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cis-Acting Sequences Involved in Human Immunodeficiency Virus Type 1 RNA Packaging

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We have previously described a series of human immunodeficiency virus type 1-based vectors in which efficient RNA encapsidation appeared to correlate with the presence of a 1.1-kb *env* gene fragment encompassing the Rev-responsive element (RRE). In this report, we explore in detail the role of the RRE and flanking *env* sequences in vector expression and RNA encapsidation. The analysis of a new series of vectors containing deletions within the *env* fragment failed to identify a discrete packaging signal, although the loss of certain sequences reduced packaging efficiency three- to fourfold. Complete removal of the *env* fragment resulted in a 100-fold decrease in the vector transduction titer but did not abolish RNA encapsidation. We conclude that the RRE and 3′ *env* sequences are not essential for human immunodeficiency virus type 1 vector encapsidation but may be important in vectors in which a heterologous gene has been placed adjacent to the 5′ packaging signal, potentially disrupting its structure.

Retroviral RNA packaging is a specific process involving interactions among *cis*-acting sequences in the RNA genome and viral structural proteins. In human immunodeficiency virus type 1 (HIV-1), an important packaging signal has been located in the 5′ untranslated region (UTR) downstream of the major splice donor, the presence of which is necessary for genomic RNA encapsidation (1, 3, 8). Differing conclusions as to whether this region is sufficient to direct the encapsidation of HIV-1-based vectors containing heterologous genes have been reached. Several groups using a transient COS-1 cell packaging system have reported the successful encapsidation of HIV-1-based vectors which contain only the long terminal repeats (LTRs), the 5′ UTR, and in some cases part of the *gag* gene (2, 6, 11, 12, 17). Using replication-competent helper virus to package HIV-1-based vectors stably expressed in $CD4^+$ T-cell lines, we were unable to demonstrate the packaging of this type of vector, although vectors which contained a 1.1-kb *env* fragment in addition were packaged with high efficiency (13). This system has the advantages of simulating expression of stably integrated provirus and rigorously excluding any possibility of DNA-mediated gene transfer, which is a problem associated with the high levels of plasmid DNA present in transient transfection systems. In addition, packaging of retroviral RNA varies significantly among different cell lines (unpublished observations).

To further define the role of the Rev-responsive element (RRE) and flanking sequences in vector RNA expression and encapsidation, we constructed a series of vectors containing deletions in the 3′ *env* sequence and studied the effects of these on RNA encapsidation by a wild-type helper virus. All vectors were derived from HXBc2, an infectious proviral clone of the human T-cell leukemia virus (HTLV) IIIB isolate (4), and contain a simian virus 40 origin of replication. Restriction sites, where given, refer to positions in the HXBc2 genome (Los Alamos database numbering, in which position 1 is the first base of the 5′ LTR). The vectors are illustrated in Fig. 1. The construction of LRPL has previously been described (13). Briefly, it contains a puromycin acetyltransferase gene (*puro*) (10) inserted at a position analogous to that of the *nef* gene, between an introduced *Not*I site (8740) (20) and an *Xho*I site (8897). The *puro* gene is expressed from the 5′ LTR as a spliced transcript. The sequences between *Cla*I (830) and *Bgl*II (7621), encoding *gag*, *pol*, and the 5′ part of *env*, have been deleted. The second *tat* and *rev* exons and the RRE are retained. A series of deletions based on LRPL were constructed as follows. *Xba*I and *Sal*I sites were introduced at either end of the RRE, at positions 7704 and 8063, respectively, by oligonucleotide-directed mutagenesis (7). The sequences of the mutagenic oligonucleotides were 5′ GGGTGCTACTTCTAGAG GTTCAATTTTTAC 3′ (*Xba*I) and 5′ CCAGAGATTTATTA GTCGACCTAGCATTCC 3′ (*Sal*I). The introduction of each mutation was confirmed by restriction analysis and DNA sequencing. Deletions spanning the 1.1-kb *env* gene fragment in LRPL were constructed by removing sequences between various restriction sites (Fig. 1). Vector plasmids containing the *puro* selectable marker were transfected into the $CD4^+$ T-cell line Jurkat-*tat* (14) by electroporation. Stable cell lines were selected by the addition of puromycin (0.5 μg/ml) to the culture medium.

The vector transduction and RNA encapsidation levels of the vectors shown in Fig. 1 were determined following infection of the vector lines with the HIV-1 isolate HTLV IIIB. The progeny virus was harvested 7 days later and filtered through a 0.45-μm-pore-size membrane. Vector transduction titers and RNA encapsidation levels were determined as follows. For vector titration on HeLa T4 cells (9), 100-mm-diameter petri dishes seeded with 5×10^5 cells were infected with dilutions of virus. Puromycin selection (0.75 μg/ml) was applied 24 h after infection. Puromycin-resistant colonies were counted 10 days later, after the cells were fixed in 4% formol saline and stained with 0.1% toluidine blue. To quantitate the levels of vector RNA packaging, the amounts of vector RNA and genomic helper virus RNA in virion particles from HTLV IIIB-infected vector lines were compared. Virion RNA was extracted and

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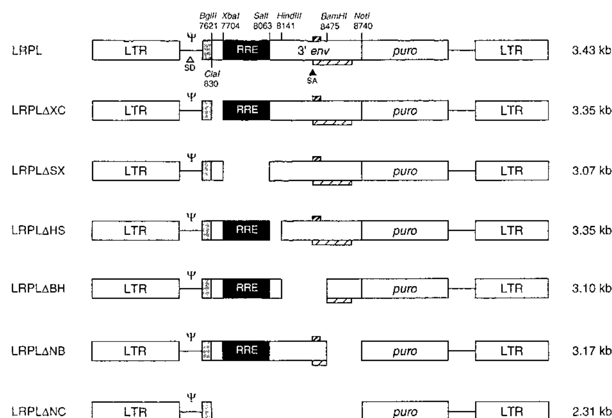

FIG. 1. HIV-1 vectors. All vectors contain the 5′ and 3′ LTRs, the 5′ UTR, and the first 43 bp of *gag*. The stippled boxes show *gag* sequences. The major splice donor (SD) and the *tat* and *rev* splice acceptors (SA) are marked. The RRE is indicated. Hatched boxes indicate the second exons of *tat* and *rev*.

normalized for reverse transcriptase activity, and duplicate slot blots were prepared and hybridized with vector-specific (*puro*) or helper virus-specific (*pol*) probes as previously described (13). Bound probe was measured with an Instant Imager (Packard), which measures radioactivity in real time. To overcome differences in the lengths and specific activities of the two probes, a reference sample in which the stoichiometry of *puro*- to *pol*-hybridizing sequences is 1:1 was included. The reference sample consisted of RNA from a puromycin-resistant HIV-1 isolate containing the *puro* gene in place of *nef*. The vector encapsidation level, expressed as a percentage of the wild-type helper virus level, was calculated with the following formula:

$$\frac{\text{PURO cpm of sample}}{\text{POL cpm of sample}} \Big/ \frac{\text{PURO cpm of reference}}{\text{POL cpm of reference}} \times 100$$

The results are shown in Table 1. All of the vectors were able to transduce target cells expressing the CD4 molecule; however, the efficiency of LRPLΔNC was reduced 100-fold. The levels of vector RNA encapsidation (Fig. 2 and Table 1) were comparable to those of the parental vector, LRPL, with the exception of LRPLΔNC. Possible reasons for the low level of LRPLΔNC encapsidation are discussed below. Vectors based on LRPL containing either the introduced *Xba*I or *Sal*I restriction site, or both, were able to transduce $CD4^+$ target cells with titers comparable to that of the parental vector (data not shown). The packaging efficiency of some vectors was reduced up to fourfold; however, none of the *env* sequences deleted

TABLE 1. Vector transduction titers and encapsidation levels

Vector	HeLa T4 (CFU/ml)[a]	Encapsidation level (%)[b]
LRPL	4.47×10^3	6.8
LRPLΔXC	1.33×10^3	3.1
LRPLΔBH	3.04×10^3	1.8
LRPLΔHS	1.05×10^4	12.0
LRPLΔSX	1.06×10^3	3.1
LRPLΔNB	3.37×10^3	3.6
LRPLΔNC	1.30×10^1	1.6

[a] Normalized for a reverse transcriptase activity of 10^4 cpm/μl. The results shown are means of three or more experiments.
[b] Relative to the level of wild-type virus.

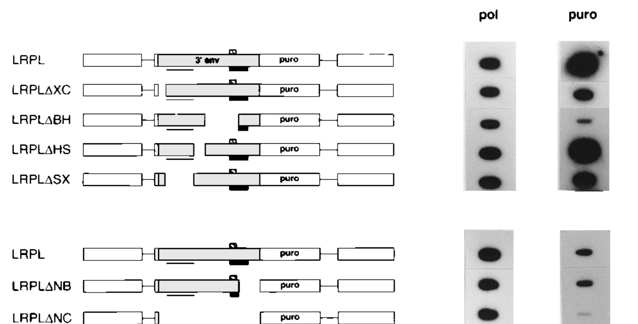

FIG. 2. Vector RNA encapsidation. Virus produced from HTLV IIIB-infected vector lines was harvested, and the virion RNA was hybridized with *puro* or *pol* gene probes.

from LRPL appeared to be essential for vector RNA encapsidation.

While quantitation of vector RNA packaging as described above indicates whether the vectors are packageable, such data do not provide information regarding the relative packaging efficiencies of different RNAs. These were determined by estimating the level of full-length (FL) vector RNA in the cells and comparing this with the amount subsequently detected in the virions by slot blot analysis. To compare the relative amounts of FL vector RNA, Northern (RNA) blots of total RNA from HTLV IIIB-infected vector lines were prepared and hybridized with a *puro* gene probe as described previously

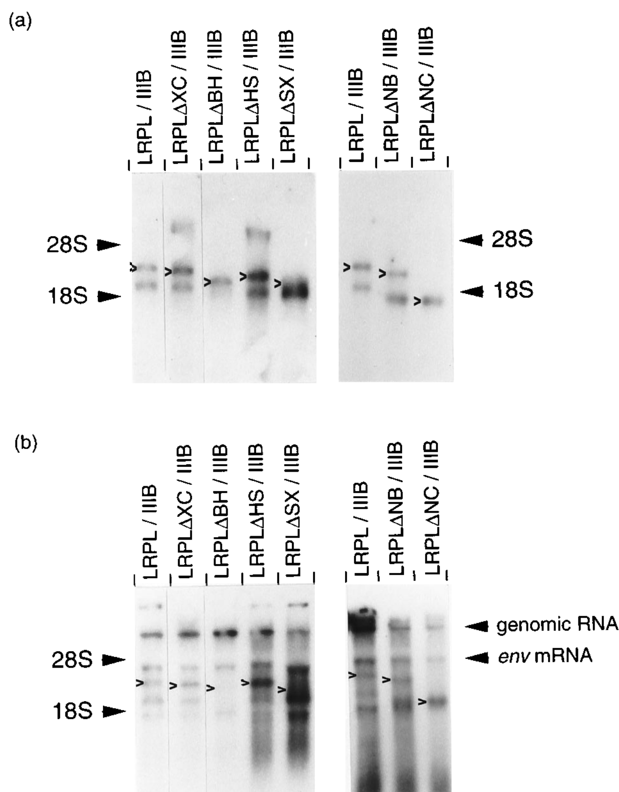

FIG. 3. Comparison of vector RNA expression levels. Total RNA from HTLV IIIB-infected vector lines was hybridized with a *puro* gene probe (a) or an LTR probe (b). Carets indicate the positions of FL vector RNA. The positions of 18S and 28S rRNAs are marked. The positions of genomic RNA and *env* mRNA are indicated.

TABLE 2. Vector expression and packaging efficiencies

Vector	Relative abundance of FL vector[a]	Encapsidation level[b]	Packaging efficiency
HTLV IIIB	100	100	1.0
LRPL	59.2	6.8	0.12
LRPLΔXC	41.7	3.1	0.07
LRPLΔBH	16.2	1.8	0.11
LRPLΔHS	81.4	12.0	0.15
LRPLΔSX	ND[c]	3.1	ND
LRPLΔNB	110	3.6	0.03
LRPLΔNC	141	1.6	0.01

[a] Expressed as a percentage of the amount of FL helper virus RNA in the infected vector lines.
[b] Expressed as a percentage of the amount of helper virus RNA present in the virions.
[c] ND, not determined.

(13) (Fig. 3a). Hybridization with an LTR probe allowed the relative levels of vector and HTLV IIIB genomic RNA to be directly compared (Fig. 3b). The packaging efficiency of the wild-type genome was assigned a value of 1.0, and the packaging efficiencies of FL vector RNAs were calculated with the following formula:

$$\frac{\text{amount of FL vector packaged}}{\text{amount of HTLV IIIB genome packaged}} \Big/ \frac{\text{amount of FL vector in the cell}}{\text{amount of HTLV IIIB genome in the cell}}$$

The results are shown in Table 2. The packaging efficiencies of the vectors containing deletions in the 1.1-kb *env* gene sequence are comparable to that of the parental vector LRPL. The variation in the RNA encapsidation levels (shown in Fig. 2 and Table 1) appears to be due to variations in the level of FL vector RNA expressed in the helper virus-infected cells (Fig. 3). Deletion of the RRE in the vector LRPLΔSX and other vectors lacking the RRE (data not shown) did not result in a reduction in vector RNA expression and encapsidation, indicating that in the absence of *cis*-repressive sequences (15), the RRE is not absolutely required for efficient expression and encapsidation of FL vector RNA, although the lack of the RRE may have led to increased splicing. The relative abundance of FL LRPLΔSX was not determined, as it was not possible to distinguish between FL and spliced vector RNAs by the method used. The vector in which the entire *env* gene has been deleted, LRPLΔNC, was transduced at very low levels and packaged with 10-fold lower efficiency than that of the parent vector, despite the relatively high level of FL vector RNA available for packaging. While it is not essential for RNA encapsidation, the *env* fragment does appear to contribute to efficient RNA packaging, and part of its effect may be due to the sequences acting as a spacer between the heterologous gene and the 5′ packaging signal.

The role of the 5′ UTR in directing encapsidation of HIV-1 vectors based on LRPL was examined by a quantitative RNase protection assay. Transient cotransfection of COS-1 cells with vector and helper virus constructs was used to compare encapsidation of LRPL and a construct lacking the 5′ UTR, pSVII-I*env*3-2. This plasmid has been previously described (18, 19). It contains the HIV-1 *rev* and *env* genes (nucleotides [nt] 5496 to 8897) under the control of 5′ LTR sequences (nt 288 to 535, corresponding to −167 to +80 with respect to the RNA start site), and the 3′ LTR is replaced by simian virus 40 polyadenylation sequences. A *gag-pol-env* expressor was used as a helper virus for the cotransfection assay. pBCCX-CSF (a kind gift from Alan Cann) contains HIV-1 JR-CSF sequences 640 to 8915 encoding the *gag*, *pol*, and *env* genes; the 5′ and 3′ LTRs have been replaced by human cytomegalovirus immediate early promoter and polyadenylation sequences, respectively. COS-1 cells were transiently transfected with vector and helper virus constructs by a DEAE-dextran transfection protocol (16). Cells and supernatants were harvested 48 h later. Cytoplasmic RNA was prepared by a standard protocol (5). For RNA extraction from virions, particles were purified from tissue culture supernatant through 20% sucrose cushions as previously described (13). Virus particles were lysed in proteinase K buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 10 mM EDTA, 1% sodium dodecyl sulfate, 100 μg of proteinase K per ml, and 100 μg of tRNA per ml) for 30 min at 37°C. After two phenol-chloroform extractions and one chloroform extraction, the RNA was precipitated with ethanol and stored at −70°C.

A DNA template, KSIIψCS, containing *Sca*I (313)-to-*Cla*I (830) sequences of HXBc2 inserted into the *Eco*RV and *Cla*I sites in the polylinker of Bluescript KSII (Stratagene) was used for synthesis of radiolabelled RNA probes. KSIIψCS was linearized with *Xba*I, and ^{32}P-labelled antisense riboprobes were synthesized with T3 RNA polymerase by using an in vitro transcription kit (Promega). Reagents for RNase protection assays were obtained from a commercially available kit (Ambion, Austin, Tex.). Cytoplasmic RNA or RNA extracted from pelleted particles, normalized for reverse transcriptase activity, was analyzed by RNase protection assay according to the manufacturer's recommended protocol. For size determination, ^{32}P-labelled RNA markers, synthesized with an RNA Century Marker template set (Ambion), were run in parallel. The predicted sizes of the protected fragments are shown in Fig. 4.

The results of a typical RNase protection assay are shown in Fig. 5. The input probe is of the expected size (Fig. 5, lane 8), and no signal is detected with the control tRNA (lane 7). Helper virus (pBCCX-CSF) bands are of the predicted sizes (Nar1-SD, 100 nt, and Nar1-*gag*, 170 nt). A fragment corresponding to FL LRPL vector RNA (375 nt) was detected in cytoplasmic RNA (Fig. 5, lane 2). Spliced RNA (289 nt) and RNA corresponding to the 3′ LTR (238 nt) were also detected (Fig. 5, lane 2). FL vector RNA was detected in particles released from cells transfected with LRPL and helper virus constructs (Fig. 5, lane 5). The level of FL vector RNA compared with that of spliced RNA was enriched in the particles by comparison with the levels present in the cells, indicating the

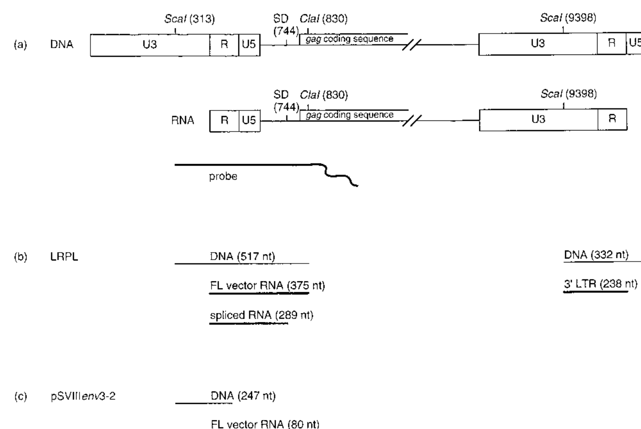

FIG. 4. Predicted sizes of protected fragments for the RNase protection assay. (a) KSIIψCS riboprobe is complementary to HIV-1 nt 313 to 830. (b) The predicted sizes of protected fragments for vector LRPL. (c) The predicted sizes of protected fragments for vector pSVIII*env*3-2.

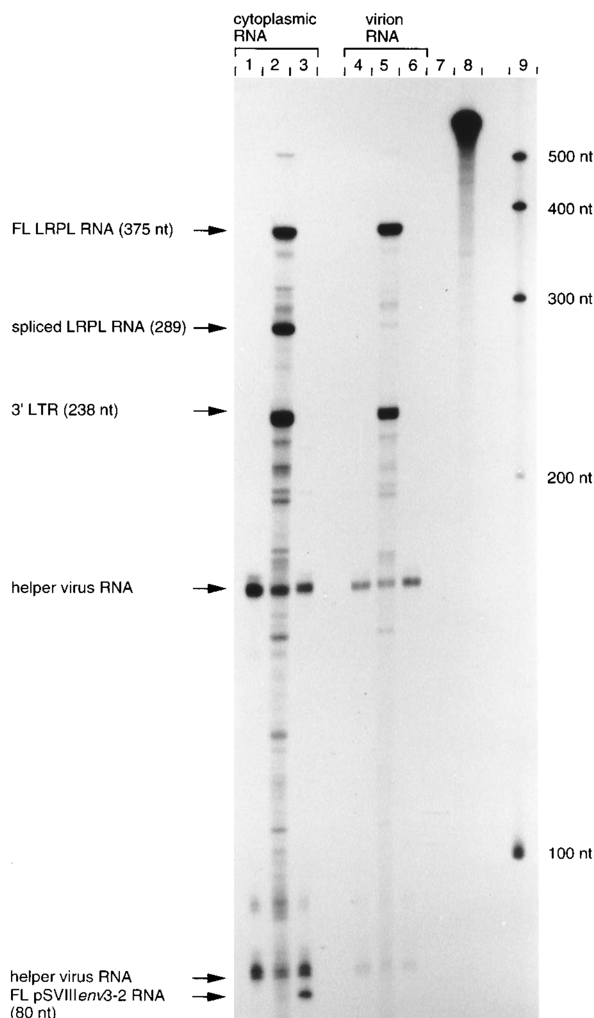

FIG. 5. Packaging of RNA into virions released from transfected COS-1 cells. Shown is an autoradiogram of gel-separated fragments of ^{32}P-labelled riboprobe KSIIψCS resulting from RNase protection with cytoplasmic RNA (lanes 1 to 3) or virion RNA (lanes 4 to 6) prepared from COS-1 cells cotransfected with LRPL and pBCCX-CSF (lanes 2 and 5), pSVIII*env*3-2 and pBCCX-CSF (lanes 3 and 6), or pBCCX-CSF alone (lanes 1 and 4). Protection with tRNA alone is shown in lane 7, and riboprobe without RNase treatment is shown in lane 8. ^{32}P-labelled RNA size markers are shown in lane 9. Fragment sizes are indicated at the left side of the panel. The positions of the protected helper virus fragments are indicated.

specificity of encapsidation of RNAs containing the 5′ ψ sequence. A fragment corresponding to the FL pSVIII*env*3-2 vector RNA (80 nt) was detected in cytoplasmic RNA (Fig. 5, lane 3). This fragment was not detected in particles released from cells cotransfected with pSVIII*env*3-2 and helper virus constructs (Fig. 5, lane 6). This result confirms the importance of the previously identified packaging signal in the 5′ UTR for encapsidation of HIV-1 RNA. The failure of the *env* gene sequences in pSVIII*env*3-2 to direct encapsidation of the vector RNA further indicates that *env* sequences are not sufficient to direct encapsidation of HIV-1 RNA.

From previously published work, it was unclear whether the different requirements for packaging vectors in COS cells and T cells reflected cell-specific phenomena or differences between transient and stable vector expression or were related to aspects of vector construction. In this report, we address the latter by characterization of the role of *env* gene sequences in vector RNA expression and encapsidation. Analysis of a series of vectors based on LRPL demonstrated no absolute requirement for the 3′ *env* region for encapsidation, although deletion of the entire region significantly inhibited packaging. That this was not completely abolished is shown by comparison with the negative control pSVIII*env*3-2, which, despite containing the entire *env* gene and being expressed at adequate levels in the cytoplasm, is completely nonpackageable. This also confirms the essential nature of the 5′ UTR in encapsidation. We previously reported that when a heterologous gene is placed near the 5′ UTR packaging signal, encapsidation is reduced or abolished (13). The findings reported here are in agreement with this. Point mutation of the *gag* ATG also appears to be detrimental. Our previous work demonstrated that some of these very poorly packageable vectors may be rendered packageable by inclusion of 3′ *env* sequences downstream of the heterologous gene. In this paper, we show that this effect is not due to the presence of an essential *cis*-acting packaging signal in *env*, as *env* sequences can be omitted entirely from a packageable vector construct. However, the same region can enhance vector packaging when placed upstream of a heterologous gene. The practical implications of these findings are that it would appear important to include viral sequences between the 5′ packaging signal and any heterologous gene in vectors based on HIV-1. In addition, vector RNA packaging may be enhanced by inclusion of the 3′ *env* region in a vector construct. Studies such as these will aid in the development of HIV-based vectors, which to date have shown relatively low infectious titers, and also in the development of packaging cell lines based on lentiviruses.

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