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-1) 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 NotI site (8740) (20) and an XhoI site (8897). The puro gene is expressed from the 5’ LTR as a spliced transcript. The sequences between Clal (830) and BglII (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. XbaI and SalI 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’ GGTTCAATTTTTAC3’ (XbaI) and 5’ CCAGAGTTATTTA GTCGACCTAGC3’ (SalI). 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-1 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 × 10⁶ cells were infected with diluted 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% formal 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-1 IIIB-infected vector lines were compared. Virion RNA was extracted and
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:

\[
\frac{\text{PURO cpm of sample}}{\text{POL cpm of sample}} / \frac{\text{PURO cpm of reference}}{\text{POL cpm of reference}} \times 100
\]

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 LRPL\textsuperscript{DNC} 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 LRPL\textsuperscript{DNC}. Possible reasons for the low level of LRPL\textsuperscript{DNC} encapsidation are discussed below. Vectors based on LRPL containing either the introduced \textit{XbaI} or \textit{SalI} restriction site, or both, were able to transduce CD4\textsuperscript{+} 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 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.

**TABLE 1. Vector transduction titers and encapsidation levels**

<table>
<thead>
<tr>
<th>Vector</th>
<th>HeLa T4 (CFU/ml)*</th>
<th>Encapsidation level (%)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>LRPL</td>
<td>(4.47 \times 10^3)</td>
<td>6.8</td>
</tr>
<tr>
<td>LRPL\textsuperscript{AXC}</td>
<td>(1.33 \times 10^3)</td>
<td>3.1</td>
</tr>
<tr>
<td>LRPL\textsuperscript{ABH}</td>
<td>(3.04 \times 10^3)</td>
<td>1.8</td>
</tr>
<tr>
<td>LRPL\textsuperscript{AHS}</td>
<td>(1.05 \times 10^4)</td>
<td>12.0</td>
</tr>
<tr>
<td>LRPL\textsuperscript{AXS}</td>
<td>(1.06 \times 10^3)</td>
<td>3.1</td>
</tr>
<tr>
<td>LRPL\textsuperscript{ANB}</td>
<td>(3.37 \times 10^4)</td>
<td>3.6</td>
</tr>
<tr>
<td>LRPL\textsuperscript{ANC}</td>
<td>(1.30 \times 10^4)</td>
<td>1.6</td>
</tr>
</tbody>
</table>

* Normalized for a reverse transcriptase activity of \(10^4\) cpm/μl. The results shown are means of three or more experiments.

* Relative to the level of wild-type virus.
TABLE 2. Vector expression and packaging efficiencies

<table>
<thead>
<tr>
<th>Vector</th>
<th>Relative abundance of FL vectora</th>
<th>Encapsulation levelb</th>
<th>Packaging efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTLV IIB</td>
<td>100</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>LRPL</td>
<td>59.2</td>
<td>6.8</td>
<td>0.12</td>
</tr>
<tr>
<td>LRPLΔXC</td>
<td>41.7</td>
<td>3.1</td>
<td>0.07</td>
</tr>
<tr>
<td>LRPLΔBH</td>
<td>16.2</td>
<td>1.8</td>
<td>0.11</td>
</tr>
<tr>
<td>LRPLΔHS</td>
<td>81.4</td>
<td>12.0</td>
<td>0.15</td>
</tr>
<tr>
<td>LRPLΔSX</td>
<td>NDc</td>
<td>3.1</td>
<td>ND</td>
</tr>
<tr>
<td>LRPLΔNB</td>
<td>110</td>
<td>3.6</td>
<td>0.03</td>
</tr>
<tr>
<td>LRPLΔNC</td>
<td>141</td>
<td>1.6</td>
<td>0.01</td>
</tr>
</tbody>
</table>

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.

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 results of 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 (NarI-SD, 100 nt, and NarI-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

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, KSIII, containing SacI (313)-to-ClaI (830) sequences of HXBc2 inserted into the EcoRV and ClaI sites in the polylinker of Bluescript KSII (Stratagene) was used for synthesis of radiolabelled DNA probes. KSIII was linearized with XbaI, and 52P-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, 32P-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 predicted sizes of protected fragments for the 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 (NarI-SD, 100 nt, and NarI-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
specificity of encapsidation of RNAs containing the 5' ψ sequence. A fragment corresponding to the FL pSVIIIψ3-2 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 pSVIIIψ3-2 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 pSVIIIψ3-2 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 pSVIIIψ3-2, 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.

This work was supported by the Medical Research Council (United Kingdom) AIDS Directed Programme and the Sykes Trust. We thank Jane Greatorex for technical assistance and the Medical Research Council (United Kingdom) AIDS Reagent Project for the cell lines Jurkat-tat and HeLa T4 and the HIV-1 isolate HTLV-IIIB. We thank Alan Cann (University of Leicester) for kindly providing the particle expressor pBCCX-CSF.

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