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NOTES

A Human Immunodeficiency Virus Type 1 (HIV-1)-Based Retroviral Vector System Utilizing Stable HIV-1 Packaging Cell Lines

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We have constructed stable human immunodeficiency virus (HIV) packaging cell lines that when transfected with an HIV-based retroviral vector produce packaged vectors capable of transducing susceptible CD4$^+$ cells. This HIV-1-based retroviral vector system has the potential for providing targeted delivery and regulated expression of immunogens or antiviral agents in CD4$^+$ cells.

The ability of retroviral vectors to integrate into the genome of target cells renders them effective gene transfer vehicles (7). Retroviral vector systems typically consist of two components: a packaging cell line and a replication-defective vector. The packaging cell line synthesizes the viral structural proteins required for assembly of infectious virus-like particles. The vector contains the *cis*-acting signals required for incorporation of the vector into virions, initiation of reverse transcription, and insertion of vector DNA into the host genome.

A human immunodeficiency virus type 1 (HIV-1)-based retroviral vector system represents a potentially attractive approach to combating HIV-1 infection in susceptible cells. HIV-1 virus-like particles containing the HIV envelope glycoprotein gp120^{Env} can be selectively targeted to CD4$^+$ cells (2). Moreover, HIV-based vector gene expression can be rendered dependent on *trans*-acting signals encoded by HIV (reviewed in reference 5). We report the development of an HIV-based retroviral vector system employing a stable HIV packaging cell line. When transfected with an HIV-derived retroviral vector, this cell line produces packaged HIV vectors that transduce CD4$^+$ cells. Furthermore, vector gene expression in the transduced cells is responsive to HIV regulatory proteins.

Construction and characterization of HIV-based packaging cell lines. All HIV-1 sequences were derived from the molecular clone HXBc2 (8), and nucleotides are numbered as described previously (24). The packaging cell expression vector pHIVΔψHYG (Fig. 1) was constructed by generating a previously described (1) 21-nucleotide (nt) deletion (nt 294 to 314) in the HXBc2 packaging (ψ) site (18) by site-directed mutagenesis using a Muta-Gene kit (Bio-Rad). In addition, the HXBc2 *nef* gene and 3′ long terminal repeat (LTR) were replaced with the hygromycin phosphotransferase (Hyg^r) gene and a synthetic polyadenylation signal. The Hyg^r-encoding plasmid pHph+1 (Boehringer Mannheim) was modified by inserting a synthetic polyadenylation signal (AATAAA) and a *Cla*I site at the Hyg^r 3′ terminus. *Nco*I sites were introduced at both the Hyg^r translation initiation codon and the HXBc2 *nef* initiation codon (nt 8422 to 8424). The altered HXBc2 and Hyg^r coding sequences were inserted into a pUC19-derived plasmid backbone, yielding pHIVΔψHYG.

Vero cells were transfected with pHIVΔψHYG by the calcium phosphate procedure (9) and cultured in the presence of 200 μg of hygromycin B (Sigma) per ml. Supernatant from hygromycin B-resistant clones was assayed for p24^{gag} production by using a commercially available kit (Coulter), and high-producer clones were subcloned. Two subclones that produced the highest levels of p24^{Gag} antigen (D3.2 and B4.7; Table 1) were assayed for supernatant reverse transcriptase activity as described previously (15). Also, virus-like particles pelleted from packaging cell supernatant were analyzed for the presence of full-length HIV RNA by reverse transcription PCR using primers from the HIV-1 *gag* coding region as described previously (20).

As a positive control in these characterization studies, Vero cells were transfected with pProNeo (Fig. 1), an infectious HXBc2-derived proviral clone carrying the neomycin phosphotransferase (Neo^r) gene in place of the *nef* gene (22). Transfected cells were selected in the presence of 600 μg of G418 (Geneticin; Gibco-BRL) per ml. The G418-resistant subclone producing the highest levels of p24^{gag} antigen (V1.8) was characterized further.

While p24^{Gag} antigen production by clones D3.2 and B4.7 was roughly comparable to the values obtained in V1.8 cells, reverse transcriptase activities in the supernatants of D3.2 and B4.7 cells were approximately 20- and 60-fold lower, respectively, than the level observed in V1.8 cell supernatant (Table 1). Virus-like particles produced by the D3.2 and B4.7 packaging cell lines contained full-length viral RNA, but at only approximately 5% of the level observed in particles produced by V1.8 cells (Table 1). This low level of packaged RNA is consistent with previous reports examining encapsidation of ψ-minus transcripts (1, 4, 13).

Expression of HIV structural proteins in the stably transfected cell lines was examined by radioimmunoprecipitation analysis. The cells were labelled overnight with [^{35}S]methionine. Cell lysates were adjusted to contain equal amounts of trichloroacetic acid-precipitable radioactivity, and HIV structural proteins were precipitated with either polyclonal antisera directed against p24^{Gag} or gp120^{Env} (both obtained from American Biotechnologies) or AIDS patient antisera. The

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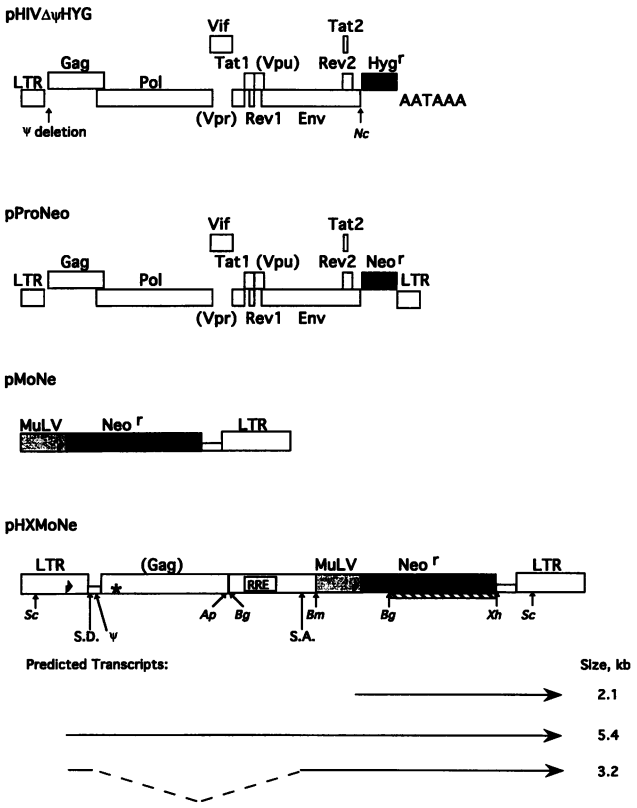

FIG. 1. Structures of packaging cell constructs and retroviral vectors. Open boxes represent HIV-derived structural regions, filled boxes denote genes encoding selectable markers (Neo^r and Hyg^r), and the shaded box represents the MoMuLV promoter. Parentheses indicate unexpressed open reading frames. In the HIV-1 proviral clone HXBc2, the *vpu* open reading frame is not expressed and the *vpr* open reading frame is truncated (24). In pHIVΔψHYG, the positions of the ψ site deletion and the synthetic polyadenylation signal (AATAAA) are indicated. In pHXMoNe, the transcription start sites are indicated by horizontal arrows (predicted transcripts are shown below the construct). The positions of the major splice donor (S.D.), ψ site, and RRE are indicated. The frameshift mutation introduced into the pHXMoNe *gag* coding sequences is depicted by an asterisk. S.A. denotes three splice acceptor sites clustered within a 30-nt region (29). The horizontal hatched bar depicts a 1-kb *Bgl*II-*Xho*I Neo^r gene fragment used as a probe in hybridization analyses. Abbreviations for relevant restriction enzyme sites: *Nc*, *Nco*I; *Sc*, *Sca*I; *Ap*, *Apa*I; *Bg*, *Bgl*II; *Bm*, *Bam*HI; *Xh*, *Xho*I.

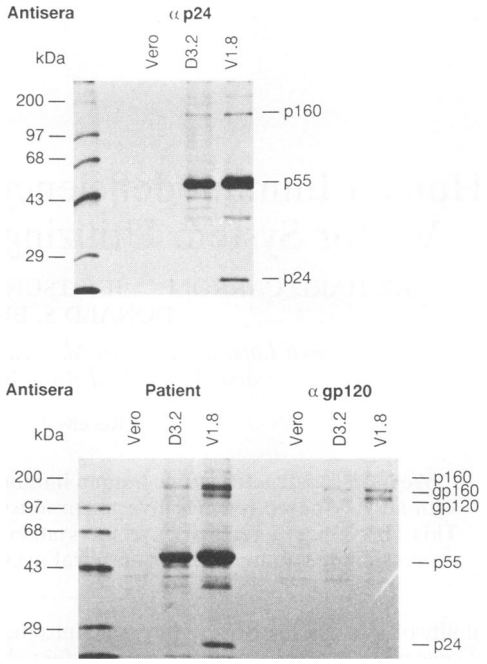

FIG. 2. Radioimmunoprecipitation analysis of stable HIV packaging cell lines. Lysates of [^{35}S]methionine-labelled D3.2 packaging cells, Vero cells, and the chronic virus-producing cell line V1.8 were incubated with AIDS patient antisera or polyclonal antisera directed against the HIV-1 envelope glycoprotein (αgp120) or capsid protein (αp24). Precipitated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. Molecular sizes are indicated, as are the positions of viral precursor and processed proteins.

results obtained for the D3.2 cell line are shown in Fig. 2. HIV precursor polyproteins p55^{Gag}, p160$^{Gag/Pol}$, and gp160^{env} were readily detected in the D3.2 line, and processing of the gp160^{Env} precursor to mature gp120^{Env} was observed. However, processed p24^{Gag} capsid protein was barely detectable in D3.2 cells, suggesting that p55^{Gag} processing was impaired in this line.

Production of transducing particles. The ability of the HIV packaging cell lines to generate transducing particles was examined by using the HIV-derived vector pHXMoNe (Fig. 1). The 5′ portion of pHXMoNe consists of a 3-kb *Xba*I-*Apa*I HXBc2 fragment ligated to an 850-nt *Bgl*II-*Bam*HI HXBc2 fragment. The *Xba*I-*Apa*I fragment contains approximately 1 kb of cellular flanking DNA, the 5′ LTR, untranslated leader sequences, and a fragment of *gag* coding sequences (ending at nt 1555). A frameshift mutation introduced at the unique *Cla*I site (nt 375 to 380) terminates *gag* translation after 21 codons. The *Bgl*II-*Bam*HI fragment contains the Rev response element (RRE [6, 26]) as well as the splice acceptors located upstream of the second *tat* and *rev* coding exons and the *nef* open reading frame (29). A 1.7-kb fragment containing the Moloney murine leukemia virus (MoMuLV) promoter (33) and Neo^r gene was inserted downstream of the *Bgl*II-*Bam*HI fragment. The polyadenylation/termination signal present in the MoMuLV LTR is absent from this fragment. The 3′ terminus of pHXMoNe consists of a portion of the HXBc2 *nef* gene (beginning at the *Xho*I site at nt 8442 to 8447), the polypurine tract, and the 3′ viral LTR.

The construct pMoNe (Fig. 1) was derived from pHXMoNe to serve as a control in transduction assays. It contains the

TABLE 1. Characterization of particles produced by packaging cell lines[a]

Cell line	p24 antigen (ng/10^6 cells/ml)	Reverse transcriptase activity (cpm/10^6 cells/ml)	Genomic RNA in viral particles[b]
V1.8	80	1.2×10^5	100.0
D3.2	68	5.2×10^3	6.0
B4.7	40	2.1×10^3	3.8
Vero	0	0	NA

[a] Values represent means of three separate experiments.

[b] Cell supernatants were normalized for p24^{Gag} content, and genomic RNA content of pelleted particles was determined by reverse transcription PCR analysis. RNA levels are expressed relative to the level detected in particles pelleted from the V1.8 line. NA, not applicable.

TABLE 2. G418 resistance in SupT1 cells transduced with HIV vectors

Cell line	Vector	Trial	Titer (transducing particles/ml)[a]
D3.2	pHXMoNe	1	10^1
		2	10^2
		3	10^1
		4	10^1
B4.7	pHXMoNe	1	10^0
		2	10^0
		3	0
D3.2/B4.7[b]	pMoNe	1	0
		2	0
		3	0
Vero	pHXMoNe	1	0
		2	0
		3	0

[a] Determined by limiting dilution analysis. Transduction was detected by the appearance of G418-resistant cells. Indicated titers represent minimum values.
[b] Two trials used pMoNe-transfected D3.2 cells, while the other used pMoNe-transfected B4.7 cells.

MoMuLV promoter, the Neo^r gene, and the 3′ HIV LTR, but it lacks the 5′ HIV LTR, untranslated leader, *gag*, and RRE regions.

Packaging cell lines D3.2 and B4.7 were transfected with pHXMoNe and selected in 600 μg of G418 per ml. G418-resistant cells were pooled, and 2×10^6 SupT1 cells were infected with 10-fold serial dilutions of filtered (0.45-μm-pore-size filter) pooled packaging cell supernatant. The SupT1 cells were placed under G418 selection and monitored for the appearance of G418-resistant cells. Transducing particle titers were defined as the lowest dilution of filtered supernatant that yielded resistant cells. It should be emphasized that these values represent minimum titers.

Transducing particle titers from pools of transfected B4.7 cells were extremely low (Table 2). In two cases, 10^0 particles per ml were produced, while in a third instance, no transducing particles were detected. Slightly higher transducing particle titers were obtained from pools of transfected D3.2 cells (Table 2). In one instance, 10^2 transducing particles per ml were produced. In three other experiments, 10^1 transducing particles per ml were generated.

The low number of transducing particles produced by pooled pHXMoNe-transfected packaging cells suggested that a minority of the cells in these populations could have produced high titers of transducing particles, events obscured by a large background of nonproducer cells. To address this issue, clonal pHXMoNe-transfected packaging cells were established and assayed for transducing particle production. While a high percentage of clonal lines produced transducing particles, none had titers higher than 10^2 particles per ml (data not shown), suggesting that the existence of high-titer packaging cell clones, while possible, was unlikely.

To exclude nonretroviral mechanisms of Neo^r gene transfer to SupT1 cells, Vero cells were transfected with pHXMoNe. The resulting cell pools contained functional vectors but were unable to generate virus-like particles. Similarly, D3.2 and B4.7 cells were transfected with pMoNe, yielding virus-like particle-producing cells containing vectors that could not be encapsidated or reverse transcribed. No transducing particles were produced by either type of cell population (Table 2), demonstrating that transduction requires both functional vectors and virus-like particle-producing cells.

Replication-competent retroviruses are frequent contaminants of retroviral vector preparations, most frequently arising through recombination between viral sequences in the packaging cell line and the vector (21). The presence of replication-competent retroviruses in supernatant from pHXMoNe-transfected packaging cells was assayed by the following method. SupT1 cells were infected with 1 ml of filtered supernatant and cultured without selection for 30 days, during which time they were monitored for syncytium formation. The 30-day culture supernatant was used to infect fresh SupT1 cells, which were assayed for $p24^{Gag}$ antigen expression 5 days later. $p24^{Gag}$ antigen levels were indistinguishable from background for all transfected packaging cell populations examined (data not shown). This assay, when performed with an HIV_{IIIB} stock, had a sensitivity threshold of 0.1 50% tissue culture infective dose per ml (data not shown). These results demonstrate that under the culture conditions used, no replication-competent retrovirus was detectable in transducing particle-producing cell lines.

Characterization of transduced SupT1 cells. SupT1 cells transduced by supernatant from pools of pHXMoNe-transfected packaging cells were examined for the presence of pHXMoNe vector DNA by Southern blotting (28). *Sca*I-digested genomic DNA was hybridized with a 1-kb probe derived from the Neo^r region of pHXMoNe. The Neo^r probe hybridizes to a 4.8-kb internal *Sca*I fragment (Fig. 1). The predicted fragment was recognized in all transduced SupT1 populations, suggesting that vector integration occurred without gross structural rearrangement (Fig. 3A). While this analysis of integration is indirect, the sequences have been retained in the cells for more than 4 months (data not shown), which strongly suggests that they have been stably transferred.

HIV vector-directed gene expression in SupT1 cells transduced by supernatant from pools of pHXMoNe-transfected packaging cells was examined by Northern (RNA) blotting analysis (28). Predicted vector transcripts are shown in Fig. 1. Total RNA was isolated by using RNAzol B (Tel-Test) and hybridized with the 1-kb Neo^r probe (Fig. 3B). The Neo^r probe hybridized strongly to a 2.1-kb band, consistent with the size predicted for MoMuLV transcripts. The Neo^r probe also recognized a less abundant transcript of approximately 3.2 kb. Spliced HIV promoter transcripts (utilizing the splice acceptors downstream of the RRE [Fig. 1]) are predicted to be 3.2 kb in size. However, the Neo^r probe did not detect the predicted 5.4-kb unspliced HIV promoter transcript.

The ability of HIV-1 regulatory gene products to modulate HIV vector-directed gene expression in transduced SupT1 cells was examined by using the *tat* expression plasmid pIIIEx-Tat (32) and the *rev* expression plasmid pCMVRev. pCMVRev consists of a cytomegalovirus promoter governing expression of a pIIIenv3-1-derived construct (31) from which 583 nt of *env* coding sequences were deleted. Transduced SupT1 cells were electroporated with either pIIIExTat, pCMVRev, or both; 48 h later, total RNA was isolated and examined by Northern blotting analysis using the Neo^r region probe (Fig. 3C).

Introduction of pCMVRev had little effect on vector transcription, while introduction of pIIIExTat resulted in a modest increase in the levels of both the 3.2- and 5.4-kb transcripts. Simultaneous introduction of both *tat* and *rev* expression plasmids into transduced SupT1 cells resulted in a marked increase in the level of unspliced 5.4-kb HIV promoter transcripts, demonstrating that vector gene expression is responsive to HIV-1 Tat and Rev.

This report demonstrates that introduction of an HIV-based retroviral vector into stable HIV-based packaging cell lines yields packaged vectors that transduce a susceptible T-cell line. While the vector titers were extremely low, it is conceivable

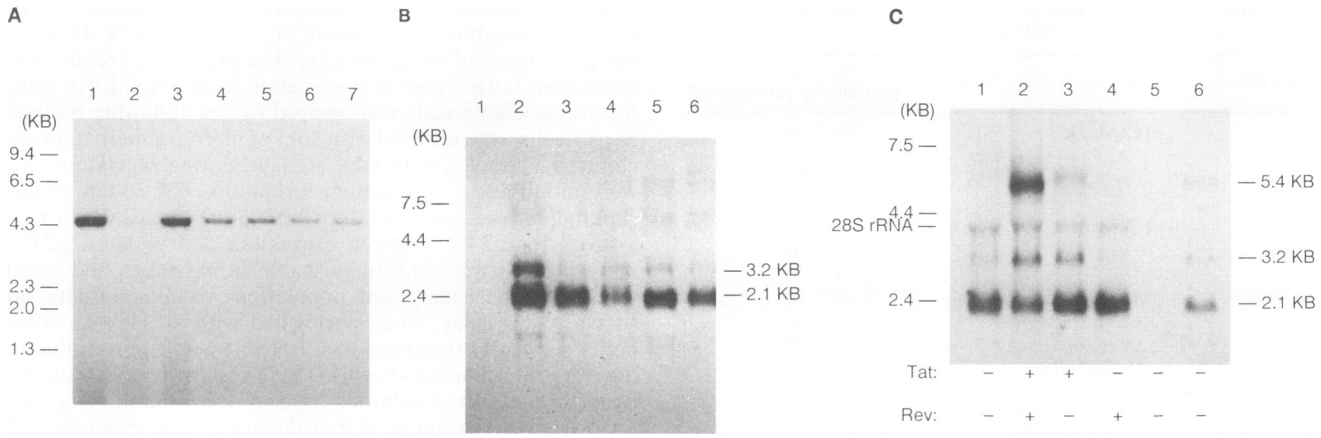

FIG. 3. Characterization of transduced SupT1 cells. (A) Southern blotting analysis of SupT1 cells transduced by supernatant from pooled pHXMoNe-transfected packaging cells. *Sca*I-digested SupT1 genomic DNA was hybridized with a 1-kb Neo^r probe (Fig. 1). Lane 1 contains *Sca*I-digested pHXMoNe vector DNA mixed with genomic DNA from untransduced SupT1 cells. Lane 2 contains genomic DNA from untransduced SupT1 cells. Lanes 3 to 7 contain genomic DNA from transduced SupT1 cells obtained from independent transduction experiments. Molecular sizes are indicated. (B) Northern blotting analysis of SupT1 cells transduced by supernatant from pooled pHXMoNe-transfected packaging cells. RNA isolated from transduced SupT1 cells was hybridized with the Neo^r probe. Lane 1 contains RNA isolated from untransduced SupT1 cells. Lanes 2 to 6 contain SupT1 RNA obtained in independent transduction experiments. Molecular sizes are indicated, as are the sizes of transcripts recognized by the Neo^r probe. (C) Modulation of vector gene expression in transduced SupT1 cells by HIV-1 regulatory gene products. Transduced SupT1 cells were electroporated with the HIV-1 *tat* expression vector pIIIExTat or the *rev* expression vector pCMVRev; 48 h postelectroporation, RNA was isolated and subjected to Northern blotting analysis using the Neo^r probe. Electroporation of *tat* or *rev* expression vectors is indicated. Lanes 1 to 4 contain RNA obtained in separate transduction experiments. Lane 5 contains RNA from untransduced SupT1 cells. Lane 6 contains RNA from a pHXMoNe-transfected packaging cell clone.

that because of the low cloning efficiency of SupT1 cells and the toxic environment created by many untransduced dying cells, many transduced cells failed to grow, resulting in an underestimation of the actual titer.

However, it is likely that other factors, in particular the lack of viral precursor protein processing in packaging cell lysates, contributed directly to the low vector titers. An accumulation of unprocessed precursor proteins in constitutive HIV particle-producing cells has been observed previously, leading the authors to suggest that cells expressing active HIV protease may be placed at a selective disadvantage (14, 17). While virion formation can occur in the absence of precursor protein processing, the released virions are not infectious (16), and they exhibit significantly reduced reverse transcriptase activity (19). This observation is consistent with our finding that supernatant reverse transcriptase activity in both B4.7 and D3.2 cells is markedly reduced and supports the contention that defective protein processing may contribute substantially to the low transducing particle titers obtained.

It is also possible that defects in vector design contributed to the low transducing particle titers. However, a comprehensive analysis of HIV vector packaging requirements showed that vectors containing the ψ site and the RRE, both of which were present in pHXMoNe, are efficiently packaged by wild type HIV-1 (25). Additionally, in the presence of an infectious HIV-1 molecular clone, pHXMoNe can be packaged to titers as high as 10^4 transducing particles per ml (22). These observations suggest that the low vector titers observed in this study are mainly due to defects in the packaging cell line rather than in pHXMoNe.

Distinct pathways can be envisioned by which HIV vectors can provide antiviral defenses. The immune response observed upon administration of HIV-like particles (10–12, 14, 27) may be augmented through the use of HIV vectors expressing immunogens or immunomodulators. Alternatively, HIV-based vectors carrying antiviral agents can enable HIV-susceptible cell populations to mount a tightly regulated, HIV-dependent antiviral response. However, the practicality of any retroviral vector systems depends on the availability of high-titer vector preparations free of replication-competent virus. While methods using transient cotransfection of vector and packaging functions (3, 23, 30) or using wild-type virus to package vector (25) have yielded higher vector titers, vectors prepared by these techniques may be unsuitable for human use protocols. The ability to select and amplify individual stable packaging cells permits the rigorous characterization of homogeneous vector-producing cells. Therefore, although the vector titers that we have reported are low, these data represent a critical step forward in production of a practical HIV-based retroviral vector system ultimately designed for human use.

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