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Analysis in Human Immunodeficiency Virus Type 1 Vectors of *cis*-Acting Sequences That Affect Gene Transfer into Human Lymphocytes

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Human immunodeficiency virus type 1 (HIV-1) can be used to generate recombinant viral vectors for delivery of heterologous genes to human CD4-positive lymphocytes. To define the *cis*-acting sequences required for efficient gene transfer, a number of HIV-1 vectors containing a previously identified packaging signal, long terminal repeats, and additional *gag*, *pol*, and *env* viral sequences were designed. By providing the viral proteins in *trans*, recombinant viruses were generated and analyzed for their abilities to transfer genes into human T lymphocytes. Inclusion of up to 653 nucleotides derived from the 5′ end of the *gag* gene in the vector improved the efficiency of gene transfer, but inclusion of additional *gag* or *pol* sequences did not further improve this efficiency. The increased efficiency of gene transfer associated with the inclusion of 5′ *gag* sequences in the vector arose, at least in part, from an increase in the packaging of vector RNA. The presence of the Rev-responsive element (RRE) increased the efficiency of transfer of vectors containing significant lengths of *gag* sequence, as expected from the Rev requirement for nucleus-to-cytoplasm transport of unspliced vector RNA containing intact packaging signals. However, the presence of a RRE did not affect the transfer efficiency of smaller vectors lacking significant lengths of *gag* sequences, arguing against a specific role for the RRE in packaging or vector transfer. These results contribute to an understanding of the minimal *cis*-acting sequences that operate in the context of HIV-1 vectors for delivering genes into human lymphocytes.

The human immunodeficiency virus type 1 (HIV-1), the etiologic agent of AIDS (3, 7, 20), is a lentivirus. One feature that distinguishes the lentiviruses from most cancer-causing retroviruses is the complexity of the viral genome. In common with all replication-competent retroviruses, the HIV-1 genome contains the *gag*, *pro*, *pol*, and *env* genes, which encode the core proteins, the virion-associated enzymes, and the envelope glycoproteins, respectively, flanked by the regulatory long terminal repeats (LTRs) (13). The LTRs include *cis*-acting sequences required for integration, transcription, and polyadenylation. HIV-1 also possesses accessory genes: *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* (25). Concerted actions of these additional genes allow modulation of viral replication. It is likely that the complex genomic organization of HIV-1 together with the ability to differentially regulate viral gene expression in a temporal manner contributes to the virus' ability to establish chronic, persistent infections.

Persistent HIV-1 infection is characterized by the slow depletion of CD4-positive T lymphocytes (3, 18, 20). HIV-1 infects CD4-bearing cells (31). This tropism is mediated by the specific high-affinity interaction between the gp120 viral envelope glycoprotein and the CD4 molecule (16, 30, 36, 40). Other potential HIV-1 target cells include the resting cells of the macrophage/monocyte lineage (21, 28).

Somatic cell gene therapy is a rapidly developing therapeutic approach proposed for the treatment of genetic disorders, cancer, and infectious diseases. Retroviral vectors have been adapted as suitable vehicles for delivering genes into eukaryotic cells, since these cells allow efficient expression of the gene to be transferred as well as its stable integration into the chromosomal DNA of the target cells. Commonly used retroviral vectors are based on the avian leukosis viruses or the mouse retrovirus, Moloney murine leukemia virus (2, 5, 8, 11, 42, 56). The relatively well understood biology of these retroviruses has made possible the development of efficient vector systems. Packaging cell lines which provide in *trans* all the proteins required for viral particle formation have been established (14, 39, 41, 50). These cells are able to package high titers of replication-defective vectors.

The chronicity and slow pathogenicity of HIV-1 infection make it a candidate viral infection that might be combated by gene therapy. The practical use of gene therapy to treat HIV-1-infected individuals will require an efficient means to deliver antiviral genes to relevant target cells. One approach to this problem is the development of HIV-1-based retroviral vector systems. The properties of HIV-1 vectors that may prove useful in this regard are (i) specific cell targeting, regulated by the gp120-CD4 interaction; (ii) HIV-specific inducibility of the HIV LTR; (iii) the ability of HIV-1 to integrate into the chromosomes of nondividing cells (33, 57); and (iv) the potential for efficient expression of multiple gene products. Replication-defective HIV-1 vectors have been previously used to study different aspects of the HIV-1 life cycle (9, 27, 43, 45). These vectors can be propagated when viral proteins are provided in *trans*, resulting in a single cycle of replication. To date, an HIV-1-based vector system with an efficiency of gene transfer comparable to that achieved by the Moloney murine leukemia virus-based system has not been developed. Such development will require a definition of the

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optimal placement of viral *cis*-acting sequences within the HIV-1 vector. There is still considerable uncertainty about the extent of the HIV-1 sequences required for optimal packaging, for example. Although part of the HIV-1 packaging signal is located between the major splice donor and the *gag* initiation codon (1, 12, 26, 32), *env* sequences overlapping the Rev-responsive element (RRE) have recently been suggested to contribute to the packaging function (48).

To investigate the contribution of *cis*-acting sequences in the *gag*, *pol*, and *env* genes to efficient gene transfer, a number of HIV-1 vectors were designed. In this paper, we report on the ability of these vectors to be packaged into virions and to mediate gene transfer and stable integration into human T-lymphocyte cell lines.

MATERIALS AND METHODS

Plasmids. The packaging system and vectors utilized in this study are depicted in Fig. 1. All HIV-1-related sequences were derived from the HXBc2 provirus (46). Sequences are numbered as by Ratner et al. (46), where +1 represents the cap site of the viral mRNA. The simian virus 40 (SV40) origin of replication is included in all plasmids, to allow plasmid propagation in COS-1 cells, which constitutively express the SV40 T antigen. In all HIV-1 vectors, polyadenylation signals are provided by sequences within the 3′ HIV-1 LTR.

Packaging system (Fig. 1A). The CMVΔP1ΔenvpA plasmid expresses the HIV-1 *gag*, *pol*, *vif*, and *tat* genes and was derived by replacement of the 5′ HIV-1 LTR on the previously described HXBΔP1Δenv plasmid (45) with the cytomegalovirus (CMV) immediate-early promoter. The HXBΔP1Δenv plasmid was partially digested with *Cla*I, which recognizes a site in the sequences flanking the 5′ LTR, and *Bss*HII (nucleotide 257, located approximately 80 bp 3′ of the LTR). After treatment with the Klenow fragment of DNA polymerase, *Xba*I linkers were ligated to the blunt ends. A 0.7-kb *Spe*I fragment derived from pcDNA I Neo (Invitrogen) that contains the CMV promoter, T7 promoter, and part of the polylinker was inserted into the *Xba*I site to create the CMVΔP1ΔenvpA plasmid.

The pSVIIIenv3-2 plasmid expresses the HIV-1 *rev* and *env* genes under the control of HIV-1 LTR sequences from −167 to +80 (53, 54). Both the CMVΔP1ΔenvpA and pSVIIIenv3-2 plasmids contain polyadenylation signals derived from SV40 and lack sequences shown to be important for efficient HIV-1 packaging (1, 32).

Vectors (Fig. 1B). The selectable marker (*neo*) in all vectors used herein encodes neomycin phosphotransferase and is located within a 0.65-kb *Bam*HI fragment containing the SL3-3 U3 LTR sequences positioned 5′ to a 1.3-kb *Sal*I fragment derived from the Loren plasmid. The Loren plasmid is a derivative of the BAG vector (45a) and was a gift from C. Cepko, Harvard Medical School. All vector plasmids contain complete 5′ LTRs and leader sequences between the 5′ LTR and *gag* gene initiation codon (nucleotide 336). Some of the vectors contain *gag* and *pol* sequences, with the number of included nucleotides appearing in the name of the vector. Thus, the v40 RSN and v40 SN vectors contain 40 *gag* bp up to the *Cla*I site (nucleotide 376), the v653 RSN and v653 SN vectors contain *gag* sequences up to the *Sph*I site (nucleotide 989), the v1216 RSN vector contains *gag* sequences up to the *Apa*I site (nucleotide 1552), the v1864 RSN vector contains *gag* and *pol* sequences up to the *Msc*I site (nucleotide 2200), and the v2731 RSN vector contains *gag* and *pol* sequences up to the *Pflm*I site (nucleotide 3067). All of the vectors with R in the name contain a HIV-1 *env* fragment from *Bgl*II (nucleotide 7199) to *Bam*HI (nucleotide 8053) inclusive of the RRE. The sequences between the *neo* gene and the 3′ LTR in the vectors are derived from the HXBc2 provirus and extend from *Bam*HI (nucleotide 8053) in the v653 RSN, v653 SN, v1216 RSN, v1864 RSN, and v2731 RSN vectors and from *Kpn*I (nucleotide 8593) in the v0 RSN, v0 SN, v40 RSN, and v40 SN vectors, to the boundary of the LTR. Further details of the construction of the vectors are available from the authors on request.

Recombinant virus production and transduction. COS-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and were plated at a seeding concentration of 1.5×10^6 to 2×10^6 cells per 100-mm-diameter dish the day before transfection. Recombinant virus was generated by cotransfection of these cells with the packaging system plasmids along with the vector, by the DEAE-dextran technique (15), and 5 μg of each plasmid. Fresh medium was placed in the COS-1 cell cultures 24 h before the virus was collected. COS-1 supernatants were harvested at approximately 65 h posttransfection and filtered (pore size, 0.45 μm; Millipore), and the reverse transcriptase activity was measured as previously described (47). Equivalent reverse transcriptase units (from 18,000 to 117,000 cpm) of supernatants were used to infect 2.5×10^5 Jurkat cells, which had been seeded into six-well culture plates, in 2.5 to 5 ml of complete medium. To assess the transduction ability of the vectors, 10-fold serial dilutions of the supernatants were applied to each well. Incubation of the recombinant viruses with the Jurkat cells was carried out for 24 h before the culture medium was changed. G418 selection was applied the following day. To this end, Jurkat cells were pelletted, resuspended in complete medium containing G418 (GIBCO) at an active concentration of 0.8 mg/ml, and dispensed into 24-well culture plates at 1×10^5 to 2×10^5 cells per well. Plates were fed every 3 to 4 days. Positive wells containing clusters of live G418-resistant cells were scored up to 45 days postinfection.

To clone the G418-resistant Jurkat cells, the transduced cells were diluted to a concentration of 0.2 viable cells per 100 μl of medium and dispensed into 96-well culture plates. Wells containing single cells were identified by phase-contrast microscopy, and individual cells were expanded in complete medium containing 0.8 mg of G418 per ml.

Measurement of vector RNA in virions and in packaging cells. Cytoplasmic RNA was extracted from COS-1 cells transfected with the packaging system and with different vectors by lysis of the cells 60 h posttransfection in a buffer containing 0.5% Nonidet P-40 and 1,000 U of RNasin (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5% [vol/vol] Nonidet P-40) per ml. Cell nuclei were removed by being spun for 2 min at 4°C in an Eppendorf microcentrifuge, and sodium dodecyl sulfate (SDS) and proteinase K were added to the cytoplasmic extracts to yield final concentrations of 0.2% and 130 μg/ml, respectively. After a 15-min incubation at 37°C, nucleic acids were purified by extraction with phenol-chloroform and stored as an ethanol precipitate (18a). Virion-associated RNA was isolated from particles released into the supernatant of the transfected cultures after a pelleting of the particles through 20% sucrose cushions, and the RNA samples were adjusted for the amount of $p24^{gag}$ present in the pellets as described previously (16a).

For RNase protection analysis, two DNA templates for in vitro synthesis of radiolabeled RNA probes were used. The pTZ18U/MB template (16a) contains a *Mro*I-*Bss*HII fragment from pHXB-SV (nucleotides 308 to 713) in an antisense orientation to a T7 RNA polymerase promoter. The *Mro*I-*Bss*HII fragment represents 5′ LTR and untranslated leader sequences upstream of the HIV-1 major splice donor site.

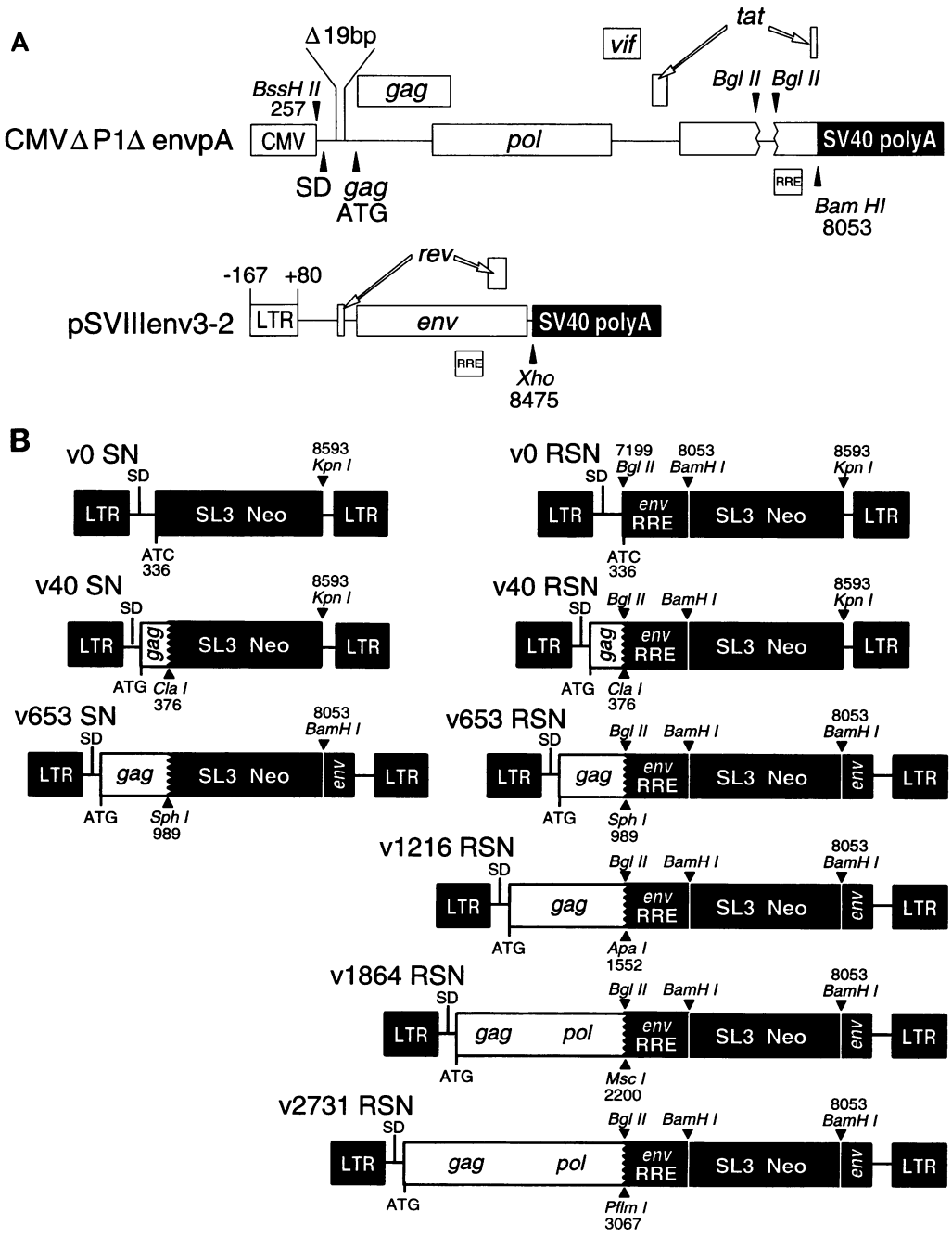

FIG. 1. Structure of the packaging system and vector plasmids. (A) Packaging system. The CMVΔP1ΔenvpA and pSVIIIenv3-2 plasmids are shown. HIV-1 genes that are functionally inactive in the HXBc2 provirus that was used to construct these plasmids are not shown. A segment of the *env* gene is deleted between the *Bgl*II sites in the CMVΔP1ΔenvpA plasmid. (B) HIV-1 vectors. The HIV-1 vectors lacking the RRE are shown on the left, with the corresponding vectors containing the RRE shown on the right. The numbers in the vector names indicate the number of *gag-pol* sequences included in the vector. In the v0 SN and v0 RSN vectors, the SL3-Neo transcription unit has been placed at the *gag* initiation codon, which has been altered to an ATC. Junctions for the other vectors are indicated by the restriction endonuclease site and nucleotide number in the parental provirus. SD, major 5′ splice donor.

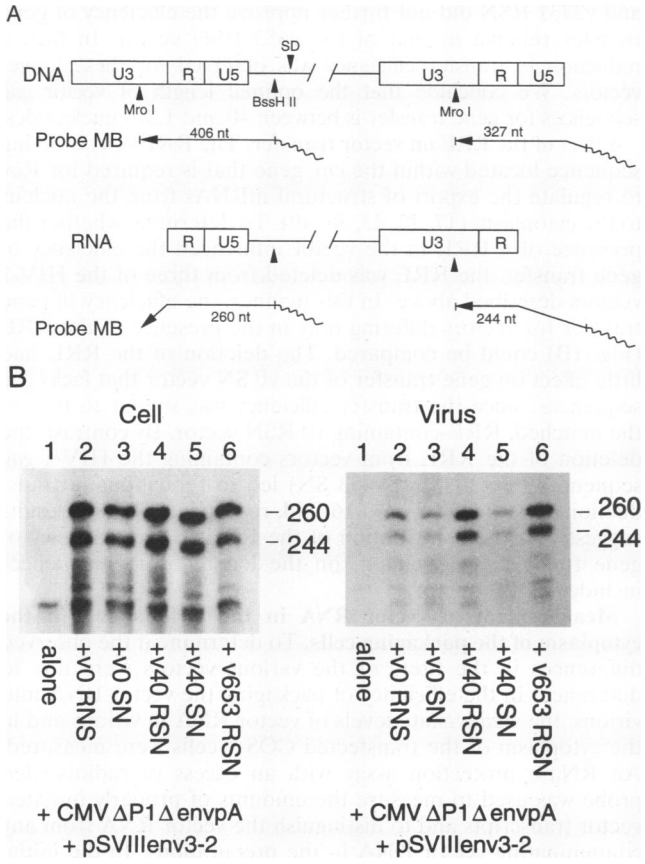

FIG. 2. RNase protection analysis of cytoplasmic and virion RNA. (A) The annealing of the MB probe with both vector DNA and RNA is shown. Annealing of the MB probe with the vector RNA is expected to yield 260- and 244-bp fragments derived from the 5′ and 3′ ends of the RNA, respectively. SD, splice donor. nt, nucleotide. (B) Protection of the 260- and 244-bp fragments by cytoplasmic RNA derived from the packaging cells or by viral RNA. The RNA samples were derived from COS-1 cells transiently transfected with plasmids expressing the packaging system alone (lane 1) or by the packaging system plus V0 RSN (lane 2), V0 SN (lane 3), v40 RSN (lane 4), v40 SN (lane 5), or V653 RSN (lane 6).

Runoff transcripts were made from pTZ18U/MB linearized with *Mro*I by using T7 RNA polymerase to yield the MB probe (Fig. 2A). The MB probe can distinguish between vector RNA and contaminating plasmid DNA but does not distinguish between unspliced and spliced vector RNA. The second probe (MX), which was synthesized from pTZ18U/MX linearized with *Mro*I, covers the major splice donor site upstream of the *gag* gene. The MX probe can therefore distinguish between unspliced and spliced vector RNA. The MX probe is complementary to 335 nucleotides of unspliced and 287 nucleotides of spliced v0 RSN and v0 SN vector RNA and to 355 nucleotides of unspliced and 287 nucleotides of spliced RNA for all other vectors. The pTZ18U/MX template for the synthesis of probe MX was constructed by inserting a *Mro*I-*Xba*I fragment (nucleotides 308 to 815) from pHXB10-LS8/9→SR (16b) into pTZ18U/PL (16a). The pHXB10-LS8/9→SR mutant has a unique *Xba*I site at the position of codons 8 and 9 of the *gag* gene (16b).

Reagents for RNase protection analysis were from a commercially available kit (Ambion, Austin, Tex.). Cytoplasmic RNA (1 μg) or RNA extracted from equivalent amounts of viral particles, as determined by $p24^{gag}$ radioimmunoassay, was incubated with 4×10^5 cpm of ^{32}P-labeled probe in 30 μl of hybridization buffer [80% formamide, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 400 mM NaOAc (pH 6.4), 1 mM EDTA] for 16 h at 45°C. Unhybridized portions of the probe were then degraded by addition of 0.15 U of RNase A and 30 U of RNase T_1 in 300 μl of RNase digestion buffer (Ambion) and incubation for 30 min at 37°C. Protected fragments were precipitated in ethanol, redissolved in RNA loading buffer, and separated in 4.5% polyacrylamide–8.3 M urea gels. For size determination, ^{32}P-labeled RNA size markers were run in parallel. The gels were dried and subjected to autoradiography and analysis with a BetaScope model 603 blot analyzer (Betagen Corporation, Waltham, Mass.).

Southern blotting. Cellular DNA isolation from G418-resistant clones was carried out with a DNA extraction kit (Stratagene). Twenty micrograms of DNA was digested with *Sac*I, subjected to electrophoresis on a 0.8% agarose gel, and transferred to a nitrocellulose filter (Schleicher & Schuell, Inc.) as described previously (55). A *Bam*HI fragment of 2 kb from the v40 SN vector containing the SL3-3 LTR and the neomycin resistance gene was used as a probe, after being labeled with [α-^{32}P]dCTP by a random priming method (19). Approximately 2×10^6 cpm of denatured probe per ml was used in the hybridization reaction mixture (Denhardt's solution, 50% formamide, 1 M NaCl, 50 mM Tris [pH 7.5], 0.1% SDS, 5 mM EDTA, 240 μg of salmon sperm DNA per ml). Hybridization was performed at 37°C for 14 h. Membranes were washed in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% SDS at room temperature for 1 h and then at 65°C for 40 min. Finally, membranes were washed in 0.1× SSC at 65°C for 1 h. All washes were performed with agitation; the buffer was changed every 20 min. Membranes were then air dried and exposed to radiographic film with an intensifying screen.

RESULTS

Packaging and vector genomes: generation of recombinant HIV-1. Almost all retroviral vector systems consist of two components: (i) the packaging component, which provides all the *trans*-acting factors required for the production of viral particles; and (ii) the retroviral vector itself, which carries the gene to be transferred, the packaging signal, and the sequences necessary for reverse transcription, vector integration, and the expression of the gene of interest.

The packaging component in our system has been separated into two different plasmids, one expressing the *gag*, *pol*, *tat*, and *vif* genes, and the other expressing the *env* and *rev* genes, as described elsewhere (45) (Fig. 1A). This system has already been shown to provide *trans*-acting viral functions that permit transfer of an HIV-1 vector without generation of replication-competent virus (45). Previous reports have demonstrated that a region between the 5′ splice site and the initiation codon of the *gag* gene is involved in the packaging of retroviral RNA into virions (1, 12, 26, 32). This packaging signal (ψ) was deleted, and the 3′ LTRs have been replaced by the polyadenylation sequence from SV40 in both of the plasmids used in the packaging system. Furthermore, in the *gag-pol*-expressing plasmid, the 5′ LTR has been replaced by the immediate-early promoter of CMV (Fig. 1A). This replacement has been found to improve the efficiency of gene transfer, even when the number of recombinant virion particles produced in the packaging cells are normalized (44).

The HIV-1 vectors used in this study are shown in Fig. 1B. All of the vectors contained the LTRs, the tRNA primer binding site, the segment encompassing the defined packaging

TABLE 1. Transduction efficiency of HIV-1 vectors in Jurkat lymphocytes

Vector	Log $TCID_{50}$[a]
v0 RSN	0.7 ± 0.4
v0 SN	1.0 ± 0.5
v40 RSN	1.9 ± 0.5
v40 SN	1.2 ± 0.6
v653 RSN	2.2 ± 0.2
v653 SN	1.0 ± 0.5
v1216 RSN	1.1 ± 0.2
v1864 RSN	1.3 ± 0.4
v2731 RSN	1.5 ± 0.2

[a] The number of tissue culture infectious doses ($TCID_{50}$) per 20,000 U of reverse transcriptase activity of recombinant virus in the transfected COS-1 supernatants was determined by 10-fold serial dilutions in duplicate in at least three separate experiments. The values shown were determined by pooling all the results and using the Reed-Muench method (29) for the calculation of $TCID_{50}$. The standard error of the mean is also shown.

signal between the 5′ splice donor and the *gag* gene initiation codon (1, 12, 26, 32), and 3′ genomic sequences between the *Kpn*I site (nucleotide 8593) and the boundary of the 3′ LTR, including the polypurine tract. Specific HIV-1 vectors included additional sequences derived from the *gag*, *pol*, or *env* genes. All vectors utilized the neomycin phosphotransferase (*neo*) gene as a marker that allowed selectable growth of cells in G418-containing medium. The *neo* gene was regulated by the U3 sequences derived from the SL3-3 ecotropic murine leukemia virus. The SL3-3 LTR has been shown to act as a constitutive promoter in human and murine T and B lymphocytes (10, 34). The polyadenylation signal for the *neo* transcript was provided by the 3′ LTR sequences, a feature that distinguishes these vectors from those previously utilized in our laboratory (45).

Effect of inclusion of *gag-pol* sequences on recombinant virus titer. To examine the effect of inclusion of *gag-pol* sequences on transfer of the HIV-1 vectors into T lymphocytes, vectors containing either no *gag-pol* sequences or up to 2,731 bp of *gag-pol* sequence (Fig. 1B) were transfected into COS-1 cells along with the two-part packaging system described above. At 65 h after transfection, equivalent amounts of reverse transcriptase activity from the transfected COS-1 supernatants were incubated with Jurkat T lymphocytes, either undiluted or in 10-fold dilutions. The cells were incubated in selection medium containing G418, and the number of wells containing surviving colonies was counted after 30 to 45 days. No G418-resistant colonies were detected when Jurkat cells were incubated with undiluted supernatants of COS-1 cells transfected with no DNA, with the packaging system alone, or with the vectors in the absence of the packaging components (data not shown).

By contrast, all of the HIV-1 vectors shown in Fig. 1B were able to generate recombinant viruses conferring G418 resistance when cotransfected into COS-1 cells with the packaging components. The titers of recombinant virus, determined by at least three independent experiments, varied among the different vectors (Table 1). The v0 RSN vector, which lacks *gag* sequences, exhibited an average titer of $10^{0.7}$. Increased efficiency of gene transfer was associated with the inclusion of additional *gag* sequences in the vectors. Relative to the v0 RSN vector, the v653 RSN vector exhibited a 32-fold increase in the efficiency of gene transfer, with the average titer for the latter vector being $10^{2.2}$. The inclusion of more than 653 nucleotides of *gag-pol* sequences in the vectors v1216 RSN, v1864 RSN, and v2731 RSN did not further improve the efficiency of gene transfer relative to that of the v653 RSN vector. In fact, a reduction in transfer efficiency was observed for these longer vectors. We conclude that the optimal length of vector *gag* sequences for gene transfer is between 40 and 1,216 nucleotides.

Effect of the RRE on vector transfer. The RRE is a *cis*-acting sequence located within the *env* gene that is required for Rev to regulate the export of structural mRNAs from the nucleus to the cytoplasm (17, 22, 23, 38, 49). To determine whether the presence of a RRE in the vector influenced the efficiency of gene transfer, the RRE was deleted from three of the HIV-1 vectors described above. In this manner, the efficiency of gene transfer for vectors differing only in the presence of the RRE (Fig. 1B) could be compared. The deletion of the RRE had little effect on gene transfer of the v0 SN vector that lacks *gag* sequences, since the transfer efficiency was similar to that of the matched, RRE-containing v0 RSN vector. By contrast, the deletion of the RRE from vectors containing the HIV-1 *gag* sequences (v40 SN and v653 SN) led to reductions in transduction efficiency of 5- and 16-fold, respectively. These results suggest that the contribution of the RRE to the efficiency of gene transfer is dependent on the length of *gag* sequences included in the vector.

Measurement of vector RNA in the virions and in the cytoplasm of the packaging cells. To determine if the observed differences in the titers of the various vectors were due to differences in the efficiency of packaging the vector RNA into virions, the steady-state levels of vector RNA in virions and in the cytoplasm of the transfected COS-1 cells were measured. An RNase protection assay with an excess of radiolabeled probe was used to measure the amounts of properly initiated vector transcripts and to distinguish the vector RNA from any contaminating vector DNA in the preparations. In the initial study, an RNA probe (probe MB) that measured total vector RNA and did not distinguish between spliced and unspliced vector RNAs was used (Fig. 2). While only small differences were observed among the levels of total cytoplasmic RNA for the vectors examined, the level of virion RNA was significantly higher for the v40 RSN and v653 RSN vectors than for the v0 RSN, v0 SN, and v40 SN vectors. The packaging efficiency, estimated by the ratio of virion to cytoplasmic RNA, of the v653 RSN vector was approximately 1.8 times that of the v40 RSN vector, consistent with the relative gene transfer efficiencies of these vectors (Table 2).

The gene transfer efficiencies of the v0 RSN and v0 SN vectors were similar, indicating that in the absence of *gag* sequences in the vector, the presence of the RRE did not increase vector titers. The packaging efficiencies of the v0 RSN and v0 SN vectors were also similar (Fig. 2 and Table 2). By contrast, when *gag* sequences were present in the vectors, the presence of the RRE increased gene transfer efficiency and apparent packaging (compare v40 RSN and v40 SN in Fig. 2 and Table 2). One explanation is that this effect results from the documented ability of Rev to enhance the cytoplasmic levels of unspliced RRE-containing messages. Since only unspliced vector RNA contains packaging signals, differences in the cytoplasmic levels of unspliced vector RNA could account for the differences in titer between v40 RSN and v40 SN or between v653 RSN and v653 SN. Alternatively, the RRE could have a direct effect on packaging that would be manifest only in the presence of other packaging signals in the *gag* gene sequences. To examine these possibilities, an RNase protection assay was performed on the cytoplasmic RNA, with a probe (probe MX) able to distinguish spliced and unspliced vector RNA. The ratio of cytoplasmic unspliced RNA to spliced RNA for each of the vectors examined is shown in

TABLE 2. Cytoplasmic and virion vector RNAs

Vector	Virion RNA/cytoplasmic RNA[a] (relative packaging efficiency[b])	Unspliced/spliced cytoplasmic RNA[c]
v0 RSN	0.08 (1.0)	0.71
v0 SN	0.09 (1.3)	0.86
v40 RSN	0.17 (2.3)	0.86
v40 SN	0.05 (0.7)	0.17
v653 RSN	0.31 (4.1)	0.84

[a] The ratio of virion to cytoplasmic vector RNA was determined by BetaScope analysis of the data in Fig. 2.

[b] Relative packaging efficiency is the total virion/cytoplasmic RNA ratio for each vector divided by that of the v0 RSN vector.

[c] The ratio of unspliced to spliced vector RNA in the cytoplasm of the packaging cells was determined by using the MX probe as described in Materials and Methods. Betascope analysis was used to measure the intensity of the protected fragments corresponding to the unspliced RNA (335 bp in length for the v0 RSN and v0 SN vectors and 355 bp in length for the other vectors) and the spliced RNA (287 bp in length).

Table 2. For the v0 RSN, v0 SN, v40 RSN, and v653 RSN vectors, the ratios of unspliced to spliced cytoplasmic RNA were comparable, approximately 0.8. For the v40 SN vector lacking the RRE, the unspliced/spliced RNA ratio was 0.17. Thus, the poor packaging and gene transfer efficiency of the v40 SN vector appear to be due to the low level of unspliced vector RNA in the cytoplasm. The positive effect of the RRE on gene transfer of *gag*-containing vectors apparently results from the effect of Rev on the accumulation of unspliced, packageable vector RNA in the cytoplasm rather than a direct effect of the RRE on packaging efficiency.

Characterization of transduced clones. An analysis of the genomic DNA from two clones derived by limiting dilution of the G418-resistant Jurkat cells infected with the v653 RSN vector was performed. Total DNA was isolated, digested with *Sac*I, and examined by Southern blotting. An approximately 2.0-kb fragment containing the SL3-3 LTR and *neo* gene was used as a probe. Figure 3 shows that the two expected *Sac*I fragments of 2.4 and 2.2 kb were observed in both clones examined. These fragments were not detected in the control Jurkat cells (data not shown). No other bands were detected in the G418-resistant clones. These results indicate that transfer of the v653 RSN vector was accomplished without gross rearrangements in the cases examined. These results confirm those of previous studies with HIV-1 vectors, which indicate that vector rearrangement occurs very rarely (9, 45, 52).

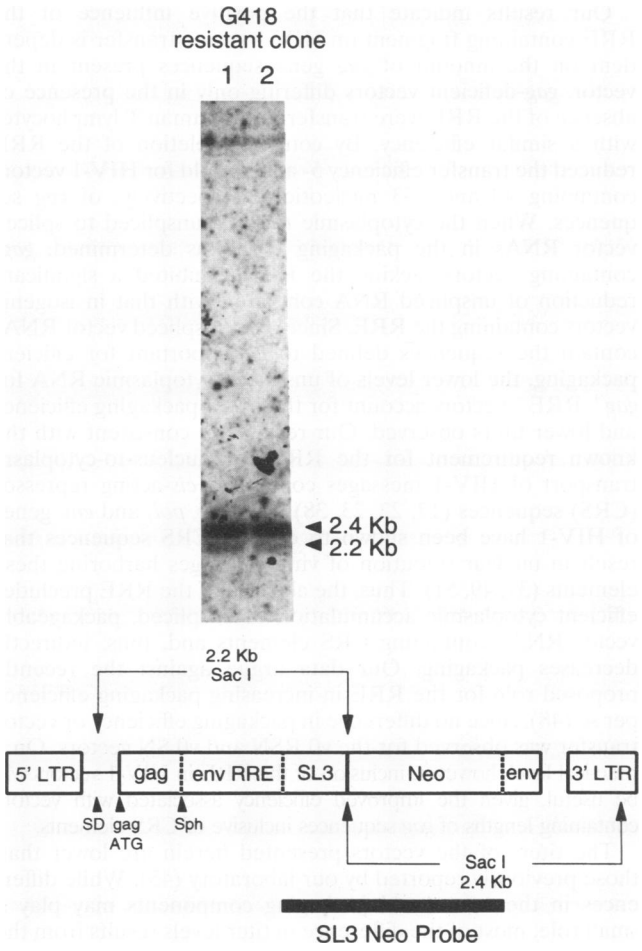

FIG. 3. Southern blot of Jurkat clones expressing the transduced *neo* gene. The position of the probe and the expected fragment size on the basis of the structure of the vector are shown. The DNA in lanes 1 and 2 was derived from independent clones. SD, splice donor.

DISCUSSION

Previous studies have shown that the HIV-1 LTRs and immediate flanking sequences, including those implicated in viral RNA packaging (1, 12, 26, 32), are sufficient for vector transfer into human T lymphocytes. Here we report on the effects of inclusion of additional sequences derived from the *gag*, *pol*, and *env* genes on the transfer efficiency of the vector. Our results with the v0 RSN and v0 SN vectors indicate that HIV-1 *gag* sequences are not absolutely required for the transfer of vectors to target lymphocytes. Inclusion of 40 and 653 nucleotides of the *gag* sequences in the vector increased the efficiency of gene transfer by 16- and 32-fold, respectively. These results are in agreement with those obtained by Buchschacher and Panganiban (9), who also observed increases in the transfer of HIV-1 vectors that included *gag* sequences. Similarly, the inclusion of *gag* sequences in Moloney murine leukemia virus vectors, while not absolutely required for packaging or gene transfer, has been reported to increase viral titers (2, 6). Our results indicate that at least part of the increase in the observed frequency of gene transfer associated with the inclusion of *gag* sequences in the vectors results from an increase in packaging of the vector RNA into virion particles. The presence of the viral *gag* sequences might promote the folding of the RNA secondary structure proposed for the packaging signal (4, 24) or might independently facilitate the interaction of the viral RNA with Gag proteins involved in particle formation. The in vitro binding of purified HIV-1 Gag protein to *gag* gene sequences has been observed (35), which supports the latter possibility. Since the increase in observed titer for vectors containing up to 40 nucleotides of *gag* sequence was greater than the observed increase in packaging efficiency, this region may contribute to functions besides packaging. For example, Gag protein interactions with *gag* sequences may play roles in addition to packaging. Sequences important for the dimerization of the viral RNA, which is important for reverse transcription, have also been mapped to a region including the 5′ end of the HIV-1 *gag* gene (55a).

Inclusion of more than 653 nucleotides of *gag-pol* sequences in the vector did not further improve gene transfer efficiency. In fact, a decrease in transfer efficiency of approximately 5- to 13-fold was observed for the v1216 RSN, v1864 RSN, and v2731 RSN vectors relative to that for the v653 RSN vector. The basis for this decrease is unknown.

Our results indicate that the positive influence of the RRE-containing fragment on HIV-1 vector transfer is dependent on the amount of *gag* gene sequences present in the vector. *gag*-deficient vectors differing only in the presence or absence of the RRE were transferred to human T lymphocytes with a similar efficiency. By contrast, deletion of the RRE reduced the transfer efficiency 5- and 16-fold for HIV-1 vectors containing 40 and 653 nucleotides, respectively, of *gag* sequences. When the cytoplasmic ratio of unspliced to spliced vector RNAs in the packaging cells was determined, *gag*-containing vectors lacking the RRE exhibited a significant reduction of unspliced RNA compared with that in isogenic vectors containing the RRE. Since only unspliced vector RNAs contain the sequences defined to be important for efficient packaging, the lower levels of unspliced cytoplasmic RNA for gag^+ RRE^- vectors account for the lower packaging efficiency and lower titers observed. Our results are consistent with the known requirement for the RRE for nucleus-to-cytoplasm transport of HIV-1 messages containing *cis*-acting repressor (CRS) sequences (17, 22, 23, 38). The *gag*, *pol*, and *env* genes of HIV-1 have been shown to contain CRS sequences that result in nuclear retention of viral messages harboring these elements (37, 49, 51). Thus, the absence of the RRE precludes efficient cytoplasmic accumulation of unspliced, packageable vector RNAs containing CRS elements and, thus, indirectly decreases packaging. Our data argue against the recently proposed role for the RRE in increasing packaging efficiency per se (48), since no difference in packaging efficiency or vector transfer was observed for the v0 RSN and v0 SN vectors. On a practical level, however, inclusion of the RRE in HIV-1 vectors will be useful, given the improved efficiency associated with vectors containing lengths of *gag* sequences inclusive of CRS elements.

The titers of the vectors presented herein are lower than those previously reported by our laboratory (45). While differences in the vector and packaging components may play a small role, most of the difference in titer levels results from the longer period of selection used in this work prior to scoring colonies. We have found that early scoring of colonies following G418 selection can overestimate recombinant virus titer, and we believe that the titers obtained in this study more accurately reflect the number of stable, long-term proviral integration and expression events. While the transient systems used herein produce recombinant viral titers too low to be practical for many purposes, the rapidity of the system has allowed quick assessment of the optimal vector construction. The establishment of stable packaging cell lines should increase the efficiency of production of recombinant viruses for future use.

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