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Research Article

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Targeted and Highly Efficient Gene Transfer into CD4$^+$ Cells by a Recombinant Human Immunodeficiency Virus Retroviral Vector

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Abstract

We have established a recombinant HIV gene transfer system based on transient expression of the HIV packaging functions and a recombinant vector genome in monkey kidney Cos cells. The recombinant HIV retroviral vector introduced the neo^R gene into CD4$^+$ cells with high efficiency, comparable to that achieved with the highest titer amphotropic murine recombinant retrovirus. Vector preparations were devoid of replication competent, infectious HIV. Gene transfer was dependent on CD4 expression, as shown by expression of the CD4 gene in HeLa cells, and could be inhibited by soluble CD4. This specific and efficient gene transfer system may be useful for development of gene therapy for which T cells are the desired targets. (*J. Clin. Invest.* 1991. 88:1043–1047.) Key words: gene transfer • gene therapy • AIDS • intracellular immunization • Cos cells

Introduction

Much effort has been focused on the development of strategies for insertion of genes into somatic cells for treatment of genetic diseases, cancer, or acquired immunodeficiency syndrome (1–3). Among the factors that limit the feasibility of such strategies is the efficiency with which genes can be transferred and expressed in human cells. Systems for the exploitation of murine retroviruses for gene transfer are the most advanced. Very useful packaging cell lines capable of generating helper-free replication incompetent, recombinant viruses that exhibit a single round of infection and gene integration have been developed (4, 5). These systems have provided the recombinant viruses used in the first protocols for in vitro gene transfer into human cells that were subsequently returned to the patient (6).

Murine retroviruses have been classified according to their host range (7); ecotropic viruses infect all murine cells and a few rat cell lines, whereas amphotropic viruses have a broad host range including human cells. The specificity is determined by the major envelope protein, gp70, through its interaction with a membrane protein that acts as a receptor to initiate the process of virus entry. The receptor for ecotropic viruses has been cloned and shown to be a transmembrane protein with multiple spanning regions (8), whereas the receptor for amphotropic viruses has yet to be identified. These receptor proteins are expressed on most differentiated cells allowing indiscriminate transfer of genes into target populations. The level of expression of the viral receptor proteins appears to vary and might limit viral entry into desired targets, e.g., T-lymphocytes or hematopoietic stem cells. These features, indiscriminate infection of many cell types but limited infection of desired targets, have retarded the development of murine retroviruses for human gene therapy.

HIV-1 enters cells by interaction of its envelope protein (gp120) with CD4, a transmembrane protein expressed on helper T-lymphocytes (9). CD4 expressing T-lymphocytes are a potentially important target for therapeutic gene transfer. Expression of various genetic elements designed to inhibit HIV replication or gene expression might create a desired state of intracellular immunization for treatment of AIDS (10, 11). We have developed a packaging system capable of generating helper virus-free, replication incompetent viruses packaged with HIV proteins. This system is similar in design to that independently developed and recently reported by others (12). In our experiments, we have shown that recombinant HIV can be used for highly efficient gene transfer into CD4$^+$ cells.

Methods

Plasmid construction. Recombinant DNA molecules were constructed by standard methods (13). The basic vectors, LN, LH, and L, are derivatives of the pMfos$^{\Delta S}$ plasmid (14). Each contains the SV40 origin and the human beta globin second intron, third exon, and polyadenylation signal (Fig. 1 *A*). LN and LH have the neo^R gene (15) and the hygromycin-resistant gene (16) as a selectable marker, respectively. CMV/LN was constructed by inserting the 0.7-kb XhoI-BamHI human cytomegalovirus (CMV)[1] immediate early promoter (17) into LN. CBH10 was constructed by inserting a 8.8-kb SacI-SacI (nucleotides 675–9428 [18]) fragment from a cloned HIV genome, BH10, into the BamHI site between the CMV promoter and the beta globin segment in CMV/LN. CGPE$^+$ was constructed by removing a 0.7-kb XhoI-SacI (nt 8927–9602) fragment from CBH10. In CGPE$^-$, the putative packaging signal sequence between the splicing donor site and the initiation site of the Gag protein (18) in CGPE$^+$ was mutated by inserting a synthetic oligonucleotide between the HphI (752) and ClaI (827) sites (Fig. 1 *B*). Nucleotides 753 to 786 were deleted and replaced with 20 bp containing desired restriction sites. Nucleotides 787 to 826 were included in

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1. *Abbreviations used in this paper:* CMV, cytomegalovirus; TK, thymidine kinase.

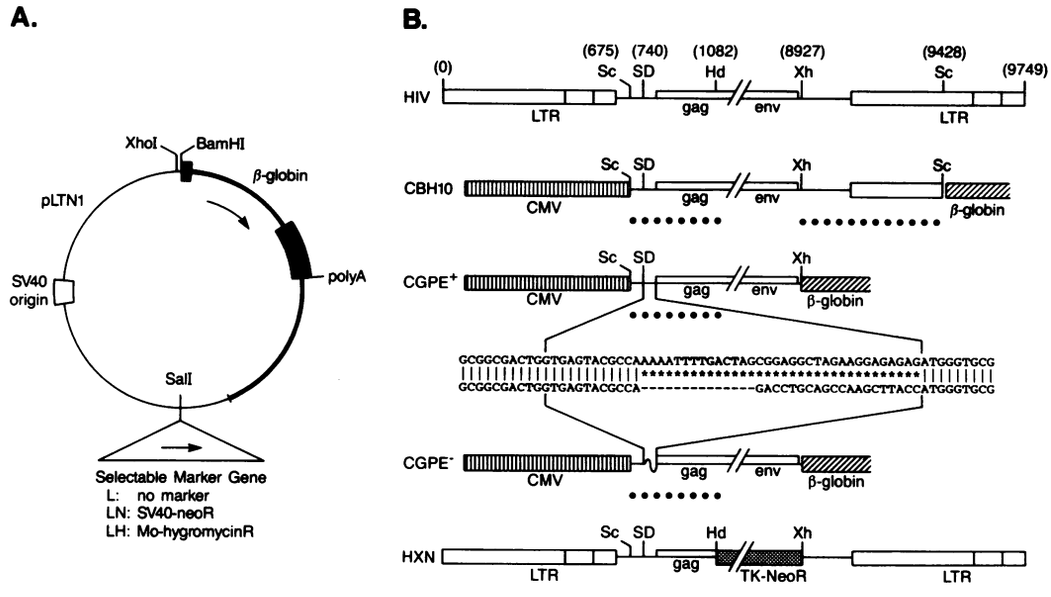


Figure 1. Structure of expression vectors. (*A*) The basic plasmids, L, LN, and LH. The thin line shows pLTN1 sequence including the SV40 origin of replication (21), while the thick line shows human beta globin sequence containing a part of the second exon, the second intron, the third exon, and the polyadenylation signal. LN has the neo^R gene driven by the SV40 early promoter, and LH has the hygromycin^R gene driven by the Moloney murine leukemia virus LTR. (*B*) Restriction maps of packaging and gene transfer plasmids. The nucleotide numbers in parethesis are according to reference 18. *Sc,* Sac I; *Hd,* Hind III; *Xh,* Xho I; *SD,* the splicing donor site. In CGPE$^-$, a mutation was introduced within the putative packaging signal sequence using a synthetic oligonucleotide fragment (see Methods). Asterics show mismatched base pairs. The dotted lines show the homologous regions between the packaging vectors and HXN. In each case the relevant fragment was subcloned between the XhoI and the BamHI site of a vector shown in *A*, as follows: CBH10, from 5′ end of CMV to 3′ Sc site into LN vector; CGPE$^+$ and CGPE$^-$, from 5′ end of CMV to Xh site into LN vector; HXN, entire fragment shown in *B* into L vector.

the synthetic oligonucleotide to recreate the wild-type sequence. The HXN vector consists of a 0.7-kb XhoI-HindIII (−189 to 1082) fragment containing the HIV-LTR, the packaging signal, and a part of Gag coding sequence, a 2.0-kb XhoI-BamHI fragment containing the bacterial neo^R gene driven by the thymidine kinase promoter (TK-NeoR) from pMC1neo (19), and a 0.8-kb XhoI-NarI (−189 to 637) fragment containing the HIV-LTR all inserted between the XhoI and BamHI sites of the L vector. CCD4/LH was constructed by inserting the CMV promoter and 1.7-kb EcoRI-BamHI fragment containing the human CD4 gene from pSP65T4.8 (20) into the LH vector.

Virus production and transduction. Cos cells, HeLa cells, and human T cells (CEM, HPB-ALL, MT-2) were grown as described (21). Cos cells (2×10^6 per 10-cm dish) were transfected with 10 μg of the packaging vector and 10 μg of the HXN plasmid by the $CaPO_4$ coprecipitation method. The transfected Cos cell conditioned culture medium was collected after 48 h and filtered through 0.22-μm pore size filters.

Human T cells (2×10^6) were incubated with 5 ml of the transfected Cos cell media and 6 μg/ml polybrene. The medium was replaced with fresh medium after 24 h, and 1,000 μg/ml active G418 was added 48 h after infection. For titering, the infected cells were dispensed into 96-cell culture plates at 1×10^2 cells/0.1 ml G418 containing medium/well. CD4$^+$ Hela cells (1×10^5 cells in a 60-mm dish) created by prior introduction of the CCD4/LH plasmid, were incubated with 3 ml of the Cos cell medium in the presence of 6 μg/ml polybrene for 24 h. The cells were replated on 10-cm dishes and cultured in the presence of 1,000 μg/ml active G418 for 2 wk.

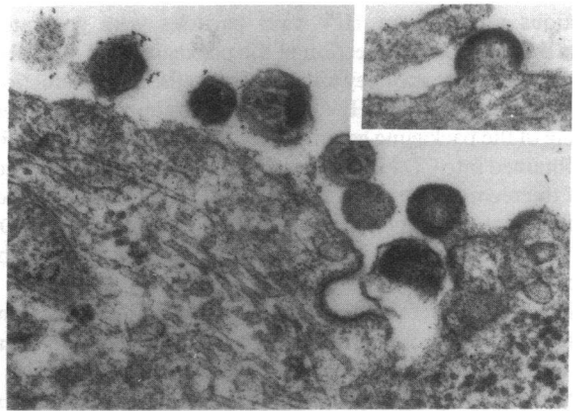
Characterization of the recombinant virus. For viral RNA analyses, viral particles from 10-ml transfected Cos cell medium were precipitated with 6% polyethylene glycol (8,000) and 0.3 M NaCl. RNA was isolated from the viral precipitate by phenol-chloroform extraction, treated with 10 U of RNase free DNaseI (Promega Biotec., Madison, WI) in the presence of 10 mM DTT and 200 U RNasin ribonuclease inhibitor (Promega Biotec.) for 30 min at 37° and reextracted. Gag protein, p24, was assayed by a radioimmunoassay kit (DuPont Co., Wilmington, DE).

Results

Production of the recombinant HIV. Cos cells were transfected with the expression vectors by the $CaPO_4$ coprecipitation method, and the cells and their medium were analyzed after 48 h. Indirect immunofluorescence staining using the monoclonal antibody to HIV gp120 (22) showed that 10 to 15% of the Cos cells transfected with either CBH10, CGPE$^+$, or CGPE$^-$ transiently expressed a high level of envelope glycoprotein (data not shown). Fig. 2 *A* shows electron micrographs of Cos cells transfected with a packaging vector, CBH10. Virus-like particles, including typical budding forms, were observed. The culture medium from transfected Cos cells was assayed for p24 gag protein by radioimmunoassay (Fig. 2 *B*). CGPE$^+$ produced about threefold more p24 than CGPE$^-$ (*1* and *3*), suggesting that the sequence immediately upstream of the gag initiation codon is important for gag gene expression. Cotransfection with the HXN plasmid increased the level of p24 in the supernatants (*2* and *4*) but particle formation did not appear to depend on the presence of the recombinant HIV genome (Fig. 2 *B*).

RNA was extracted from viral particles precipitated with polyethylene glycol, treated with DNaseI, and slot-blotted onto nylon paper (Fig. 2 *B*). Hybridization with the Neo probe showed that recombinant HIV-Neo RNA from the HXN plasmid was associated with particles. The blot was also hybridized with the HIV coding sequence probe, H4. Comparable amounts of RNA transcripts from either CGPE$^+$ or CGPE$^-$ were detected in the viral particle fraction. The amount of neo^R hybridizing sequences recovered from viral particles was comparable to that found in supernatant from ecotropic Moloney retroviral-producing cells having a functional neo^R titer of 10^6 on 3T3 cells.

A.



B.

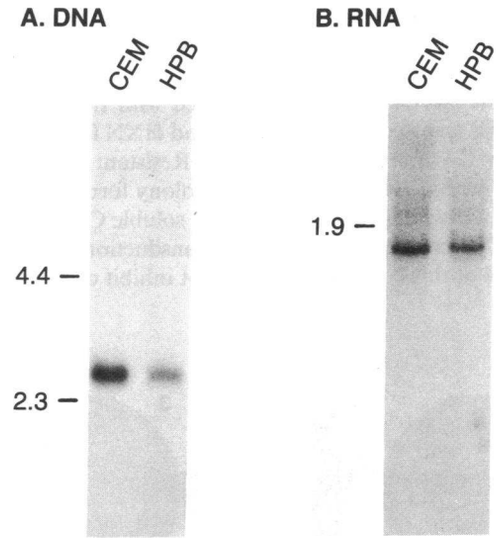
Plasmids	p24 (ng/ml)	RNA	
		Neo	H4
1. CGPE$^+$	5.6		
2. CGPE$^+$ + HXN	12.8		
3. CGPE$^-$	2.0		
4. CGPE$^-$ + HXN	7.7		
5. BTat + HXN	0.0		

Figure 2. Characterization of the recombinant HIV. (*A*) Electronmicrographs of Cos cell transfected with the packaging vector CBH10 (magnification, 36,360). The inset shows a typical budding form. (*B*) Detection of the p24 gag protein and RNA in the supernatants from transfected Cos cells. The supernatants were filtered and assayed by a p24 RIA kit (DuPont Co.). RNA associated with viral particles was slot-blotted and hybridized with the Neo probe (a 0.9-kb Pst fragment from pSV2neo [15]) or the H4 probe (a 4.5-kb ScaI-ScaI (nt 2903–7431) fragment from BH10). BTat is a pLTN1 based HIV Tat expression vector driven by the B19 parvovirus promoter.

Assay for replication competent virus. The HTLV-1 transformed human T cell line, MT-2, is highly sensitive to cytopathic effects of HIV. At 5 d after incubation, extensive syncytium formation was observed among MT-2 cells cultured with the supernatant from Cos cells transfected with CBH10 + HXN. Although the frequency of two recombination events is usually very low (5, 23), this assay is sufficiently sensitive to detect infectious virus arising from the double recombination that is necessary to generate an intact HIV genome with both LTRs from the RNA transcripts of these two plasmids. In contrast, we could not detect infectious, cytopathic HIV virus in the medium from Cos cells transfected with CGPE$^+$ + HXN or CGPE$^-$ + HXN after 3 mo of culture with MT-2 cells. With these combination, there is only one homologous region within the packaging and HIV neo^R vectors substantially reducing the probability of generation of wild-type virus by recombination.

Highly efficient transduction of human T cells. Two human CD4$^+$ T cell lines, CEM and HPB-ALL, were incubated with the medium from Cos cells transfected with HXN and CGPE$^+$ and were selected for G418 resistance for 2 wk. Southern blot analysis with the Neo probe showed a 2.4-kb SacI fragment in two polyclonal T cell populations (Fig. 3 *A*), indicating integration of an intact proviral genome in most or all infected cells. Northern blot analysis of total cellular RNA showed a single 1.6-kb band derived from the TK promoter of the integrated provirus. Transcripts directed from the HIV-LTR were not detected.

A limiting dilution technique was used to quantitate neo^R transduction efficiency of T cells. CEM cells were incubated with tenfold serial dilutions of the conditioned media from Cos cells transfected with CGPE$^+$ + HXN or from a high titer amphotropic retrovirus-producing cell line, N263A2 (24). The infected cells were dispensed into 96-microtiter wells at 100 cells per well and cultured under selection with G418. Wells containing resistant cells were counted 14 d after infection (Table I). Transduction efficiency on CEM cells by the undiluted Cos cell medium was equivalent to that of a murine amphotrophic virus stock having a titer of 5.0×10^7 on 3T3 cells.



C.

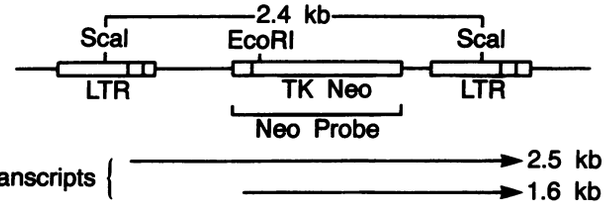


Figure 3. DNA and RNA analyses of human T cell lines (CEM and HPB-ALL) transduced with recombinant HIV. (*A*) Southern blotting. Genomic DNA from G418 resistant T cells was digested with ScaI and blot hybridized with the Neo probe. The variation in band intensity reflects differences in the amount of DNA in the two lanes as shown by ethidium staining of the gel before transfer. (*B*) Northern blotting. Total RNA was blot hybridized with the Neo probe. (*C*) A restriction map of the recombinant HIV-Neo provirus.

Table I. Comparison of Transduction Efficiency of the Conditioned Medium from Cos Cells Producing Recombinant HIV and N263A2 Cells Producing Amphotropic Moloney Retrovirus

Producing cells	Virus	Dilution	Titer on 3T3 cells	No. of wells with G418-resistant cells/96 wells
Cos	Recombinant HIV	1		66
		10^{-1}		12
N263A2*	Amphotropic	1	5.2×10^8	90
	Moloney virus	10^{-1}	5.2×10^7	56
		10^{-2}	5.2×10^6	12
		10^{-3}	5.2×10^5	0

CEM cells incubated with the conditioned medium from virus producing cells were dispensed into 96-well culture plates at 1.0×10^2 cells in 0.1 ml of media with 1,000 μg/ml G418 per well. Wells containing G418-resistant cells were counted 14 d after infection. * Reference 24.

Targeted transduction of CD4⁺ cells. A HeLa cell subline (T4H) expressing CD4 molecules was established by stable transfection with the CD4 vector (CCD4/LH). T4H cells and $CD4^-$ parental HeLa cells were incubated with the medium from Cos cells transfected with $CGPE^+$ and HXN followed by selection for G418 resistance for 2 wk. Resistant clones appeared only with T4H cells (Fig. 4 *A*). Colony formation was inhibited in a dose-dependent manner by soluble CD4; 10 μg/ml soluble CD4 completely blocked the transduction (Fig. 4 *B*). Soluble CD4 at this concentration did not inhibit cell growth.

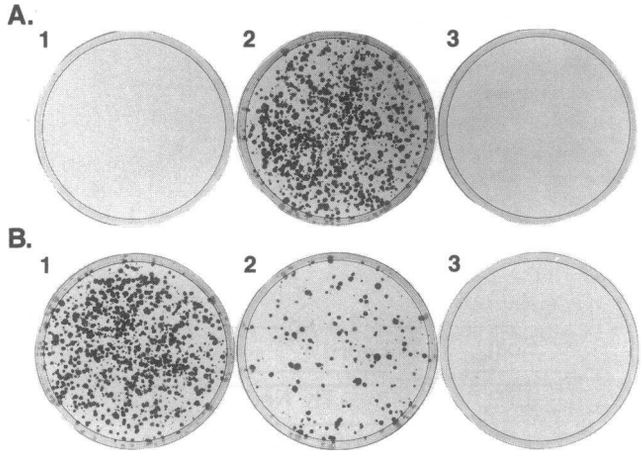


Figure 4. Transduction of $CD4^+$ HeLa cells. (*A*) Transduction specific for $CD4^+$ cells with recombinant HIV. $CD4^-$ parental HeLa cells (1) and $CD4^+$ HeLa cells (T4H) (2, 3) were exposed to transfected Cos cell medium. Plasmid DNA used for transfection of Cos cells were $CGPE^+$ + HXN and LN + HXN. (*B*) Inhibition of the transduction with soluble CD4. T4H cells were transduced with the transfected Cos cell medium ($CGPE^+$ + HXN) in the presence of 0 μg/ml (*1*), 2 μg/ml (*2*), and 10 μg/ml (*3*) recombinant soluble CD4. Infected HeLa cells were selected for G418 resistance for 2 wk and stained with crystal violet.

Discussion

Infectious recombinant HIV viral particles were generated within 48 h after cotransfection of the packaging and recombinant genomes into monkey kidney (Cos) cells. The production of replication competent HIV was avoided by appropriate engineering of the packaging genome. Highly efficient gene transfer was obtained into CD4 expressing T cells. The infectious capacity of the recombinant HIV vector could be extended to other cell types by prior introduction and expression of the CD4 gene and HIV-mediated gene transfer could be blocked by soluble CD4.

There are several advantages to this HIV based gene transfer system compared with the murine retrovirus system. First, gene insertion is directed to CD4 expressing T cells, a desirable target for gene transfer purposes. In complex mixtures of cells in vitro or in vivo, those cells lacking CD4 surface protein would be immune from gene transfer. Second, the system is highly efficient. The biologically effective neo^R titer of the recombinant HIV vector on T cells is equivalent to that of a murine amphotrophic retrovirus that has a neo^R titer on 3T3 cells of 5×10^7. Producer clones that generate recombinant retrovirus with this titer are rarely achieved by conventional strategies. Third, the process of identifying useful producer clones of murine retroviruses requires screening before an appropriate clone is identified. Many months may be required. In contrast, the HIV recombinant system depends on cotransfection of the helper and recombinant genome into monkey kidney cells with harvest of the culture medium containing viral particles only 48 h later.

Recently, Poznansky et al. (12) have described a defective HIV system similar to that which we have independently developed. Both systems are based on transient expression of HIV packaging functions in Cos cells cotransfected with a separate vector capable of generating a packagable HIV genome containing the neo^R gene. The major difference between the two systems is in the design of the expression vectors. Poznansky et al. (12) used two separate plasmids, one containing the gag and pol region and a second containing the envelope region, driven in each case by the promoter within the 5′ HIV LTR. RNA processing signals were derived from the SV40 genome. Generation of replication competent infectious HIV is highly improbable in this system and indeed could not be detected using susceptible T cells as a target. We achieved safety with respect to generation of infectious HIV by eliminating overlapping regions from the packaging and gene transfer vectors and by using a CMV promoter rather than the HIV LTR.

The promoter sequences of the recombinant HIV vector are inactive after gene transfer into target cells (Fig. 3 *B*). Inactivation reflects the lack of TAT, the transacting protein required for RNA production from the HIV promoter (25). Self-inactivating Moloney based vectors have been derived by mutation of the 3′ LTR in an effort to minimize promoter and/or enhancer interference that compromises expression of transcriptional units located between the LTRs after proviral integration (26–28). One advantage of the HIV recombinant system for the study of gene regulation may be the natural silencing of the LTR in target cells. The host range of the recombinant HIV virus can be extended by expression of CD4 in desired targets as we have shown.

T-lymphocytes are an important potential target for thera-

peutic gene transfer both in vitro and in vivo. Treatment of acquired immunodeficiency syndrome has been proposed by expression of antiviral sequences in lymphocytes to produce a state of intracellular immunization (10). The first attempt at human gene therapy has involved the introduction of the human adenosine deaminase gene into T-lymphocytes from a patient with severe combined immunodeficiency syndrome (29). Insertion and expression of anti-tumor cytokines in tumor infiltrating lymphocytes has been proposed as a treatment modality for cancer (6). The recombinant HIV system that we have developed may be useful for these therapeutic applications.

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