Human immunodeficiency virus type 2 lentiviral vectors: packaging signal and splice donor in expression and encapsidation

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Retroviral vectors provide the means for gene transfer with long-term expression. The lentivirus subgroup of retroviruses, such as human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2), possesses a number of regulatory and accessory genes and other special elements. These features can be exploited to design vectors for transducing non-dividing as well as dividing cells with the potential for regulated transgene expression. Encapsulation of the transgene RNA in lentiviral vectors is determined by the leader sequence-based multipartite packaging signal. Embedded in the packaging signal is a major splice donor site that, this study shows, is not by itself essential for transgene expression or encapsidation. We designed HIV-2 vectors that contained all the sequence elements thought to be necessary and sufficient for vector RNA encapsidation. Unexpectedly, despite abundant expression, only a small fraction of the transgene RNA was encapsidated and the titre of the vector was low. Redesign of the vector with a mutant splice donor resulted in increased vector RNA encapsidation and yielded vectors with high titre. Inefficient encapsidation by the conventionally designed vector was not due to suboptimal Rev responsive element (RRE)–Rev function. Varying the length of RRE in the vector did not change vector RNA encapsidation, nor did the introduction of a synthetic intron into the mutant vector. The vector RNA with the intact splice donor may have been excessively spliced, decreasing the amount of packageable RNA. A titre of 10^6 transducing units (TU)/ml was readily obtained for vectors with the neo or GFP transgene, and the vector could be concentrated to a titre of 1–5 × 10^7 TU/ml.

Introduction

Human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) possess a number of regulatory and accessory genes and other special elements which can be exploited for designing gene transfer vectors (Naldini et al., 1996; Arya & Gallo, 1996a; Verma & Somia, 1997; Amado & Chen, 1999). These lentiviruses are uniquely able to infect quiescent cells. This is partly because they possess the accessory gene vpr/vpx and the matrix protein with nuclear localization signals. Thus, they provide the means to transduce transgenes into non-dividing as well as dividing cells (Naldini et al., 1996; Akkina et al., 1996; Blomer et al., 1997; Kafri et al., 1997; Zufferey et al., 1997; Mochizuki et al., 1998). The other regulatory genes provide the opportunity to regulate, positively or negatively, transgene expression. Recent design of self-inactivating (SIN) HIV-1 vectors (Miyoshi et al., 1998; Zufferey et al., 1998) and of packaging cell lines (Corbeau et al., 1996; Srinivasakumar et al., 1997; Kaul et al., 1998; Kafri et al., 1999) further enhances the usefulness of these vectors. Moreover, HIV-2-derived vectors may have advantages over HIV-1-derived vectors. The desirable karyophilic nuclear import function of HIV-2 is encoded by the single-function vpx (Fletcher et al., 1996). The same function in HIV-1 is encoded by vpr, which also causes undesirable cell cycle arrest (DiMarzio et al., 1995; Rogel et al., 1995; Mahalingam et al., 1997). If accessory genes of HIV-1 and HIV-2 play a role in their ability to transduce non-dividing cells of certain lineages, HIV-2 vectors may be simpler to design as the cell cycle arrest and nuclear import functions are already segregated in this lentivirus. Furthermore, animal models for testing HIV-2 vectors exist which may not be available for testing HIV-1 vectors. HIV-2 is generally less pathogenic than HIV-1; thus it is safer to handle in the design and production stage and presumably also in clinical practice. For gene therapy of HIV-1 infection, HIV-2 vectors will be

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better in being less likely to generate recombinants with the resident HIV-1 genome because of their sequence divergence. Moreover, HIV-2 may itself down regulate HIV-1 (Arya et al., 1994; Arya & Gallo, 1996b; Al-Harthi et al., 1998; Browning et al., 1999).

Encapsidation of transgene RNA by a retroviral vector is governed by the packaging signal located in the leader sequence. The packaging signal of HIV-1 and HIV-2 is multiparticle with subelements located upstream (exonic) and downstream (intronic) of the splice donor site in the leader sequence (Lever et al., 1989; Aldovini & Young, 1990; Luban & Goff, 1994; Garzino-Demo et al., 1995; McBride & Panganiban, 1996; McCann & Lever, 1997; Arya et al., 1998; Poeschla et al., 1998). Inclusion of the 5’ end of the gag gene is thought to enhance RNA encapsidation (Luban & Goff, 1994; Berkowitz et al., 1995; Parolin et al., 1994; McBride et al., 1997; Miller, 1997). However, gag of HIV-1, and presumably also of HIV-2, contains inhibitory/instability or cis-acting repressive (INS/CRS) sequences. These sequences downregulate expression post-transcriptionally, in part by causing nuclear retention of the transcripts accompanied by their splicing and/or degradation. This negative effect can be overcome by providing the Rev responsive element (RRE) in cis and reo in trans (Maldarelli et al., 1991; Schwartz et al., 1992; Malim & Cullen, 1993; Schneider et al., 1997).

We have previously presented our studies on the mapping of the HIV-2 packaging signal and the requirement for helper virus-free encapsidation (Garzino-Demo et al., 1996; Arya et al., 1998; Sadaie et al., 1998). We report here that despite the provision of the required sequence elements, only a small fraction of the HIV-2 vector RNA was encapsidated by HIV-2. The vector RNA appeared to be spliced before it could be encapsidated. Modification of the splice donor resulted in increased packaging of the vector RNA. Vectors with or without the internal cytomegalovirus (CMV) early gene promoter were competent in transducing target cells. An uncentenrated vector titre of 10^5 transducing units (TU)/ml was readily obtained for vectors containing the neo or the green fluorescent protein (GFP) transgene. Transduction-competent vectors containing a number of other transgenes representing different disease entities and potential target cells have also been created.

Methods

Molecular cloning. A stepwise strategy was used to construct HIV-2 vectors. A minimal vector was created by deleting the central portion (nt 505–8766) of a biologically active proviral clone of HIV-2(ST) (Kumar et al., 1990; Arya & Sadaie, 1993). It was modified by insertion of a synthetic linker to reconstitute the leader sequence up to the gag ATG at nt 548. A segment of 400 nt of the gag along with a synthetic stop placed in-frame immediately after the gag ATG was inserted into this clone. A synthetic linker with multiple cloning sites and a cassette of the marker neo gene linked to the picornavirus internal ribosomal entry site (IRES) was then inserted into this clone downstream of the gag sequence. This vector was further modified by addition of fragments of 300, 528 and 790 nt from the envelope region containing RRE to create vectors pSGT-5(RRE/neo), pSGT-5(RRE2/neo) and pSGT-5(RRE3/neo), respectively. To create the vector pSGT-5(SDM/neo), the splice donor in pSGT-5(RRE/neo) was mutated from G-AAGGTA-A to G-GATATC-A to make it diverge from the consensus. Corresponding clones with a cassette of the GFP gene linked to the CMV promoter were created by replacement of the IRES-neo cassette.

The wild-type HIV-2 (ROD) proviral clone (pROD) and its truncated version pROD(S3D6) have been described previously (Arya et al., 1998). Clone pROD(S3D6) is a deletion mutant of pROD where the subelements of the packaging signal located upstream (nt 300–459) and downstream (nt 482–538) of the splice donor have been deleted, but the splice donor site itself is preserved. It is replication defective, but produces all the proteins needed for packaging (Arya et al., 1998). Clone pROD(S3D6/EM) was created by deleting nt 6370–6640 of the env region from the clone pROD(S3D6) and substituting them with a synthetic linker with multiple stop codons in all three open reading frames in the amino terminus region of the env gene. Clones pCM-ROD(S3D6) and pCM-ROD(S3D6/EM) were derivative clones of pROD(S3D6) and pROD(S3D6/EM) where the 5’-LTR had been substituted with the CMV promoter and the 3’-LTR with a heterologous pA signal. The VSV-G clone was kindly provided by Inder Verma of the Salk Institute (La Jolla, CA, USA) with additional advice from Didier Trono, now at the University of Geneva (Naldini et al., 1996). Flossie Wong-Staal of the University of California, San Diego, kindly provided the HIV-2 vector clone termed pLACG (Poeschla et al., 1998).

DNA-mediated transfection. Human epithelioid 293T cells were transfected by the calcium phosphate protocol (Arya & Gallo, 1988; Arya, 1993). Typically, 1 x 10^5 cells from subconfluent monolayer culture were transfected with 10 µg of the vector DNA and 4–10 µg of the cotransfecting DNA. Cultures were incubated with calcium-DNA aggregates overnight, washed and reincubated with fresh medium. Cells and culture supernatant were harvested 3 days after transfection.

RNA analysis. For cellular RNA, transfected cells were lysed with Trizol reagent (Life Technologies) and RNA was recovered by isopropanol precipitation. RNA was further purified by extraction with phenol–chloroform and re-precipitated with ethanol. It was then digested with DNase in excess and re-extracted and ethanol precipitated (Arya et al., 1998). Cytoplasmic RNA was prepared by lysing the cells with Triton X-100 in a hypotonic buffer and removing the nuclei by centrifugation. The cytoplasmic extract was mixed with Trizol reagent, extracted with chloroform and RNA was precipitated with ethanol. The RNA was further purified by DNase treatment, phenol–chloroform extraction and ethanol precipitation. The recovery of RNA was quantified by absorbance at 260 nm. Viral RNA was prepared from partially purified virus particles. Culture supernatant was layered on a column of 20% (v/v) glycerol in a Beckman SW41 rotor tube and centrifuged at 32,000 r.p.m. for 1.5 h (Arya et al., 1998). The pellet was lysed with Trizol reagent and viral RNA extracted and DNase treated as described above. Final RNA was dissolved in 100 µl of 10 mM Tris–HCl (pH 7.8)–1 mM EDTA.

The abundance of vector RNA was estimated by slot-blot hybridization. Aliquots of cellular RNA (20 µg) or viral RNA (50 µl) were denatured and two dilutions (1:1 and 1:5) were slot-blotted and hybridized with the 3²P-labelled neo probe. For Northern blot analysis, about 20 µg of cellular RNA was electrophoresed in denaturing formaldehyde–agarose gels, transferred to a nylon membrane by electroblotting and blot-hybridized with the neo probe. The filters were exposed to an X-ray film and subsequently to an imaging screen. The abundance of RNA was quantified by integrating the intensity of the bands with a PhosphorImager (Molecular Dynamics). Most results reported here...
represents multiple independent transfections done with cells at different passages.

- **p27 antigen capture assay.** The level of p27 viral antigen was determined with a commercially available ELISA kit (Coulter). Briefly, serial dilutions of the vector sample were lysed and applied to the mouse anti-SIV p27 antibody-coated wells of the microtitre plate. After washing, biotinylated anti-mouse antibody was added to the wells and binding quantified with the streptavidin-conjugated horseradish peroxidase reaction.

- **Transduction and vector titration.** The titre of the vector was determined by transducing 293 or 293T cells. Concentrated vector was obtained by pelleting the particles from the supernatant of the transduced culture by two cycles of ultracentrifugation at 50,000 g for 1 h and resuspension in 1/200–1/500 of the original volume. Serial dilutions of the supernatant with or without prior concentration were applied to the target cells to be transduced. Cultures of about 5 × 10^4 cells were incubated with the serial dilutions of the vector along with 8 μg/ml polybrene for 16–20 h. To score for neo gene expression, cultures were incubated with G418 until there were no viable cells in the control cultures (12–16 days) and the surviving colonies were counted. To score for GFP gene expression, transduced cultures were washed, cells detached from the surface with mild EDTA treatment, fixed with paraformaldehyde and analysed for GFP expression by flow cytometry.

**Results**

**Vector design**

We designed HIV-2 transfer vectors to contain all the cis-acting sequence elements thought to be necessary and sufficient for efficient encapsidation and subsequent transduction. The basic vector contained 5’ and 3’ LTRs and the complete leader sequence with an intact splice donor. In addition, it also contained the first 400 nt of the gag sequence with a stop codon and a frame shift and the IRES–translation of the cloning therapeutic genes upstream, allowing the independent expression of the vector RNA. This vector was included in anticipation of overcoming any negative effect of additional sequences and created the vector pSGT-5(SDM-RRE) which was transcomplemented with either the native envelope (pCM-ENV) or with VSV-G (pCM-VSV-G). Again, while there was an abundant expression of the vector RNA in the cotransfected cultures, the vector RNA was poorly encapsidated (Fig. 2).

The reference vector pSGT-5(RRE/RN) contained 300 nt of envelope sequence corresponding to RRE. With the objective of improving the vector RNA encapsidation, we tested the encapsidation of RNA transcribed by the second vector that contained 528 nt of RRE [pSGT-5(RRE2/RN)]. This vector was thus cotransfected with the helper virus clone pROD(SD36) or with pROD(SD36/EM) transcomplemented with either the native envelope or VSV-G. When the particle and cellular RNAs were analysed by hybridization with the neo probe, intense signals were obtained for the cellular RNAs and only faint signals for the particle RNA (Fig. 2). These results showed that the vector RNA was abundantly expressed in the transduced cells but only a minor amount of it was encapsidated into particles.

Because the vectors contained a strong splice donor within the packaging signal, we wondered if the poor encapsidation was due to the excessive splicing of the vector RNA. We therefore mutated the splice donor away from the consensus sequences and created the vector pSGT-5(SDM/RRE/RN). The results of the transfection of this vector with different helper virus clones are included in Fig. 2. Hybridization of the cellular RNA with the neo probe showed that this vector synthesized abundant quantities of vector RNA in transfected cells and this abundance was roughly equivalent to the RNA synthesized by the reference vector pSGT-5(RRE/RN) or its RRE2 derivative. Apparently, the mutation of the splice donor did not affect the expression of the vector. However, there was a marked effect on encapsidation. The particle RNA gave clear hybridization signals showing that the vector RNA synthesized by the mutant vector was encapsidated. Comparison of the hybridization signals for the particle RNA suggested...
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Fig. 1. For legend see facing page.
HIV-2 lentiviral vectors

Fig. 2. Expression and encapsidation of vector RNA. Vectors carrying the neo transgene were cotransfected into human epithelioid 293T cells with (a) the packaging signal-deleted helper virus clone pROD(SD36) or (b) env-truncated clone pROD(SD36/EM) complemented with the HIV-2 envelope or with (c) VSV-G clone. Culture supernatants were collected, virus particles were partially purified by glycerol gradient centrifugation, and analysed for Neo RNA by slot-blot hybridization with the neo-specific probe. Cells were also analysed for intracellular Neo RNA expression. Band intensities were quantified by PhosphorImager measurements. Blots from a representative experiment are shown. The relative band intensities obtained with the particle RNAs for multiple independent transfections are presented in Table 2.

Table 1. Secretion of viral antigen p27 from the transfected cells

Cultures of 293T cells were cotransfected with the indicated vectors as described in Fig. 2. Supernatants were assayed for viral p27 by the ELISA antigen capture assay and values normalized with respect to the reference vector pSGT-5(RRE/RN). The levels of p27 in the culture medium ranged from 40 to 200 ng/ml, depending on the experiment. They were within a factor of two of each other for the set of vectors in any experiment.

<table>
<thead>
<tr>
<th>Relative p27 for vectors cotransfected with:</th>
<th>pROD(SD36)</th>
<th>pROD(SD36/EM) + pCM-ENV</th>
<th>pROD(SD36/EM) + pCM-VSV-G</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSGT-5(RRE/RN)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>pSGT-5(RRE2/RN)</td>
<td>0.6 ± 0.2</td>
<td>1.1 ± 0.1</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>pSGT-5(SDM/RRE/RN)</td>
<td>1.6 ± 0.4</td>
<td>0.8 ± 0.4</td>
<td>1.2 ± 0.4</td>
</tr>
</tbody>
</table>

Fig. 1. Genetic structure of HIV-2 vectors and helper virus packaging clones. The reference vector, pSGT-5 (RRE/RN), contained the HIV-2(ST) long terminal repeat (LTR), leader sequence (nt 340–548) with a splice donor (SD) at nt 470, the first 400 nt of the gag sequence where the Gag initiator codon is immediately followed by a stop codon and a frame shift, and 300 nt of the envelope region corresponding to the RRE region. It also contained a cassette of the marker neomycin-resistance (neo) gene linked to the picornavirus independent ribosomal entry site (IRES). Vectors designated RRE2 and RRE3 contained 528 nt and 790 nt of the envelope fragment with RRE, respectively. pSGT-5(SDM/RRE/RN) vectors differed from pSGT-5(RRE/RN) in having the splice donor site mutated away from the consensus and are denoted by a cross. pSGT-3(SL) is a minimal vector containing only the LTRs and the leader sequence. Vectors with the marker GFP gene are the corresponding vectors where the IRES–neo cassette has been replaced with the cassette of GFP linked to the CMV promoter (CM–GFP). Helper virus packaging clone pROD(SD36) is a derivative of the replication-competent HIV-2(ROD) provirus where both elements of the packaging signal have been deleted, but the splice donor is preserved. pROD(SD36/EM) is a double mutant with the deletion of the packaging signal and truncation of the envelope gene. The truncation was achieved by introduction of a synthetic stop codon, a short deletion and a frame shift. pCM-ENV and pCM-VSV-G contain the HIV-2 env and the VSV-G gene directed by the CMV promoter.
Table 2. Encapsidation of the Neo vector RNA

Cultures of 293T cells were cotransfected with the indicated vectors. Supernatants were collected and virus particles were purified by glycerol gradient centrifugation. They were lysed, RNA was isolated, slot-blotted and hybridized with the neo probe as described in Fig. 2. Band intensities were quantified by PhosphorImager analysis and normalized with respect to the reference vector pSGT-5(RRE/RN).

<table>
<thead>
<tr>
<th>Vector</th>
<th>Relative Neo RNA encapsidation of vectors cotransfected with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pROD(SD36)</td>
</tr>
<tr>
<td>pSGT-5(RRE/RN)</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>pSGT-5(RRE2/RN)</td>
<td>1 ± 0.2</td>
</tr>
<tr>
<td>pSGT-5(SDM/RRE/RN)</td>
<td>9.3 ± 1</td>
</tr>
</tbody>
</table>

Fig. 3. Cytoplasmic expression and encapsidation of vector RNA. Cultures of human epithelioid 293T cells were cotransfected with the indicated vectors and the packaging clone pROD(SD36); cells were processed for the isolation of the cytoplasmic RNA and supernatant for the particle RNA. The RNAs were hybridized with the neo probe as described in the legend to Fig. 2.

that the mutant vector [pSGT-5(SDM/RRE/RN)] encapsidated 7 to 10 times more RNA than the reference vector [pSGT-5(RRE/RN)] (Table 2). This difference in encapsidation was observed regardless of whether the helper virus carried the envelope in cis [pROD(SD36)] or in trans [pROD(SD36/EM) plus pCM-ENV] or was pseudotyped with VSV-G [pROD(SD36/EM) plus pCM-VSV-G] (Fig. 2, Table 2). Both clones produced similar amounts of virus particles as judged by the supernatant p27 levels (Table 1).

We also analysed the abundance of the vector RNA in the cytoplasmic fraction of the transfected cells (Fig. 3). As with the previous analysis, hybridization of the cytoplasmic RNA from cells transfected with different vectors gave signals of equivalent intensities with the neo probe. The intensity of the hybridization signal of the particle RNA for the mutant vector pSGT-5(SDM/RRE/RN) was about 8- to 10-fold the intensity for the reference vector pSGT-5(RRE/RN). The data displayed in Fig. 3 were obtained with the packaging clone pCM-ROD(SD36). Similar results were obtained with the packaging clones pCM-ROD(SD36/EM) plus pCM-VSV-G.

We then analysed by Northern blot hybridization the nature of the RNA synthesized by the reference and the splice donor mutant vector (Fig. 4). The expected size of the transcript from these vectors is about 3.2–3.5 kb, if no splicing of RNA occurred. The Northern blot analysis showed that the predominant RNA species produced by the reference vector [pSGT-5(RRE/RN)] and its derivative pSGT-5(RRE2/RN) was about 2.0 kb and the minor species was about 3.2–3.5 kb. In contrast, the predominant form of RNA produced by splice donor-mutant vector [pSGT-5(SDM/RRE/RN)] was of the expected size of about 3.5 kb, with only little RNA species of 2.0 kb. However, this vector also produced some 2.5 kb RNA species. The results were consistent with the supposition that
Table 3. Titre of HIV-2 neo-transgene vectors

Serial dilutions of vectors produced by cotransfection of vector and helper plasmids were applied to monolayer cultures of 293 cells. Cultures were incubated in the presence of G418 (0.75 mg/ml), until there were no viable cells in the control untransduced cultures (12–16 days). G418-resistant colonies were counted and the titre computed. Data represent two independent transfections and subsequent titrations.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Helper plasmid</th>
<th>Titre (neo⁶/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSGT-5(RRE/RN)</td>
<td>pROD(SD36/EM1) + VSV-G</td>
<td>1.4 ± 0.7 x 10⁴</td>
</tr>
<tr>
<td>pSGT-5(RRE2/RN)</td>
<td>pROD(SD36/EM1) + VSV-G</td>
<td>1.6 ± 1.0 x 10⁴</td>
</tr>
<tr>
<td>pSGT-5(SDM/RRE/RN)</td>
<td>pROD(SD36/EM1) + VSV-G</td>
<td>3.0 ± 1.0 x 10⁵</td>
</tr>
<tr>
<td>pSGT-3(SL)</td>
<td>pROD(SD36/EM1) + VSV-G</td>
<td>0</td>
</tr>
<tr>
<td>pSGT-5(RRE/RN)</td>
<td>pROD(SD36/EM1) + None</td>
<td>0</td>
</tr>
<tr>
<td>pSGT-5(SDM/RRE/RN)</td>
<td>pROD(SD36/EM1) + None</td>
<td>0</td>
</tr>
<tr>
<td>pSGT-5(RRE/RN)</td>
<td>None + VSV-G</td>
<td>0</td>
</tr>
<tr>
<td>pSGT-5(SDM/RRE/RN)</td>
<td>None + VSV-G</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4. Effect of RRE and synthetic intron on the titre of GFP-transgene vectors

Serial dilutions of vectors produced by cotransfection of the vector and packaging plasmids were applied to monolayer cultures of 293T cells for 14–16 h, washed and incubated for 3 days. Cells were fixed and GFP-expressing cells were analysed by flow cytometry. Data represent two independent experiments. Amount of p27 core protein was estimated by ELISA.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Helper plasmid</th>
<th>Titre (TU/ml)</th>
<th>Titre (TU/ng p27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSGT-5(RRE/CM-GFP)</td>
<td>pCM-ROD(SD36/EM1) + VSV-G</td>
<td>4.2 ± 2.0 x 10⁴</td>
<td>&lt;1 x 10⁵</td>
</tr>
<tr>
<td>pSGT-5(SDM/RRE/CM-GFP)</td>
<td>pCM-ROD(SD36/EM1) + VSV-G</td>
<td>3.2 ± 1.3 x 10⁵</td>
<td>2.1 x 10⁵</td>
</tr>
<tr>
<td>pSGT-5(SDM/RRE2/CM-GFP)</td>
<td>pCM-ROD(SD36/EM1) + VSV-G</td>
<td>4.3 ± 1.7 x 10⁵</td>
<td>0.7 x 10⁵</td>
</tr>
<tr>
<td>pSGT-5(SDM/RRE3/CM-GFP)</td>
<td>pCM-ROD(SD36/EM1) + VSV-G</td>
<td>3.4 ± 2.0 x 10⁵</td>
<td>1.6 x 10⁵</td>
</tr>
<tr>
<td>pSGT-5(SDM/SI/RRE/CM-GFP)</td>
<td>pCM-ROD(SD36/EM1) + VSV-G</td>
<td>2.2 ± 1.2 x 10⁵</td>
<td>1.2 x 10⁵</td>
</tr>
<tr>
<td>pSGT-3(SL)</td>
<td>pCM-ROD(SD36/EM1) + VSV-G</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pSGT-5(RRE/CM-GFP)</td>
<td>pCM-ROD(SD36/EM1) + None</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pSGT-5(SDM/RRE/CM-GFP)</td>
<td>pCM-ROD(SD36/EM1) + None</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pSGT-5(RRE/CM-GFP)</td>
<td>None + VSV-G</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pSGT-5(SDM/CM-GFP)</td>
<td>None + VSV-G</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

A greater fraction of the RNA synthesized by the reference vector pSGT-5(RRE/RN) was being spliced from 3·2 kb species to 2·0 kb. Some of RNA synthesized by the splice donor-mutant vector may have been spliced from 3·5 kb to 2·5 kb.

**Vector-mediated transduction**

To determine if the particles produced by different vectors were competent in transducing target cells, they were titrated on 293 cells as targets and scored for G418-resistant colonies (Table 3). The results showed that the vector produced competent particles and that the titre of the particles produced by the splice donor mutant vector [pSGT-5(SDM/RRE/RN)] was nearly 20-fold higher than the titre of the particles produced by the reference vector [pSGT-5(RRE/RN)]. The ability of the vectors to transduce target cells depended on their being packaged with the helper virus and the envelope clone. In their absence, transduction competent vector was not obtained. This also shows that there was no carry-over of vector DNA from the primary transfection.

To further confirm these observations, and as a prelude to in vivo marking studies, a new set of vectors containing GFP gene as a marker gene was created (see Fig. 1). This set included the reference vector pSGT-5(RRE/CM-GFP), its splice donor mutant pSGT-5(SDM/RRE/CM-GFP) and vectors with different lengths of RRE. An additional vector termed pSGT-5(SDM/SI/RRE/CM-GFP) contained a short synthetic intron of 50 nt downstream of the packaging signal. These vectors were cotransfected with the helper virus clone pCM-ROD(SD36/EM1) and pCM-VSV-G and the resulting vector particles were titrated on 293T cells (Table 4). The results showed that transduction with the splice donor-mutant vector
Table 5. Comparison of HIV-2 GFP vectors of two different designs under identical experimental conditions

<table>
<thead>
<tr>
<th>Vector</th>
<th>Titre (TU/ml)</th>
<th>Titre (TU/ng p27)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unconcentrated</td>
<td>Concentrated</td>
</tr>
<tr>
<td>pLAGC/CM-GFP(←)</td>
<td>$5.8 \pm 0.8 \times 10^3$</td>
<td>$4.3 \pm 0.4 \times 10^3$</td>
</tr>
<tr>
<td>pSGT-5(SDM/RRE/CM-GFP)</td>
<td>$1.1 \pm 0.1 \times 10^3$</td>
<td>$1.3 \pm 0.2 \times 10^3$</td>
</tr>
<tr>
<td>pSGT-5(SL)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

[pSGT-5(SDM/RRE/CM-GFP)] gave a titre that was about 10-fold (range 8–20) higher than the titre of the reference vector with the intact splice donor [pSGT-5(RRE/CM-GFP)]. Increasing the length of RRE did not markedly change the titre. Similarly, the insertion of a synthetic intron into the mutant vector did not have significant effect. All vectors produced roughly equivalent amounts of particles as determined by the supernatant p27 antigen (not shown).

We also compared the titre of the splice donor-mutant vector with that of the HIV-2 vector created by Flossie Wong-Staal and her colleagues. Their vector, termed pLAGC, differs from our vector pSGT-5(SDM/RR/CM-GFP) in that it had intact splice donor and the CMV–GFP cassette was in the antisense configuration relative to the 5’ LTR. In addition, their vector is derived from HIV-2(KR) and ours from HIV-2(ST). When packaged with our helper virus and pseudotyped with VSV-G, pSGT-5(SDM/RR/M-GFP) vector had a titre 15- to 30-fold higher than the titre of pLAGC vector (Table 5).

Discussion

We have previously reported that the cis-acting sequence elements both upstream and downstream of the major splice donor in the leader of HIV-2 were required for encapsidation of vector RNA (Garzino-Demo et al., 1995; Arya et al., 1998). Our reference HIV-2 vector described in this report contained all the sequence elements thought to be necessary and sufficient for efficient encapsidation and competent vector production. Despite the presence of required elements and of abundant expression in transfected cells, the reference vector RNA was poorly encapsidated. This was not due to suboptimal activity of the Rev–RRE axis. The Rev in our helper virus was functional, as it produced p27 core antigen intracellularly and it produced supernatant particles with p27 (Arya et al., 1998). The RRE in our vectors corresponded to the sequence previously shown to be optimal for folding for Rev response (Dillon et al., 1990). Furthermore, increasing its length in the vector did not improve the amount of vector RNA encapsidated or the vector titre.

We then considered the possibility that the vector RNA was being excessively spliced, affecting its packaging. Splicing of viral RNA, a normal process in virus replication, involves the major splice donor in the leader and a number of splice acceptors downstream. It apparently is a controlled process in virus replication where sufficient viral genomic RNA is left unspliced to be packaged for the production of the progeny virus. In our vectors, the splice donor, located within the packaging signal, could utilize a cryptic splice acceptor resulting in reduction of the amount of vector RNA available for packaging. The results with the splice donor mutant vector support this possibility. The titre of the vector with the splice donor mutation was uniformly higher than the titre of the unmutated vector. This was the case for both Neo and GFP vectors. However, other explanations are possible. For HIV-1 and equine infectious anaemia virus, the splice donor itself has been reported to have a negative effect on viral gene expression, which is overcome by Rev–RRE (Tan et al., 1996; Borg et al., 1997). The Rev–RRE was functional in our vectors. Thus, this mechanism may not be important here. In addition, the introduction of a splice-donor as a part of a synthetic intron had only a minimal effect on vector titre. For HIV-1 vectors, others have reported the curious observation that a deletion of the splice donor markedly reduces vector RNA expression but has no effect on vector titre (Cui et al., 1999). One interpretation of this result is that with the HIV-1 vector, RNA is always in excess such that even when its abundance in the packaging cell is reduced because of splice donor mutation, there is no effect on vector RNA encapsidation or titre.

Successful packaging of some HIV-2 vectors with an intact splice donor has been reported previously (McCann & Lever, 1997; Poeschla et al., 1998). We do not know if a modification of the splice donor in those vectors also will not enhance encapsidation and vector titre. When we directly compared the
vector with an intact splice donor provided by F. Wong-Staal and colleagues with our vector with the mutated splice donor, the titre of the vector with the splice donor mutation was 15- to 30-fold higher. Thus, the splice donor mutation was advantageous. Furthermore, in situations where vector mobilization is desired, such as gene therapy of HIV infection, splice donor-mutant vector will have an added advantage. It will curtail vector RNA splicing and promote encapsidation and vector production. This will occur only in HIV-infected cells and not in uninfected cells as only infected cells can provide the packaging machinery in trans.

We think it is important to recognize the significant role of the splice donor in vector design. With a splice donor in place in the vector, a cryptic splice acceptor brought in with a transgene, or sequences surrounding it, will undermine the integrity of the vector and reduce the titre by a log or more. While the importance of a log difference may be debatable, it would seem desirable to take advantage of this observation in designing vectors, especially considering that low titre is one of the major shortcomings of the presently designed lentiviral vectors. With the splice donor mutated, we have generated HIV-2 vectors with a number of transgenes representing several disease models, including aromatic amino acid decarboxylase (AADC) gene for Parkinson’s disease, Bax gene for tumour apoptosis, α-galactosidase A gene (AGA) for Fabry disease and the chemokine (RANTES) gene for HIV infection. These vectors successfully transduced appropriate target cells, thus setting the stage for in vivo studies.

Participation of Ms Heidi Brown, a student at Brown University, and of Ms Priya Kundra, then a student at Duke University and now at the Virginia Commonwealth University School of Medicine, was made possible by an award by the Student Research Training Program (SRTP) of the National Cancer Institute. We acknowledge the participation of Ms Maryam Zamani (then a student at Georgetown University and now Maryam Zamani, MD) as an SRTP awardee and of Ms Taetia Phillips (then a student at the University of the Virgin Islands) as a MARC awardee. We thank Ms Cynthia Ginsberg (a student at Stanford University) and Ms Suzie Chang (a student at the Georgetown University) for assistance with the manuscript.

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Received 25 August 2000; Accepted 12 October 2000