig. 10C to F). The kinetics of both the RT activity and cell viability of three representative CEMss clones are reported in Fig. 10A and B. It is evident that the HIV superinfection did not significantly affect the growth of the F12-HIV-expressing clones even at the highest MOI. Clone 15 is representative of 5 of the 25 transduced CEMss clones superinfected at the highest MOI in which a few RT-positive samples were detected (Fig. 10B). In the remaining CEMss clones, very low or negative RT values were detectable, whatever the MOI used and the day after superinfection considered.

Thus, as already described (7), the expression of the F12-HIV genome strongly inhibited the replication of a wild-type superinfecting HIV, without affecting the CD4 HIV receptor exposure. The interfering property was, thus, also preserved when the F12-HIV genome lacking its *nef* gene was inserted into the N2 vector and transduced by recombinant retroviral infection.

DISCUSSION

Several experimental strategies aimed at blocking HIV replication have been proposed so far, mostly on the basis of immunotherapeutic or chemotherapeutic approaches. The goal of a more recently developed alternative anti-HIV experimental design was to render the target cell resistant to HIV replication through the induction of intracellular immunization (1). Inhibition of the superinfecting HIV was, in fact, demonstrated in cells expressing HIV-negative *trans*-dominant proteins (2, 12, 17, 21, 26, 34, 41), ribozymes directed against HIV sequences (18, 33, 42, 44, 45), *tat-rev* decoys (35, 36), or antisense RNAs (4, 13, 28, 29, 32). Moreover, true anti-HIV intracellular immunization was achieved by transfecting HIV-susceptible cells with DNA coding for either an anti-gp160 single-chain antibody (22) or a monoclonal anti-*rev* single-chain variable region (6).

We previously showed a strong inhibition of the superinfecting HIV in cells expressing the F12-HIV genome (7, 8, 9, 38), a functionally defective HIV-1 variant. We demonstrated that, in $CD4^-$ parental Hut-78/F12 cells, the retrotranscription of

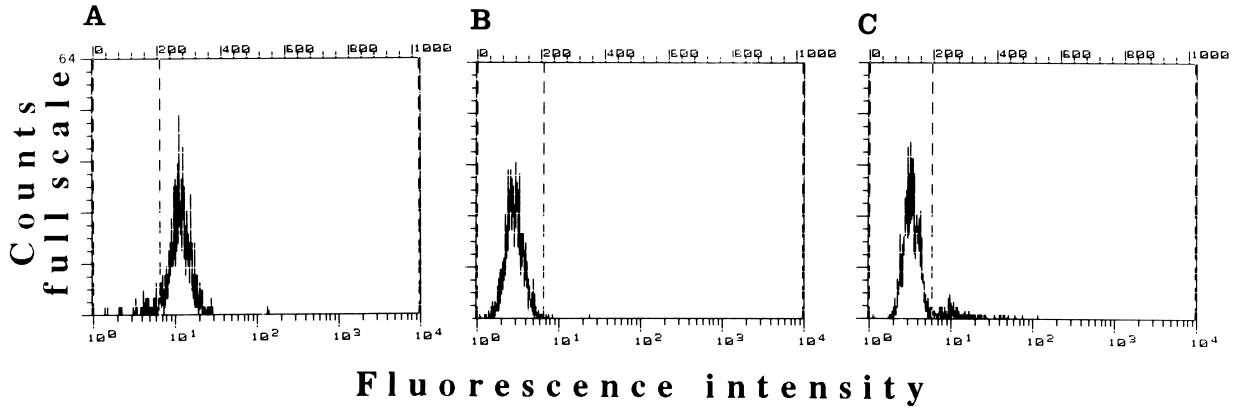

FIG. 9. FACS analysis for the presence of intracytoplasmic F12-HIV *gag* proteins in fresh human PBLs 5 days after the end of the cocultivations with PA317 clone 12 (C). The slopes of anti-*gag*-treated Hut-78/F12 cells (A) (positive control) and N2-transduced PBLs (B) (negative control) are also reported. Percentages of positive cells are (A) 96%, (B) 0.7%, and (C) 5.95%.

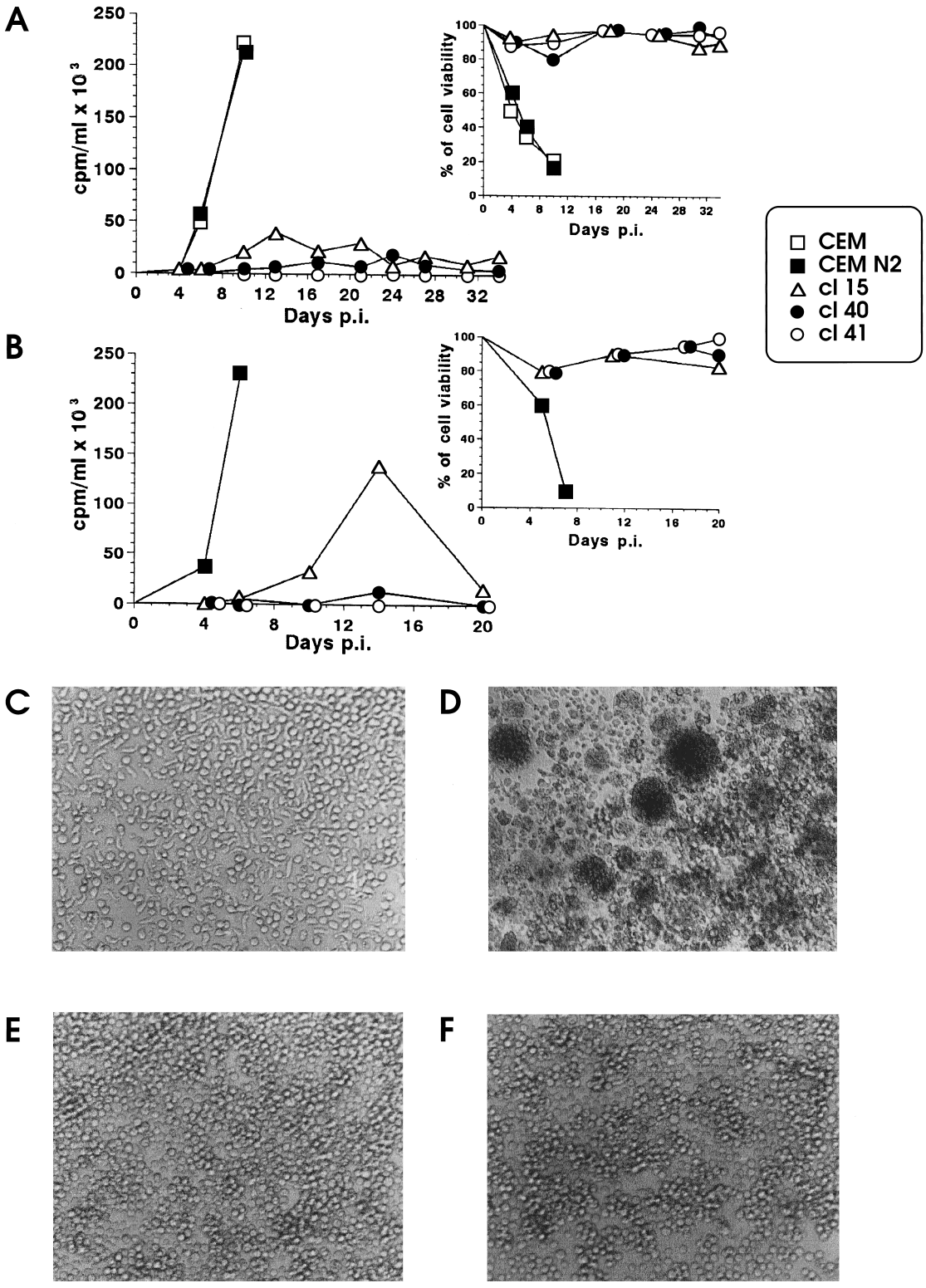

FIG. 10. HIV-1 superinfection. Representative CEMss clones (cl. 15, 40, and 41) transduced with the N2/F12-HIV *nef*− AS vector were superinfected with either (A) 5×10^3 $TCID_{50}/10^6$ cells or (B) 10^5 $TCID_{50}/10^6$ cells. As controls, both parental CEMss and N2-transduced CEMss cells were utilized (the latter cells only at the highest MOI). RT values are given as counts per minute per milliliter of culture supernatants at different days postinfection (p.i.). In the smaller graphs at the right, the cell viability of HIV superinfected cells is reported. Syncytia were detectable in N2-transduced CEMss cell cultures (D) but not in clone 40 cell cultures (F) 5 days after HIV-1 infection with 10^5 $TCID_{50}/10^6$ cells. Uninfected N2-transduced CEMss (C) and clone 40 (E) cell cultures are also shown.

the superinfecting HIV nucleic acid may begin, but only an incomplete and labile cDNA could be detected (38). In contrast, when the CD4 exposure was retained (e.g., in F12-HIV-expressing HeLa $CD4^+$ cells as well as in N2/F12-HIV-transduced CEMss clones) complete forms of cDNA were detected after the HIV superinfection (7 and data not shown). The evidence that the superinfecting HIV DNA disappears after a few days and that the interference could not be overcome, even when an HIV infectious molecular clone was transfected (7), strongly suggests that a postretrotranscriptional blockage is the basis of the F12-HIV-induced interference. Mutagenesis experiments are in progress in order to establish which F12-HIV gene(s) expression is necessary for the interference induction.

In order to assess whether F12-HIV-induced homologous viral interference could be reproduced in human cells that support HIV replication in vivo, we attempted to transduce human $CD4^+$ T cells with the F12-HIV genome within a Moloney MLV-based (N2) retroviral vector. In this paper, the generation of infectious recombinant retroviruses originating from the insertion of a *nef*-deleted HIV provirus in the N2 retroviral vector is described. In contrast, no transduced cells were obtained by using N2/F12-HIV retroviral constructs in which the F12-HIV genome lacking part of its 5′ LTR (corresponding to the negative regulatory elements) and of its 3′ LTR [encompassing the poly(A) signal] were inserted in both sense and AS orientations with respect to the N2 retroviral vector. Similar negative results were also obtained when the F12-HIV *nef*− genome was inserted in the same orientation as that of the N2 retroviral vector (not shown).

Molecular analyses performed on packaging and CEMss clones and on T lymphocytes transduced by the N2/F12-HIV *nef*− AS retroviral construct demonstrate the following. (i) The molecular construct was stably integrated in the host cell DNA. (ii) No unexpected RNA species were detected in either murine or human transduced cells. It is worth noting that no spliced RNAs promoted by the MLV N2 5′ LTR were detected in the transduced cells. As already observed in F12-HIV-transfected HeLa $CD4^+$ clones (7), the double-spliced F12-HIV RNA signal in the transduced CEMss clones appears fainter than those from the parental Hut-78/F12 cells. Conversely, the expression of both unspliced and single-spliced F12-HIV transcripts appeared as strong as those in the parental Hut-78/F12 cell clone. (iii) The lack of the *nef* gene did not seem to modify the protein pattern of the F12-HIV genome as detected by the radioimmunoprecipitation assay (Fig. 8).

The inhibition of challenging HIV-1 in CEMss clones transduced with the N2/F12-HIV *nef*− AS vector is strong at both MOI tested (Fig. 10). It should be stressed that also in CEMss clones whose supernatants show occasional positive points in RT assay (as in clone 15 at the highest MOI), neither syncytia nor the impairment of cell viability was ever observed. This experimental evidence indicates that the lack of most of the *nef* gene and of the whole 3′ LTR does not modify the peculiarities of the F12-HIV genome, i.e., its nonproductivity and, more noteworthy, its ability to interfere with the replication cycle of superinfecting HIV.

In spite of their relatively low titers, retrovirions released by the producer PA317 clones were able to infect human target cells quite efficiently, as demonstrated by the large polyclonality of transduced Neo^r CEMss and Hut-78 cells, although a fraction of Neo^r CEMss cells seems unable to express the F12-HIV genome. This could be due to small genetic rearrangements and/or deletions not detectable by Southern blot analysis. Alternatively, it is possible that, in the transduced CEMss cells that do not express the F12-HIV genome, the molecular construct was integrated in a genomic region that permitted N2 5′ LTR transcription but not F12-HIV 5′ LTR transcription on the opposite strand.

Additional problems, mainly concerning F12-HIV RNA polyadenylation and the possible occurrence of recombinatorial events, still remain unresolved. The expression of the G418 resistance gene inserted in the orientation opposite to that of the retroviral vector without additional polyadenylation signals has already been reported (20). Recently, a report was published demonstrating that retroviral transcripts could be polyadenylated either in the cellular sequences adjacent to the provirus or with cryptic signals within viral sequences (37). We hypothesize that the F12-HIV transcripts can be polyadenylated with either favorable cellular flanking sequences or the AAATAA sequence (35 nt upstream from the *Bss*HII site) that is present in the U3 region of the N2 5′ LTR (minus strand) (39), which might work as a cryptic polyadenylation signal. However, it has been demonstrated that the polyadenylation signal is not per se sufficient for RNA polyadenylation. In fact, a GU-rich sequence downstream from the polyadenylation signal is required for efficient polyadenylation (27). The fact that only 35 to 55% of the Neo^r CEMss cells transduced with the N2/F12-HIV *nef*− AS construct express F12-HIV proteins could also be because of the availability of such sequences in the cellular DNA close to the integration site.

We cannot formally exclude that recombination events might eventually occur between the transduced F12-HIV genome and the replication-competent superinfecting HIV. The most undesirable result would be the reversion of the F12-HIV phenotype to a productive HIV. However, the N2/F12-HIV *nef*− AS construct lacks most of the *nef* gene and the whole 3′ LTR, which are necessary for the replicative cycle. Furthermore, the genetic determinants responsible for nonproductivity are scattered along the genome (i.e., the *pol*, *vif*, and *env* genes) so that a high number of independent recombination events are needed to revert to the nonproducing phenotype. In addition, in a cell population homogeneously expressing the F12-HIV genome, any escaping replication-competent HIV would most likely be blocked by the interfering action of the same F12-HIV expressed by any other transduced cells. This is consistent with the evidence that, in HIV-1-superinfected HeLa $CD4^+$ cells expressing the full-length F12-HIV provirus, there was no infectious HIV-1 release over 3 months of continuous CEMss coculturing (unpublished observations).

Most of the anti-HIV strategies approached so far are based on the use of either chemical or biological reagents whose anti-HIV mechanism of action is well known. This is clearly an advantage when description and monitoring of the anti-HIV effects are needed. However, the actual potentialities of such well-known anti-HIV reagents do not seem to be sufficient to generate a resolvent anti-HIV therapy. This demonstrates that, in some instances, the level of knowledge reached so far regarding HIV biology and pathogenesis does not yet allow for a successful resolution to the clinical problem of a cure for AIDS. Thus, attempts to explore new strategies, even those involving anti-HIV reagents whose mechanism of action is not yet completely defined (as in F12-HIV-induced interference), could allow new insights into HIV biology and, thus, will increase the possibility of successfully fighting the spread of HIV.

The described susceptibility to HIV superinfection of an already chronically infected HIV cell line (15) enforces our conviction that F12-HIV-induced interference could be the result of an exclusive property of this HIV-1 variant genome. This is an additional matter that encourages us to continue our efforts to propose the use of the F12-HIV model in gene therapy.

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