Functional replacement of the R region of simian immunodeficiency virus-based vectors by heterologous elements

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Substitution of lentiviral cis-acting elements by heterologous sequences might allow the safety of lentiviral vectors to be enhanced by reducing the risk of homologous recombinand vector mobilization. Therefore, a substitution and deletion analysis of the R region of simian immunodeficiency virus (SIV)-based vectors was performed and the effect of the modifications on packaging and transfer by SIV and human immunodeficiency virus type 1 (HIV-1) particles was analysed. Deletion of the first 7 nt of R reduced vector titres by 10- to 20-fold, whilst deletion of the entire R region led to vector titres that were 1500-fold lower. Replacement of the R region of SIV-based vectors by HIV-1 or Moloney murine sarcoma virus R regions partially restored vector titres. A non-retroviral cellular sequence was also functional, although to a lesser extent. In the absence of tat, modification of the R region had only minor effects on cytoplasmic RNA stability, steady-state levels of vector RNA and packaging, consistent with the known primary function of R during reverse transcription. Although the SIV R region of SIV-based vectors could be replaced functionally by heterologous sequences, the same modifications of R led to a severe replication defect in the context of a replication-competent SIV. As SIV-based vectors containing the HIV-1 R region were transferred less efficiently by HIV-1 particles than wild-type SIV vectors, a match between R and cis-acting elements of the vector construct seems to be more important than a match between R and the Gag or Pol proteins of the vector particle.

INTRODUCTION

Homology of the 5′ and 3′ R regions located at the start and the end of the genomic retroviral RNA, respectively, is important for strand transfer during reverse transcription (Gilboa et al., 1979; Swanstrom et al., 1981; Coffin, 1990). Therefore, the R regions are essential cis-acting elements that have to be maintained by retroviral vectors. The length of the R regions of different retroviruses ranges from 16 bases of Mouse mammary tumor virus (MMTV) to 247 bases in human T-cell leukemia virus type 2 (Sodroski et al., 1984; Moore et al., 1987). As strand transfer can be initiated by shorter R regions (Lobel & Goff, 1985; Klaver & Berkhour, 1994; Dang & Hu, 2001), its length might also depend on other functions residing in R such as polyadenylation (Guntaka, 1993; Berkhour et al., 1995a). Lentiviral R regions also contain the transactivation response element (TAR) (Coffin, 1990; Rabson & Graves, 1997). Binding of Tat to TAR leads to transcriptional activation of the lentiviral promotor located in the U3 region of the 5′ LTR of the proviral DNA (reviewed by Cullen, 1992; Daelemans et al., 2000). In addition, the R region of Human immunodeficiency virus 1 (HIV-1) has been reported to enhance packaging efficiency (Das et al., 1997, 1998; McBride et al., 1997; Clever et al., 1999; Helga-Maria et al., 1999). The multiple functions of R at different steps of the virus life cycle render modifications of the R region of retroviral vectors difficult. In attempts to reduce the risk of vector mobilization (Bukovsky et al., 1999), we previously generated simian immunodeficiency virus (SIV)-based vectors with mutated primer-binding sites (Hansen et al., 2001). The resulting defect in the initiation of reverse transcription was complemented by providing a matched artificial tRNA during vector production. As the artificial tRNA was lacking in vector-transduced cells, mobilization of the mutated vector by the parental virus was reduced, although not completely abolished (Grunwald et al., 2004). Formation of heterozygous particles containing genomic RNA of the parental virus and genomic RNA of the vector could lead to strand transfer of minus-strand strong-stop DNA (−ssDNA) of the parental virus to the 3′ end of the vector RNA and thus result in transfer of the vector RNA by the parental virus. Reducing the homology of the vector R region and the

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parental virus might prevent this strand transfer and therefore reduce the risk of vector mobilization further. We were interested in determining whether the entire R region or parts of it could generally be replaced by heterologous sequences, despite its multiple functions. Therefore, we analysed the effect of different deletion and replacement mutants of the 5’ and 3’ R regions of SIV on vector titres, RNA packaging and replication of full-length SIV.

METHODS

Plasmids. The vector AR53 (see Fig. 2a) is based on the SIV vector ViGΔBH described previously (Schnell et al., 2000). To create AR53, a 447 nt DNA fragment was deleted by restriction digest with SpeI and BpEI. In addition, the SIV enhancer of the 5’ U3 region upstream of the TATA box was replaced by the human cytomegalovirus (HCMV) immediate-early enhancer spanning nt 324 to 27. To insert the HCMV enhancer via NdeI and NarI, this region was amplified from pcDNA3.1 (Invitrogen) by overlap-extension PCR with the primers CMVs (5’-GATCCGCATATGTTTAAACGCGGCC- GCAAGTAGCGCCCCATATTGACGT-3’, sense), CMVSp1a (5’-GACA- GTGATATTTATATGACCCTCCACGCCGACGCT-3’, antisense), SIV-TATAs (5’-GGAGGTTCTTATAATATCAGTCTTGC-3’, sense) and S1122a (5’-ACTCAGAGGCTTCTACTCTCTG-3’, antisense). The 5’ R region was deleted by overlap-extension PCR with the primers pBR5s (5’-GATGACTGAGCGAGAGGGGAAGCCGA-3’, sense), SIV-TATA-Sala (5’-ACTAGTTTAAACTGTCAGGAGAAGCCGAAT- GCAGGTATATTTA-3’, antisense), SIV-U5-Sals (5’-GCGGCTCCTCCTCCACGATC-3’, sense), SIV-R-Sals (5’-GAGTCAGTGAGCGAGGAAGCGGA-3’, antisense) and S1122a. The final PCR product was inserted into the NdeI and NarI restriction sites, thereby replacing the R region by restriction sites of SalI and SpeI. To delete the 3’ R region, a U3/S U5 fragment was amplified with the primers SIV-U5-Xhos (5’-ACTAGTTTAAACTGTCAGGAGAAGCCGAAT- GCAGGTATATTTA-3’, antisense), SIV-R-Sals (5’-GCGGCTCCTCCTCCACGATC-3’, sense) and S1122a. The final PCR product was inserted into the NdeI and NarI restriction sites, thereby replacing the R region by restriction sites of SalI and SpeI. The 5’ R region was amplified from pHIT-G (Malim et al., 1988; Fouchier et al., 1997; Wagner et al., 2000), pSEAP2 was obtained from Applied Biosystems. All full-length SIV constructs (CSVΔenv, MR-CSVΔenv and HR-SVΔenv) were derived from SIVΔNU (Gundlach et al., 1997). To replace the SIV R region and/or enhancer of U3, LTRs containing the desired heterologous sequences were amplified by overlap-extension PCR. A detailed cloning strategy and the sequences of the PCR primers can be obtained from the authors on request. pAenv corresponds to AenvGFP, which has been described previously (Schnell et al., 2000).

Cell culture. 293T (DuBridge et al., 1987) and SMAGI (Chackerian et al., 1995) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U penicillin ml\(^{-1}\) and 100 mg streptomycin ml\(^{-1}\). 293 cells were also grown in DMEM medium, but supplemented with 5% FCS, 100 U penicillin ml\(^{-1}\) and 100 mg streptomycin ml\(^{-1}\). CEMx174-SEAP cells (Means et al., 1997) were propagated in RPMI 1640 supplemented with 10% FCS, 100 U penicillin ml\(^{-1}\) and 100 mg streptomycin ml\(^{-1}\).

Production of vector particles. Vector-containing particles were produced by transfection of 293T cells using the calcium phosphate co-precipitation method as described previously (Soneoka et al., 1995). Vector plasmid (5 µg) was co-transfected with 3 µg gag-pol plasmid and 2 µg VSV-G and rev expression plasmids in a 25 cm\(^2\) flask. The supernatant of the transfected cells was passed through a 0.45 µm filter and immediately used for further applications. The Phospha-Light kit (Tropix) was used to analyse the activity of the secreted alkaline phosphatase (SEAP) in the supernatant.

Transduction of target cells. To determine vector titres, 293 cells were seeded in 24-well plates at a density of 1 × 10\(^5\) cells per well. After 1 day, the medium was replaced and the cells were incubated for 2–4 h with serial dilutions of vector particles in a total volume of 200 µl per well. Fresh medium was added and the number of GFP-positive cells was determined 2 days after infection to calculate GFP-forming units (GFU) ml\(^{-1}\).

Packaging assay. Vector particles were produced as described above and concentrated from approximately 5 ml transfection supernatants by ultracentrifugation through a 30% sucrose cushion. RNA was isolated from the concentrated particles and the cytoplasm of transfected cells using the QIAMP Viral RNA Mini kit and the RNeasy Mini kit (Qiagen), respectively, and eluted in 50 µl. After DNase treatment (TURBO DNA-free kit; Ambion), 1 µl particle RNA and 500 ng cytoplasmic RNA were used for quantitative RT-PCR with the primers S1115a (5’-TCTCGAGCTAGCGCTAGTGC- AGGG-3’, sense) and S1329a (5’-GACGGAGGTTTCTTACGCCG-3’, antisense) and the TaqMan probe S1229 (5’-CCCTCTTCCTTCCG- GCCTCTCCAGG-3’). For wild type, vector RNA copy numbers of ~7 × 10\(^5\) (µg cytoplasmic RNA)\(^{-1}\) and 1 × 10\(^6\) (ml supernatant of transfected cells)\(^{-1}\) were obtained. The packaging efficiency was calculated as the ratio of copy numbers in the particles to the cytoplasmic RNA copy numbers. The ratio of the wild-type vector was set to 100%, and for all other vectors of the same transfection experiment the packaging efficiency was expressed relative to the wild-type vector. At least three independent transfections were performed for each vector. Omitting the reverse transcriptase from the RT-PCR was used to confirm elimination of transfected plasmid DNA by the attachment site of the 3’ LTR. To produce a packaging-negative control plasmid (Fig. 1a), the target sequence of the RT-PCR used for the packaging assay was cloned into pCR2.1-TOPO (Invitrogen). From this intermediate plasmid, an EcoRI fragment was inserted into the EcoRI site of pIRE2-EGFP (enhanced green fluorescent protein; Invitrogen) resulting in pSD-EGFPs. The expression plasmids of vesicular stomatitis virus G protein (VSV-G) (pHIT-G) and rev (pRev) and the codon-optimized gag-pol expression plasmids of SIV and HIV (Sgp\(^{\text{pp}}\), Hgp\(^{\text{pp}}\)) have been described previously (Malim et al., 1988; Fouchier et al., 1997; Wagner et al., 2000).

pSEAP2 was obtained from Applied Biosystems. All full-length SIV constructs (CSVΔenv, MR-CSVΔenv and HR-SVΔenv) were derived from SIVΔNU (Gundlach et al., 1997). To replace the SIV R region and/or enhancer of U3, LTRs containing the desired heterologous sequences were amplified by overlap-extension PCR. A detailed cloning strategy and the sequences of the PCR primers can be obtained from the authors on request. pAenv corresponds to AenvGFP, which has been described previously (Schnell et al., 2000).

S. Brandt and others

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DNase treatment. Reverse transcriptase activity of the vector particle preparation was determined using the Reverse Transcriptase Assay chemiluminescent kit (Roche), confirming that the different transfected vector plasmids did not lead to a systematic bias in particle production.

**Stability assay.** Cytoplasmic vector RNA copy numbers determined during the packaging assay were taken as genomic vector RNA steady-state levels. To compare steady-state levels of the different vectors, cytoplasmic vector RNA copy numbers were normalized to the wild-type vector included in the same transfection experiment. To determine the stability of vector transcripts, 293T cells were transfected by calcium phosphate co-precipitation with 5 μg of the indicated vector construct and 10 μg carrier DNA. After 18 h, cells were split 1:2, seeded in six-well plates and incubated for 8 h. Half of the transfected cells were treated with 50 μg α-amanitin (AppliChem) ml⁻¹ as described previously (Lee et al., 2004). After 9 h, RNA was isolated from the transfected cells, treated with DNase and 500 ng was used for TaqMan RT-PCR as described above. The pre-GAPDH quantitative RT-PCR was carried out with the primers preGAP-DHE6s (5’-CCACCAACTGCTTAGCACC-3’, sense) and preGAP-DHE6a (5’-CTCCCCACCTTGAAAGGAAT-3’, antisense).

**Production of virus stocks and titre determination.** For generation of virus stocks, 293T cells were transfected by calcium phosphate co-precipitation with proviral SIV constructs containing different R regions and/or enhancers. Cell-culture supernatant was harvested 48 h after transfection, passed through a 0.45 μm filter and stored in aliquots at −80 °C. Titres of the virus stocks were determined on sMAGI indicator cells (Chackerian et al., 1995) in a single-cycle replication assay. For infection, 3 × 10⁵ sMAGI cells were seeded in 24-well plates and treated with 200 μl serially diluted virus. After incubation for 2–4 h, 1 ml fresh medium was added. Two days later, cells were washed with PBS, fixed with 0.5 % glutaraldehyde in PBS followed by a second washing step and stained for β-galactosidase with 5-bromo-4-chloro-3-indolyl β-D-galactopyrano-

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**Fig. 1.** Influence of the first 7 nt of the R region on vector titre and packaging. (a) Maps of the SIV vector constructs containing wild-type R regions (R53), a deletion of the first 7 nt of the 3’ R region (R53Δ7) or deletion of the first 7 nt from both R regions (R53Δ7). R53 is a self-inactivating vector with deletion of the TATA box and enhancer elements from the 3’ U3 region. The HCMV enhancer replaces the enhancer of the SIV 5’ U3 region. SIV sequences are numbered according to SIVmac239 (GenBank accession no. M33262). SIV sequences deleted from R53 to generate R53Δ7 and R53Δ7 are given after the Δ sign. EGFP, expression cassette for the enhanced GFP under the control of the murine leukemia virus (MLV) promoter; PBS, primer-binding site; Ψ, major packaging signal; RRE, Rev-responsive element; PPT, polypurine tract. pSD-EGFPs containing the target sequence for the quantitative RT-PCR was used as a negative control in the packaging assay. (b) Titres of the indicated vectors transferred by SIV or HIV-1 particles. The results are shown as means ± SD of at least five independent experiments. GFU, GFP-forming units. (c) Packaging efficiency. Vector copy numbers in the cytoplasm of transfected 293T cells and of corresponding vector particles were determined by quantitative real-time RT-PCR. The packaging efficiency of different vectors was calculated as the ratio of copy numbers in the particles to the cytoplasmic RNA copy numbers normalized to the same ratio determined for wild-type vector (set at 100 %). Results are displayed as means of at least three independent experiments. Error bars represent 95 % confidence intervals (t-test). A significant reduction in packaging efficiency (P < 0.05) relative to wild-type vector is indicated by an asterisk.
side (X-Gal). Virus titre was calculated after counting blue cells as β-galactosidase-forming units ml⁻¹. The background level of β-galactosidase-forming units obtained for supernatants of pDenv-transfected cells was 5 × 10².

Western blot analysis. To analyse gag expression, virus stocks were concentrated by ultracentrifugation through a 30% sucrose cushion and virus pellets were resuspended in 100 µl lysis buffer (Boehringer). Viral proteins were separated by 10% SDS-PAGE and incubated with SIVmac251 Gag monoclonal antibody (diluted 1:2000; National Institute of Health AIDS Research and Reference Reagent Program, catalogue no. 2321) after transfer on to a nitrocellulose membrane. Secondary antibodies were horseradish peroxidase-conjugated rabbit anti-mouse IgG (diluted 1:2000; DakoCytomation). Blots were developed using enhanced chemiluminescence (Chemiluminescent Substrate, Roche) and visualized by exposure to autoradiography films.

Virus replication assay. Replication of SIV variants containing different R regions and/or enhancers was analysed by determining the activity of SEAP after infection of CEMx174-SEAP cells (Means et al., 1997). For infection, 1×10⁶ pelleted cells were incubated for 2–4 h with 500 µl virus stock, followed by the addition of 4.5 ml medium. Cells were passaged for 7 weeks and the SEAP activity of an aliquot was measured using the Phospha-Light kit (Tropix) three times per week. Cells killed due to SIV replication were replaced by fresh CEMx174-SEAP cells. Viral RNA levels in the supernatant of these cultures were determined after concentration of viral particles by ultracentrifugation by quantitative real-time RT-PCR using primers and probes described for the packaging assay. The sensitivity of the assay was <10² copies per PCR, corresponding to <10³ copies (ml culture supernatant)⁻¹.

RESULTS

Production of SIV vectors in the absence of tat

To study the role of heterologous R regions on vector titres and packaging independent of confounding effects of Tat–TAR interaction, we first generated SIV vectors, in which the enhancer of SIV was replaced by the HCMV immediate-early enhancer spanning nt 2342 to 227. The SIV TATA box was not modified (Fig. 1a). Infectious vector particles were produced by co-transfection of the R5 vector with codon-optimized gag-pol expression plasmids of SIV or HIV-1, a VSV-G expression plasmid and a rev expression plasmid. To ensure comparable transfection efficiency, a plasmid encoding SEAP (pSEAP2) was
also co-transfected, followed by determination of SEAP activity (data not shown). After transduction of 293T cells, vector titres in the range of \(10^5\) infectious units (IU) ml\(^{-1}\) were observed (Table 1). Although titres could be enhanced by co-transfection of a tat expression plasmid, titres were sufficiently high for subsequent studies, even in the absence of tat.

**Homology in the first 7 nt of the 5’ and 3’ R region is not required for vector infectivity**

Mutation of the first nucleotides of one HIV-1 R region, resulting in a mismatch during first-strand transfer of reverse transcription, has been shown to lead to vector titres that were reduced 100-fold (Ohi & Clever, 2000). We

**Table 1. Influence of tat on vector titre**

293T cells were co-transfected with the vector R53, pHIT-G, gag-pol expression plasmids of SIV or HIV-1 and pcRev in the presence or absence of a tat expression plasmid. Vector titres in the supernatant of transfected cells were determined on 293T cells. The means ± SD of at least five experiments are shown. GFU, GFP-forming units.

<table>
<thead>
<tr>
<th>SIV particles</th>
<th>HIV-1 particles</th>
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<tbody>
<tr>
<td>(-tat)</td>
<td>(+tat)</td>
</tr>
<tr>
<td>(1.55 \times 10^5 ± 8.87 \times 10^4)</td>
<td>(4.33 \times 10^5 ± 1.43 \times 10^5)</td>
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therefore deleted the first 7 nt of the SIV R region of the 3’ LTR (R5R3Δ7 in Fig. 1a), which resulted in a 10-fold reduction in vector titre (Fig. 1b). Deletion of the first 7 nt of the R regions of the 5’ and 3’ LTR (R53Δ7 in Fig. 1a) restored the match between the 5’ and 3’ R region, but did not restore vector titres (Fig. 1b).

The first 7 nt of the 5’ R region are involved in packaging into SIV but not into HIV-1 particles

As the R region of HIV-1 also contributes to packaging of the HIV-1 genome, we analysed the packaging efficiency of the vectors R5R3Δ7 and R53Δ7. Using a quantitative real-time RT-PCR, genomic vector RNA copy numbers were determined in the cytoplasm of transfected cells and in viral particles released from these cells. Viral particles were enriched by ultracentrifugation through a 30% sucrose cushion. The ratio of vector copy numbers in the particle preparation to cytoplasmic vector RNA copies for the wild-type vector was set at 100%. Production of comparable amounts of SIV and HIV-1 particles for the different vector constructs was confirmed by measuring the reverse transcriptase activity of the particle preparations (data not shown). To determine the background level of non-specific packaging, cells were also transfected with pSD-EGFPS, an expression plasmid encoding EGFP fused to the fragment of the 5’ leader sequence of SIV targeted by the PCR (Fig. 1a). The packaging efficiency of pSD-EGFPS was 0.1%. In contrast, deletion of the first 7 nt of the 3’ R region did not reduce packaging into SIV or HIV-1 particles (Fig. 1c). However, a 2- to 3-fold reduction in RNA packaging into SIV particles, but not HIV-1 particles was observed if the 5’ R region also contained the 7 nt deletion (Fig. 1c).

Deleting the entire R region of one or both LTRs results in a marked titre reduction with minor effects on RNA packaging

To confirm the expected importance of the entire R for vector infectivity, we generated vectors with a complete deletion of R in the 3’ and/or 5’ LTR (Fig. 2a). Transfer efficiency of the R-deleted vectors by SIV or HIV-1 particles was reduced by up to 1500-fold (Fig. 2b). In contrast, packaging of the vectors with deletion of the 3’ LTR and of both R regions by SIV were only reduced by approximately 2-2-fold. A twofold reduction in packaging by HIV-1 particles was observed after deletion of the 5’ R region and both R regions (Fig. 2c).

The R region of SIV vectors can be replaced functionally by heterologous sequences

Heterologous R regions of HIV-1 or MoMSV were inserted into the R-deleted vectors to rescue the marked defect in vector infectivity (Fig. 3a). In comparison with the vectors with deletions in both R regions, insertion of the HIV-1 and MoMSV R regions enhanced transfer of the vector by SIV particles 50- and 20-fold, respectively (Fig. 3b). Transfer of the R-complemented SIV vector by HIV-1 particles was approximately 56- to 100-fold more efficient in comparison with the R-deleted vector. The R regions of SIV were also replaced by the leader sequence of the human TBP. As this sequence does not contain a poly(A) signal, the last 53 nt of SIV R containing the SIV poly(A) signal were not deleted. Insertion of the TBP leader sequence replacing R also enhanced vector titres, although this was less efficient than the HIV-1 and MoMSV R region. Insertion of the HIV-1 R region into the SIV vector did not lead to a detectable increase in packaging of the genome into HIV-1 particles. Packaging of the other R-complemented vectors by SIV or HIV-1 particles was reduced approximately twofold, similar to the R-deleted vectors (Fig. 3c).

Modifications of the R region have only minor effects on vector stability and steady-state levels

Complementation of the SIV R region by heterologous sequences did not restore vector infectivity completely. Deletion of the first 7 nt of R also led to reduced vector titres, despite complete homology between the 5’ and 3’ R region. As determination of packaging efficiency does not provide information on genomic RNA steady-state levels and its stability, these parameters were also determined. Steady-state levels were analysed by quantification of vector RNA in the cytoplasm of 293T cells 48 h after transfection by RT-PCR. To investigate vector stability, transfected 293T cells were treated with α-amanitin, an inhibitor of cellular RNA polymerase II. At 36 h post-transfection, cytoplasmic vector copies were determined by quantitative RT-PCR and compared with vector copy numbers of untreated cells. Inhibition of RNA polymerase II was confirmed by analysing total pre-GAPDH mRNA levels. Treatment with α-amanitin resulted in a 92–99 % decrease in GAPDH transcripts. Neither deletion of the first 7 nt nor deletion of the entire R region reduced steady-state levels or the stability of genomic vector RNA by more than a factor of 2 (Table 2).

Full-length SIV containing heterologous R regions shows titres comparable to corresponding vectors, but is defective in long-term replication

As the R region of SIV vectors could be replaced functionally by heterologous sequences, we also analysed the function of HIV and MoMSV R in the context of full-length SIV. Both R regions of the proviral nef-deleted SIV plasmid SIVΔNU were replaced by the sequences indicated in Fig. 4(a). As the MoMSV R region contains no TAR element, the enhancers of both U3 regions were replaced by the HCMV enhancer, as described for the vectors. To ensure that any effect observed for this SIV construct was not caused by the HCMV enhancer, an SIV variant was constructed in which only the enhancer of both LTRs was replaced by the enhancer of HCMV. All constructs revealed gag expression in Western blot analyses of virus particles (Fig. 4b). However, the amount of Gag from MR-CSVΔnef contain-
Table 2. Influence of the R region on stability and steady-state levels of SIV vector transcripts

293T cells transfected with the indicated vectors were treated with α-amanitin for 9 h or left untreated. Cytoplasmic RNA was isolated and used for quantitative RT-PCR. Stability was calculated as the ratio of vector RNA copy numbers in α-amanitin-treated versus vector copy numbers in untreated cells. NA, Not applicable.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Stability ± SD (%)</th>
<th>Steady-state level ± SD (%)</th>
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<tbody>
<tr>
<td>R53</td>
<td>34.09 ± 9.51</td>
<td>100</td>
</tr>
<tr>
<td>R5R3Δ7</td>
<td>22.29 ± 7.76</td>
<td>96.5 ± 55.7</td>
</tr>
<tr>
<td>R5Δ7</td>
<td>18.20 ± 2.21</td>
<td>75.8 ± 31.1</td>
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<td>HR</td>
<td>19.48 ± 5.31</td>
<td>62.3 ± 22.7</td>
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<tr>
<td>MR</td>
<td>21.31 ± 12.67</td>
<td>46.4 ± 9.7</td>
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<tr>
<td>TBPR</td>
<td>21.94 ± 2.99</td>
<td>59.6 ± 31.2</td>
</tr>
<tr>
<td>R5ΔR3</td>
<td>21.94 ± 1.47</td>
<td>76.2 ± 22.6</td>
</tr>
<tr>
<td>R3ΔR5</td>
<td>21.67 ± 5.78</td>
<td>101.7 ± 45.7</td>
</tr>
<tr>
<td>ΔR53</td>
<td>23.75 ± 3.81</td>
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</tr>
<tr>
<td>pre-GAPDH†</td>
<td>4.81 ± 3.00</td>
<td>NA</td>
</tr>
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</table>

*Steady-state level of cytoplasmic vector RNA was measured as described for the packaging assay by quantitative RT-PCR 48 h after transfection. Copy numbers for steady-state levels of vectors with a modified R region were normalized to the wild-type vector, which was set at 100%. The means ± SD of three independent experiments are shown.

†The stability of pre-GAPDH mRNA levels was determined in the same way using total cellular RNA instead of cytoplasmic RNA.

Fig. 4. Expression of gag from proviral SIV constructs containing different R regions. (a) Map of proviral SIV plasmids. All plasmids were derived from SIVΔNU, which contains wild-type R regions. SIV LTR sequences exchanged in the derivates are indicated by shaded boxes. Enhancer elements of both U3 regions were replaced by the enhancer of the HCMV immediate-early enhancer (CMV) in CSVΔnef and MR-CSVΔnef. The R region of MoMSV (MR) was inserted instead of the SIV R region in MR-CSVΔnef. HR-SVΔnef contains the R region of HIV-1 (HR). (b) 293T cells were transfected with the different proviral plasmids and viral particles released into the supernatant were pelleted by ultracentrifugation. The Gag content of viral particles was analysed by Western blot analysis using an anti-capsid antibody. Arrows indicate bands for the Gag precursor protein and capsid (CA). Supernatants from mock-transfected 293T cells were processed in parallel as a negative control.

DISCUSSION

The R region is a cis-acting sequence important in many steps of the lentiviral life cycle, such as transactivation of transcription, polyadenylation and first-strand transfer during reverse transcription. Therefore, it is necessary to maintain these functions in lentiviral vectors. However, it could be advantageous to modify the R region of primer-complemented vectors to reduce the risk of vector mobilization. Therefore, we analysed the influence of truncated or heterologous R regions on the infectivity of SIV-based vectors lacking tat. Deletion of the first 7 nt of the 3′ R region resulted in a 10-fold titre reduction (Fig. 1B). As this deletion caused a mismatch between −ssDNA and the 3′ acceptor R region during first-strand transfer of reverse transcription, we had expected a stronger decrease in vector infectivity. Remaining vector infectivity should be due to first-strand transfer prior to completion of reverse transcription of the entire 5′ R region, as described previously.
for HIV-1, Moloney murine leukemia virus and spleen necrosis virus (Lobel & Goff, 1985; Ramsey & Panganiban, 1993; Klaver & Berkhout, 1994; Kulpa et al., 1997). The results therefore suggest that most SIV virions (90%) reverse transcribe the entire 5’ R region before transfer of −ssDNA to the 3’ R region. However, premature strand transfer seems to be more frequent in SIV compared with HIV-1, as mutating 4 nt of the first 10 nt of the R region of HIV-1 led to a 100-fold reduction in virus titre (Ohl & Clever, 2000). Mutating both R regions of HIV-1 restored vector infectivity (Ohl & Clever, 2000). For SIV, deletion of the first 7 nt of both R regions, preventing a mismatch between the 5’ and 3’ R regions, did not restore the titre to wild-type levels. Reducing the length of the R region from 177 to 170 nt should not reduce strand transfer, as studies using an infectious HIV-1 clone in which the 3’ R region was truncated from its 3’ end to 30 nt worked efficiently (Berkhout et al., 1995b). Similar results were obtained by Dang & Hu (2001) who reduced the length of the R region of Murine leukemia virus (MLV) vectors to 12 nt without a significant effect on vector titres. As truncation of the 5’ end of both R regions of SIV by 7 nt results in a 10- to 20-fold decrease in vector titre, there might either be a preference for specific nucleotide sequences at the 5’ end of the RNA template during reverse transcription or this part of the R region may be involved in other steps of the virus life cycle.

Replacing the SIV R region by heterologous sequences increased vector infectivity 10- to 100-fold in comparison with the vector with deletion of both R regions, with higher transfer rates using HIV than SIV particles (Figs 2b and 3b). Thus, the SIV R region could be replaced functionally by heterologous sequences, although the titres were reduced compared with the wild-type vector. In attempts to analyse the reason for this titre reduction, we explored the influence of R modifications on vector packaging, as the lower stem of TAR (McBride et al., 1997; Das et al., 1998; Clever et al., 1999; Helga-Maria et al., 1999) and the intact structure of the poly(A) hairpin (Das et al., 1997; Clever et al., 1999) have been reported to enhance packaging of HIV-1. A small effect of SIV R on packaging efficiency was observed for some R mutants (Figs 1c, 2c and 3c); this was lower than that described previously for HIV-1 (Helga-Maria et al., 1999), but was in accordance with the results of other groups (McBride et al., 1997; Das et al., 1997, 1998; Clever et al., 1999). Clever et al. (2002) proposed that the entire HIV-1 leader contributes to overall packaging efficiency. Whilst the stem–loops SL1 to SL4 act through the binding of Gag, the TAR, poly(A) and U5-PBS hairpins enhance packaging in a sequence-independent manner by forming a complex secondary structure. Similarly, our results are also consistent with the hypothesis that the secondary structure of the SIV TAR and poly(A) hairpin can affect the major packaging signal of SIV located upstream of (Strappe et al., 2003) or around (Patel et al., 2003) the major splice donor leading to an increase in packaging efficiency.

We also investigated the influence of R mutations on vector RNA stability and steady-state levels. As shown in Table 2, the reduction in vector RNA stability and steady-state levels by modified R regions was less than twofold, comparable to the results of other groups after mutating HIV-1 TAR (Helga-Maria et al., 1999). Even removing the poly(A) signal by deletion of the 3’ R region did not decrease vector stability markedly. However, there is evidence that retroviral polyadenylation at the virus-encoded poly(A) signal is only
of minor importance. About 15% readthrough of transcripts has been reported for *Avian leukaosis virus*, which occurs during retrovirus infection by polyadenylation of viral RNA in cellular sequences adjacent to the provirus or increased usage of cryptic sites within viral sequences. Furthermore, mutation of the poly(A) signal, leading to less than 1% correct polyadenylation, had almost no effect on virus replication (Swain & Coffin, 1989, 1993). Use of cryptic cleavage sites could be a possible explanation for maintained vector stability after poly(A) site deletion. There is a poly(A) site variant (AGUAAA), usually used by MMTV, approximately 300 bp downstream of the original cleavage site in the backbone of the vector plasmid.

As there was only a mild effect of R on vector packaging and stability, the reason for different infectivities between SIV vectors containing wild-type or heterologous R regions remains unclear. Non-viral sequences, consisting of overlapping GFP sequences, can mediate first-strand transfer of reverse transcription efficiently in the context of an MLV vector and resulted in only a 5-4-fold titre reduction (Cheslock *et al.*, 2000). However, replacing the R region of an MLV vector by the HIV-1 R region or by an artificial sequence markedly reduced or even abolished first-strand transfer, suggesting that this process is more complex than a simple hybridization (Allain *et al.*, 1998). Furthermore, Topping *et al.* (1998) described a base pairing-independent mechanism for strand transfer in MLV, as the transferred −ssDNA is preferentially directed to the 3′ U3/R junction, although more extensive sequence homology was available downstream of this position. They suggested that the higher-order structure of the genomic RNA was responsible for this preference. In addition, there is evidence for involvement of the upper portion of HIV-1 TAR in initiation and completion of reverse transcription (Harrich *et al.*, 1996, 2000; Berkhout *et al.*, 2001). This function of the TAR element is more dependent on its structure than on the TAR sequence, with the exception of the loop sequence, which should be maintained. For SIV TAR, a similar involvement in reverse transcription is possible. The assumption that R regions that are more distantly related to SIV have less-conserved secondary structures could explain the reductions in vector infectivity that we observed for the vectors with the modified R regions. The R region truncated by only 7 nt should form almost the same TAR element and showed only a modest titre reduction, whilst the secondary structure of the non-viral TBP sequence probably differs considerably from SIV TAR and displayed the greatest decrease in vector infectivity. Therefore, it might be important to mimic the SIV R secondary structure to improve the titre of vectors with heterologous R regions.

Passaging of replication-competent SIV containing heterologous R regions might allow adaptation of the heterologous R region. The analysis of such compensatory mutations should provide clues to the molecular basis of the reduced vector titres. Therefore, we also analysed the effect of heterologous R regions on replication of a nef deletion mutant of SIV. As the MoMSV R region does not contain TAR, the HCMV promoter/enhancer region was inserted into the U3 region of SIV to enhance basal transcription. Replacing just the U3 region by the heterologous promoter/enhancer resulted in a replication-competent virus, as observed previously (Chang *et al.*, 1993; Guan *et al.*, 2001; Verhoeft *et al.*, 2001; Sommer *et al.*, 2004). However, when the R region of SIV was also replaced by the R region of MoMSV, the titre in the single-cycle replication assay was severely impaired and replication-competent virus could not be recovered. Western blot analyses after transient transfection of the chimeric proviral DNA (MR-CSVΔnef) revealed reduced particle production, suggesting inefficient transcription due to the absence of TAR. Replacing the SIV R region by the R region of HIV-1 did not seem to impair particle production, but reduced the infectious titre in the single-cycle replication assay. Consistent with inefficient reverse transcription, exchange of the SIV R region for the R region of HIV-1 reduced the infectious titre of the SIV vector and the viral construct to a similar extent. Although infectious SIV containing the R region of HIV-1 could be produced by transient transfection, no replication-competent virus grew during a 7-week-culture period (Fig. 5). Reduction of virus infectivity in single-cycle replication assays by more than 30- to 40-fold might lead to cell growth exceeding virus replication and thus extinction of the virus from the culture. However, as the influence of R on vector titres was analysed in the absence of tat, whilst the virus replication assay was performed with a functional tat gene, we cannot exclude the possibility that SIV depends to a greater degree on the R region than the SIV vector.

Comparison of packaging and transfer of SIV vectors with modified R regions by SIV and HIV-1 particles also provided important information. SIV-based vectors containing the HIV-1 R region were transferred by HIV-1 particles less efficiently than wild-type SIV vectors. This suggests that a match between R and cis-acting elements of the vector construct seems to be more important than a match between R and the Gag or Pol proteins of the vector particle.

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