Forced recombination of Ψ-modified murine leukaemia virus-based vectors with murine leukaemia-like and VL30 murine endogenous retroviruses

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Co-encapsulation of retroviral RNAs into virus particles allows for the generation of recombinant proviruses through events of template switching during reverse transcription. By use of a forced recombination system based on recombinational rescue of replication-defective primer binding site-impaired Akv–MLV-derived vectors, we here examine putative genetic interactions between vector RNAs and copackaged endogenous retroviral RNAs of the murine leukaemia virus (MLV) and VL30 retroelement families. We show (i) that MLV recombination is not blocked by nonhomology within the 5’ untranslated region harbouring the supposed RNA dimer-forming cis-elements and (ii) that copackaged retroviral RNAs can recombine despite pronounced sequence dissimilarity at the cross-over site(s) and within parts of the genome involved in RNA dimerization, encapsidation and strand transferring during reverse transcription. We note that recombination-based rescue of primer binding site knock-out retroviral vectors may constitute a sensitive assay to register putative genetic interactions involving endogenous retroviral RNAs present in cells of various species.

Introduction

Retroviruses replicate through a double-stranded DNA intermediate which is stably integrated into the host genome as proviral DNA. Upon infection of germ cells, proviruses are transmitted through the germ line and may persist as virus entities and Mendelian genes in the genome for multiple generations. Such elements of the genome, usually referred to as endogenous retroviruses (ERVs), are often replication-defective due to the accumulation of mutations and, in order to spread, rely on concomitant replication of helper viruses. ERV-derived RNA may thus hitchhike with virus particles released from the host cell (Patience et al., 1998; Scadden et al., 1990), provided that structural cis-elements within the ERV packaging signal facilitate recognition by the helper virus RNA encapsidation machinery. Among a panel of known murine ERVs, members of the murine leukaemia virus (MLV)-related and VL30 ERV families are selectively included in MLV virions (Chakraborty et al., 1994; Mikkelsen et al., 1996; Patience et al., 1998; Purcell et al., 1996; Scolnick et al., 1979).

Encapsulation of two genomic RNA molecules into budding retrovirus particles allows for recombination during reverse transcription within the viral core particle internalized in the cytoplasm of the newly infected cell. Retrovirus recombination occurs primarily by template switching of nascent minus-strand DNA (Anderson et al., 1998; Coffin, 1979; Hu & Temin, 1990a, b, 1992; Hu et al., 1997; Stuhlmann & Berg, 1992), but may involve also events of plus-strand recombination (Junghans et al., 1982; Mikkelsen et al., 1998a). Coexistence and occasional copackaging of retroviral RNAs of exogenous and endogenous origin allow for the generation of recombinant proviruses harbouring sequences of both parental origins. In such a scenario, events of template switching during DNA synthesis may facilitate recombinational patch repair of virus mutations by substituting defective segments of the genome with functional sequence patches provided by the copackaged endogenous virus. Previously reported examples of ERV-based recombinational reversion include patch repair of virus mutants harbouring (i) modifications of the integrase
RNA dimerization and encapsidation are tightly coupled events of retrovirus replication. Whereas copackaging of retroviral RNAs is a proven prerequisite for recombination (Hu & Temin, 1990a), there remains an open question of whether RNA dimer formation is required for packaging and/or genetic interactions between copackaged RNAs. For all retroviruses studied, the primary determinants of RNA dimerization and encapsidation have been mapped to the packaging signal (Ψ) within the 5' untranslated region (5' UTR) situated downstream from the PBS and upstream from the gag initiation codon. A specific stem–loop structure in the 5' UTR facilitates synthetic RNA dimer formation in vitro through intermolecular ‘kissing’ of conserved palindromic loop motifs (Clever et al., 1996; Fosse et al., 1996; Girard et al., 1995; Haddrick et al., 1996; Laugherea & Jetté, 1994; Paillart et al., 1996b, 1997). Within a 5' UTR recombination window, this kissing-loop sequence is a hotspot for nascent minus-strand DNA template switching between vector donor RNA and endogenous virus-derived acceptor RNA templates (Mikkelsen et al., 1996, 1998a, 1998b). Although the kissing-loop interaction may constitute only one of several intermolecular interactions required for RNA dimerization in vitro (Berkhout & van Wamel, 1996; Clever & Parslow, 1997; Haddrick et al., 1996; Laugherea et al., 1997; Lear et al., 1995; Paillart et al., 1996a, 1996b, 1997), this result proposes that the kissing-loop sequence induces reverse transcriptase-mediated homologous recombination and raises the question of whether kissing-loop-defective viruses are less recombinogenic within the 5' UTR dimerization and recombination window. Recent findings suggest that the frequency of recombination elsewhere in the genome, within the coding regions of different human immunodeficiency virus type 1 (HIV-1) subtype RNAs, is not significantly affected by a lack of kissing-loop homology between interacting viral RNAs (St Louis et al., 1998).

We have in previous work studied retrovirus recombination by a forced recombination approach based on the interaction between replication-defective MLV-based vectors and an MLV-like endogenous retrovirus (MLEV), which is encapsidated into virus particles derived from murine fibroblast-derived packaging cells (Mikkelsen et al., 1996, 1998a, b). This approach, based on single-cycle vector replication of PBS-defective vectors, allows for studies of template shifting events within the primary dimerization region, enabling us to study if alterations and nonhomologies within the primary packaging and dimerization region influence local recombination processes. Here, we examine putative genetic interactions between vector RNAs and copackaged ERV RNAs derived from MLEV and the VL30 retroelement family. Akv and MLEV harbour highly similar packaging and terminal repeat (R) sequences (Mikkelsen 1998a, b), whereas members of the VL30 family contain packaging and R sequences that are not related to the corresponding regions of Akv (Adams et al., 1988; Hodgson et al., 1990). We describe modes of genetic interactions that take place in spite of dissimilarities in parts of the genome involved in intermolecular RNA recognition, encapsidation and strand transferring during reverse transcription.

Methods

- Vector construction. All vector constructions were derived from the Akv–MLV-based retrovirus tvPBSPro, harbouring the neomycin resistance gene (Lund et al., 1993). The wild-type construct will here be referred to as pPBSPro244Ψ. A kissing-loop-deficient vector, pPBSProALK, was generated by two-step PCR-mediated site-directed mutagenesis performed on pPBSPro244Ψ. Briefly, the 5' LTR, PBS–Pro and part of the 5' UTR were PCR-amplified from pPBSPro244Ψ, generating a fragment harbouring the desired 16 bp deletion within the proposed kissing-loop dimerization sequence (Girard et al., 1995). The amplified sequence was cloned by standard procedures into the appropriate position of pPBSPro244Ψ. Similarly, to create a Ψ-defective vector, pPBSProΨ−, a 163 bp sequence harbouring all putative packaging and dimerization elements was removed by PCR-mediated site-specific mutagenesis. A 22 bp linker (5' GCGCCGCTCGAGAAGCTAGA3'), harbouring restriction sites for NsiI, NsiI and Xbal was introduced at the site of the deleted Ψ element, whereas a 33 bp Akv segment downstream from the PBS was maintained in the resulting vector to allow proper initiation of reverse transcription. To make a packagable vector which shared only limited Ψ sequence identity with Akv, a putative packaging and dimerization element from mouse VL30 was PCR-amplified from RNA derived from virus particles produced by Ψ−2 packaging cells (Hatzoglou et al., 1990) and cloned into NsiI/Xbal-digested pPBSProΨ−. Primers for amplification of VL30 Ψ were designed on the basis of the sequence given by Adams et al. (1988). The amplified 436 bp fragment (GenBank accession no. AF166260) shared 94% sequence identity with the corresponding region of a previously published VL30 sequence (Adams et al., 1988). The resulting vector was designated pPBSPro436VL30Ψ−. To detect a potential effect of the restriction sites flanking a Ψ insert in pPBSProΨ−, a PCR-amplified 201 bp Akv Ψ fragment was cloned into NsiI/Xbal-digested pPBSProΨ−, giving rise to pPBSProΨ−link. The modified PBS–Umu sequence, designed to be an unlikely match for the 3' end of any known ΨRNA, was previously introduced into the wild-type vector by a two-step PCR procedure to generate pPBSUmu244Ψ− (Mikkelsen et al., 1996). PBS–Pro in pPBSProALK and pPBSPro436VL30Ψ− was similarly replaced by the PBS–Umu modification to generate pPBSUmuALK and pPBSUmu436VL30Ψ−.

- Cells, transfections and virus infections. Ψ−2 packaging cells (Mann et al., 1983) and NIH 3T3 target cells were grown in Dulbecco's modified Eagle medium with Glutamax-1 supplemented with 10% newborn calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated at 37°C in 90% relative humidity and 5% CO₂. Transfections of Ψ−2 cells and selection for stably integrated vectors have been done.
been described previously (Mikkelsen et al., 1996). Briefly, 10 µg vector plasmid DNA was transfected into Ψ-2 cells seeded at 5 × 10^6 cells/cm² on the day before transfection. Two days after transfection, G418-containing medium was added to select for stably integrated vectors. G418-resistant colonies appearing after 12 days of selection were pooled. To measure transductional efficiencies, viruses were harvested from medium left on confluent cultures of stably integrated producer cells for 24 h. Virus-containing medium was centrifuged, filtered, serially diluted and transferred to recipient cells (NIH 3T3 cells seeded at 5 × 10^5 cells/cm² 1 day prior to infection) in the presence of Polybrene (6 µg/ml). Selective medium was added 2 days after infection and resistant colonies were counted, individually isolated and expanded after 10 days of G418-selection.

**Proval DNA sequence analysis.** Genomic DNA from G418-resistant clones was prepared as previously described (Lund et al., 1993). Sequence analysis of individual transduced vector sequences was performed on a PCR product encompassing part of the 5′ LTR, the PBS, the 5′ UTR and the upstream part of the neo gene. PCR was performed with the following oligonucleotides: ON1 (5′-TCATAAGAGTCGTAG-CCAGCTAATCTGAG 3′), matching Akv MLV positions 7836–7865 (Van Beveren et al., 1985), and ON2 (5′-GCCGCCCCTGCTGCTGACAGCCGAAACAC 3′), matching neo positions 1656–1683 (Beck et al., 1982). The resulting PCR product was sequenced by use of an upstream primer (ON3, 5′-TCCGATCTGGTGCTGTCACTCTGG 3′) matching Akv MLV positions 69–90 (Van Beveren et al., 1985), and for relevant clones using a downstream primer (ON4, 5′-CTTCCCTTACAGCCCTTGGCG 3′) matching neo positions 1223–1244 (Auerswald et al., 1981). The 3′ LTR of transduced proviruses harbouring the originally mutated PBS–Umu was PCR-amplified with a primer (ON5, 5′-GGGACCTGACATAGCGGTG 3′) matching Akv MLV positions 3008–3029 and a primer (ON6, 5′-AATGAAAGACCCCCGAGGG 3′) specifically recognizing MLVE/LV30 molecular markers within U5 (a region corresponding to positions 127–144 in Akv–MLV; Van Beveren et al., 1985). Amplified fragments were sequenced with ON6.

**Generation and PCR-based screening of colony pools.** In some experiments, colonies of recipient cells appearing after infection and subsequent G418-selection were pooled to allow for a PCR-based screening for recombinant proviruses among a large number of transduced proviral sequences. Genomic DNA was prepared from individually expanded colony pools each obtained by pooling all G418-resistant colonies (on average 25 colonies for PBS–Umu244Ψ and nine colonies for PBS–UmuAKL) obtained on a single plate. In PCR amplifications specific for Akv–MLEV and Akv–VL30 recombinants, the neo-specific ON2 was used together with ON7 (5′-GTCTTTCTATTGGAGTGTTCCA 3′), matching MLVE-derived PBS–Gln (Mikkelsen et al., 1996), and ON8 (5′-TGTTGACATTGCGG 3′), matching mouse VL30-derived PBS–Gly (Adams et al., 1988). ON9 (5′-GCCGCTGTA-CCGTATTC 3′) and ON10 (5′-GCCGCTGTAACCTATTC 3′) specifically recognizing PBS–Umu and an MLEV marker within R (Mikkelsen et al., 1990), respectively, were used to screen for Akv–MLEV recombinants that harboured PBS–Umu. ON11 (5′-TCAG-ACACTCAAGTCCCGGAG 3′), matching VL30 U5 positions 449–470 (Adams et al., 1988), was used together with ON5 to specifically amplify 3′ LTRs harbouring VL30-derived sequences.

**Results**

Ψ'-modified vectors

Four different Ψ'-modified vector constructs harbouring the wild-type PBS–Pro were generated (Fig. 1), all derived from an Akv–MLV-based vector containing the neomycin resistance gene. This vector, designated pPBSPro244Ψ, harbour Ψ and R regions both similar to the corresponding regions of MLEV and distinct from the analogous cis-acting regions in endogenous VL30. A Ψ'-deficient vector, pPBSProΨ−, was generated by replacing a 183 bp 5′ UTR sequence harbouring all essential packaging and dimerization elements with a 22 bp cloning linker sequence. In this vector, a 33 bp Akv segment downstream from the PBS was maintained to allow proper initiation of reverse transcription. To make a packagable vector which shared Ψ'-similarity with endogenous VL30 and only limited similarity with the corresponding region in the MLEV recombination partner, a putative packaging and dimerization element from mouse VL30 was PCR-amplified from RNA in virus particles produced by Ψ-2 packaging cells (Hatzoglou et al., 1990) and cloned into the linker of pPBSProΨ−. The resulting vector, pPBSPro436VL30Ψ, harboured within the introduced 436 bp VL30 fragment sequences compatible with a triple GACC stem–loop structure which is likely required for proper RNA encapsidation in analogy with the conserved double GACC stem–loop structure found in spleen necrosis virus (Yang & Temin, 1994) and MLV (Mougel et al., 1996). As a control, a PCR-amplified 201 bp Akv 5′ UTR fragment was recloned into the Ψ-deficient vector to generate pPBSPro276Ψlink (Fig. 1). Finally, a kissing-loop-deficient Akv Ψ-based vector, pPBSProAKL, was generated to test the effect of removing the primary recombinogetic site of the MLV 5′ UTR. In this vector, a 16 bp deletion encompassing the 6 bp loop sequence and the residues forming the upper stem (5 bp on each side of the loop sequence) was introduced.

Transduction of the Ψ'-deficient vector, PBSProΨ−, was strongly diminished as compared to the wild-type vector (Fig. 1). Functionality of the vector was partly restored by reininsertion of the original MLV Ψ into the linker of pPBSProΨ−. PBSPro276Ψlink thus replicated one order of magnitude less efficiently than the wild-type vector, most likely due to the presence of the linker in a critical position of the leader region or, alternatively, due to complications in RNA folding caused by the 18 bp direct repeat flanking the Xbal site (Fig. 1). A transduction titre 200-fold lower than the wild-type vector was obtained for the vector harbouring the inserted VL30 Ψ sequence. This titre, comparable with the titre of PBSPro276Ψlink, demonstrated the functionality of the VL30-derived 5′ UTR insertion. The transduction efficiency of the kissing-loop-deficient vector was reduced approximately 60-fold relative to the wild-type vector, in agreement with the notion that the MLV kissing-loop sequence is involved in but not essential for virus replication (Mougel et al., 1996; Fisher & Goff, 1998).

Recombinational rescue of PBS- and Ψ'-modified vectors detected by single-colony analysis

To examine the effect of altering Ψ on forced recombination with endogenous viral RNA derived from MLEV and VL30 retroelements, the PBS–Pro sequences of wild-type, VL30Ψ-
harbouring and kissing-loop-deficient vectors were modified to PBS–Umux, designed to be an unlikely match for any known tRNA molecule (Mikkelsen et al., 1996). PBS–Umux vectors were stably transfected into Ψ-2 cells and viruses transferred to NIH 3T3 cells. We have previously found that the replication efficiency of PBS-modified vectors is reduced five orders of magnitude as compared to the wild-type (Mikkelsen et al., 1996). It was therefore found less relevant to study transduction of a Ψ-deficient vector harbouring severe modifications also within the PBS.

As expected, the transduction efficiency was strongly reduced for all PBS/Ψ-modified vectors (Fig. 2). We previously found that the mutated and defective PBS in vectors similar to PBSUmu244Ψ in 32 out of 60 analysed transduction events was repaired during transduction to perfectly match the 3′-end of a glutamine tRNA primer (Mikkelsen et al., 1996). Sequence analysis of the PBS–Gln-harbouring proviruses demonstrated that PBS-modified vectors were transduced through initial priming of cDNA synthesis on a copackaged PBS–Gln-containing MLEV transcript. Following interstrand minus-strand strong-stop transfer and continued minus-strand DNA synthesis through the selective marker gene, template switching was selectively observed during minus-strand synthesis within the 5′ UTR to obtain the perfect PBS–Gln complementarity facilitating second-strand transfer (Fig. 3 a).

In this study, 19, 8 and 11 individually transduced proviruses originating from transfer of PBSUmu244Ψ, PBS-UmuAKL and PBSUmu436VL30Ψ, respectively were analysed (Fig. 2). The upstream LTR, PBS and 5′ UTR of these proviral sequences were PCR-amplified by use of primers matching Akv U3 and neo sequences and resulting products were sequenced with nested primers. A total of five transduced proviruses were found to harbour a PBS derived from an endogenous virus through a mechanism involving 5′ UTR minus-strand recombination (Fig. 2, transduction pathways a1, b1 and c1). For PBSUmu244Ψ and PBSUmuAKL, MLEV served as the PBS donor in patch repair of the PBS mutation (Fig. 3 a), whereas endogenous VL30 was identified as the recombination partner and donor of PBS–Gly in transduction of Umu436VL30Ψ (Fig. 3 b).

According to the generally accepted model for reverse transcription (Gilboa et al., 1979), R and U5 regions copied during minus-strand strong-stop DNA synthesis are transferred to the 3′-end of the genome and eventually duplicated.
Recombination of Ψ-modified MLV vectors

<table>
<thead>
<tr>
<th>TRANSDUCTION TITER (CFU/ml)</th>
<th>TRANSNUCED PBS</th>
<th>TRANSNUCTION PATHWAY</th>
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<tbody>
<tr>
<td></td>
<td>PBS-Gln 3/19</td>
<td>α1 5' UTR minus strand (MLEV) 3/3</td>
</tr>
<tr>
<td></td>
<td>PBS-Gly 0/19</td>
<td>α2 R-U5-mediated (MLEV) 1/16</td>
</tr>
<tr>
<td></td>
<td>PBS-Umu 16/19</td>
<td>α3 Unknown 15/16</td>
</tr>
<tr>
<td></td>
<td>NASA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PBS-Gln 1/8</td>
<td>b1 5' UTR minus strand (MLEV) 1/1</td>
</tr>
<tr>
<td></td>
<td>PBS-Gly 0/8</td>
<td>b2 R-U5-mediated (MLEV) 3/7</td>
</tr>
<tr>
<td></td>
<td>PBS-Umu 7/8</td>
<td>b3 Unknown 4/7</td>
</tr>
<tr>
<td></td>
<td>NASA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PBS-Gln 0/11</td>
<td>c1 5' UTR minus strand (VL30) 1/1</td>
</tr>
<tr>
<td></td>
<td>PBS-Gly 1/11</td>
<td>c2 R-U5-mediated (MLEV) 2/10</td>
</tr>
<tr>
<td></td>
<td>PBS-Umu 10/11</td>
<td>c3 Unknown 8/10</td>
</tr>
</tbody>
</table>

**Fig. 2.** Transduction of PBS- and Ψ-modified MLV vectors. Wild-type PBS-Pro sequences were in AkvΨ-, VL30Ψ- and AkvΨΔKL-based vectors exchanged with the nonfunctional PBS-Umu by site-specific mutagenesis. Ratios of transduced PBS sequences are given as numbers of transduced proviral sequences, PBS–Gln (from MLEV) (Mikkelsen et al., 1996), PBS–Gly (from endogenous VL30) or PBS–Umu (from vector) relative to the total number of single colonies analysed. The transduction pathway was determined by sequencing of relevant proviral segments (see text for details). The recombination partners are given in parentheses below types of recombination. ‘Unknown’ refers to hitherto unidentified rescue pathways.

Rare recombination events detected by PCR screening of colony pools

Since the VL30 Ψ-based PBS–Umu vector may be rescued through 5' UTR minus-strand recombination with endogenous VL30 (Fig. 3b), we next set out to elucidate whether Akv Ψ-based vectors (PBSUmu244Ψ and PBSUmuAKL) in rare cases can be rescued through genetic interactions with copackaged endogenous VL30 RNA. To this end, we performed multiple transduction series of Akv-derived vectors harbouring PBS–Umu and subsequently pooled all G418-resistant NIH 3T3 cells for PCR screening.
Fig. 3. For legend see facing page.
Table 1. Recombinant proviral fragments obtained by PCR-based screening of colony pools

<table>
<thead>
<tr>
<th>Vector construct</th>
<th>No. of colony pools</th>
<th>Average no. of colonies per dish</th>
<th>Recombination with MLEV</th>
<th>Recombination with endogenous VL30</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPBSUmu244Ψ</td>
<td>31</td>
<td>25</td>
<td>15/31 R–PBSGln–neo*</td>
<td>0/31 neo–U5§</td>
</tr>
<tr>
<td>pPBSUmuAKL</td>
<td>27</td>
<td>9</td>
<td>8/27 R–PBSUmu†</td>
<td>1/27 neo–U5§§</td>
</tr>
</tbody>
</table>

* PBSGln–neo, primers ON7 and ON2; indicative of 5’ UTR minus-strand recombination with MLEV.
† R–PBSUmu, primers ON9 and ON10; indicative of genetic interactions with MLEV facilitated by PBS read-through and subsequent R–U5-mediated second-strand transfer.
‡ PBSGly–neo, primers ON8 and ON2; indicative of 5’ UTR minus-strand recombination with endogenous VL30 harbouring PBS–Gly.
§ neo–U5 (3’ LTR), primers ON5 and ON11; indicative of 5’ UTR minus-strand or second-strand transfer recombination with endogenous VL30.
|| Indicative of R–U5-mediated second-strand transfer recombination, since no PBSGly–neo fragment was obtained by screening colony pools.
¶ Proviral sequence and cross-over sites are given in Fig. 4.
# By PCR screening, evidence for 5’ UTR minus-strand recombination with VL30 was found within the same pool. Considering the rarity of recombination with VL30, VL30-derived sequences found in both the 5’ UTR and the 3’ LTR most likely originated from the same clone and thus the same event of transduction (5’ UTR minus-strand recombination).

In initial control experiments, colony pools were screened by PCR with MLEV- and vector-specific primers to detect rescue of PBS-impaired vectors by genetic interactions with MLEV. With primers matching PBS–Gln and sequences within neo, template switching within the 5’ UTR (Fig. 3a) was registered in 15 out of 31 pools (representing almost 800 transduction events; 31 × 25 colonies, Table 1) for PBSUmu244Ψ and in eight out of 27 pools (approximately 250 transduction events; 27 × 9 colonies, Table 1) for PBSUmuAKL. Proviruses that originate through PBS read-through and subsequent R–U5-mediated second-strand transfer (Fig. 3c) are most often characterized by harbouring the original mutated PBS flanked upstream by ERV-derived R and U5 sequences (Mikkelsen et al., 1998a). By use of primers specifically matching PBS–Umu and genetic MLEV markers within the R region, several examples of such recombinant proviruses could as expected be found among the transduced proviral sequences (Table 1).

To test for exchange of the impaired PBS of Akv Ψ-based...
Fig. 4. Nonhomologous minus-strand recombination. Schematic representation of template switching events and position of cross-over sites identified by PCR-screening of colony pools (Table 1). Minus-strand DNA synthesis is initiated on copackaged endogenous VL30 RNA. Following homologous transfer of minus-strand strong-stop DNA, nonhomologous template switching is forced prior to copying of neo. Details of the strand transfer reaction are given in the right panel (3' UTR minus-strand recombination). PBS–Gly complementarity in the second-strand transfer of reverse transcription is obtained by nonhomologous strand transfer within the 5' UTR after copying of the neo gene. Cross-over occurs from a donor template site within the bacterial Tn5 promoter upstream of neo to an acceptor site within the VL30 Ψ region (details given in the left panel; 5' UTR minus-strand recombination). VL30 sequences given in left and right panels were obtained by RT–PCR on virion RNA followed by sequence analysis and were from Adams et al. (1988), respectively. Relevant sequences of donor and acceptor templates and the recombinant proviral sequences are given at the bottom of each panel. Hatched boxes indicate the 2 nt sequence identity regions at the cross-over sites. Thin lines, RNA; thick lines, DNA; dotted lines, template shift.

Sequences of VL30 origin within the 3' LTR were detected in one of 31 PBSUmu244Ψ colony pools (Table 1) and in one of 27 PBSUmuAKL pools (Table 1 and Fig. 4), indicative of initiation of minus-strand DNA synthesis on VL30 and transfer of resulting strong-stop DNA to the 3' end of either vector or VL30 RNA. Sequence analysis of the amplified provirus fragments showed that the VL30 3'-end in both cases served as acceptor template in transfer of VL30-derived minus-strand strong-stop DNA, possibly reflecting that transfer to the nonhomologous R region of Akv was disfavoured. Intrastrand transfer was thus followed by a template switch to allow minus-strand synthesis through the neo gene (Fig. 4, right panel). Also in this event of nonhomologous recombination, 2 nt sequence identity between donor and acceptor template mediated strand transfer (Fig. 4). Endogenous VL30-derived PBS–Gly and 3' LTR sequences were detected within the same pool of colonies. Considering the rarity of recombination with within the 5' UTR make this type of VL30-based recombinational repair less frequent.
VL30, these sequences most likely originated from the same provirus generated by 5’ UTR minus-strand recombination with VL30.

**Discussion**

Within the framework of a defined recombination system, we seek in this report to determine whether template switching between retrovirus-derived sequences can be modulated by altering structural features of the primary packaging and dimerization element. We show that PBS-modified MLV vectors harbouring Akv- or VL30-derived Ψ elements, some deficient of the kissing-loop dimerization domain, are rescued by initiation of reverse transcription on MLEV or VL30 genomic RNAs copackaged with vector RNA into Ψ-2-derived virus particles and subsequent (i) template switching of growing minus-strand DNA within the 5’ UTR or (ii) read-through of the PBS followed by R–U5-mediated second-strand transfer.

In single-cycle vector replication, deletion of the MLV kissing-loop leads to a 70- and 20-fold titre reduction for PBS–Pro and PBS–Umu vectors, respectively. Previous studies of RNA dimer formation have suggested that the kissing stem–loop in MLV and HIV-1 is crucial for dimerization in vitro (Clever et al., 1996; Girard et al., 1995; Laughrea & Jette, 1994; Paillart et al., 1994; Prats et al., 1990; Skripkin et al., 1994), but not essential for virus replication in vivo (Berkhout & van Wamel, 1996; Prats et al., 1995; Laughrea & Jette, 1994; Paillart et al., 1996a). It remains uncertain whether RNA dimerization is crucial for packaging and whether copackaged monomers subsequently may serve as functional substrates for reverse transcription and recombination. We show here that homologous recombination between Akv and MLEV can occur within the 5’ UTR despite kissing-loop nonhomology, supporting the notion that the kissing-loop, albeit serving as a recombination hotspot sequence within the 5’ UTR window (Mikkelsen et al., 1996, 1998b), is not an essential cis-element for retrovirus recombination. Also, we demonstrate that virus genomes harbouring distinct Ψ regions may interact during events of R–U5-mediated second-strand transfer, supporting that a lack of identity within the putative packaging and dimerization elements of heterologous recombination partners (Akv–VL30 and VL30–MLEV, respectively), does not impede a recombination-based exchange of genetic information at alternative sites of the two copackaged genomes. Interestingly, St Louis et al. (1998) recently reported that nonhomology within kissing-loop sequences of different HIV-1 subtypes does not constitute a major obstacle to recombination following postulated RNA heterodimer formation, and thus that a direct loop–loop interaction is not a prerequisite for template switching at alternative sites of the genome. If dimer formation is indeed required for RNA packaging, these and our findings may infer that intermolecular RNA interactions elsewhere in the genome outside the leader region can assist or partly assume a heterodimerization function, facilitating in the present report Akv vector recombination with MLEV or VL30. Previous work with HIV-1 and MLV-based systems seems to support this notion (Berkhout & van Wamel, 1996; Tchénio & Heidmann, 1995).

One single event of 5’ UTR minus-strand recombination with endogenous VL30 was detected by PCR screening of more than 1000 transduced Akv 244Ψ and ΔKL proviruses. This provirus was generated by synthesis of minus-strand strong stop on VL30, intrasubstrate transfer, nonhomologous switching of the nascent minus-strand to the vector, subsequent copying of neo, and nonhomologous template switching within the 5’ UTR to obtain perfect PBS complementarity in second-strand transfer (Fig. 4). The recombinant provirus was characterized by sequence analysis of PCR amplicons generated from the same colony pool in two distinct PCR reactions used in screening of multiple transduced sequences (Table 1) and we can therefore rule out that the detected event was a result of recombination during PCR amplification. This finding thus indicates that a modification of the PBS can be overcome by a transductional repair pathway involving (i) copackaging of two heterologous RNA templates, (ii) homologous intramolecular minus-strand strong-stop transfer, and (iii) nonhomologous template switching events downstream from neo and subsequently within the 5’ UTR.

Torrent et al. (1994) previously reported that a small packaging signal derived from rat VL30 facilitates efficient encapsidation of recombinant RNA into MLV particles. Insertion of non-murine heterologous packaging and dimerization sequences into retrovirus vectors may improve safety by rendering the vectors less recombinogenic. Nonhomologous minus-strand DNA transfer has previously been found to mediate recombination between distinct viruses harbouring similar packaging signals and divergent R regions (Yin et al., 1997). In addition, vector RNAs harbouring encapsidation elements of distinct retrovirus origins have been found to interact genetically during minus-strand synthesis, suggesting that homology within the presumed primary dimerization region is dispensable for the generation of recombinant proviruses (Yin & Hu, 1997). Expanding on these crucial observations, we show here that copackaging of Ψ- and R-distinct retrovirus-derived sequences followed by two events of nonhomologous template switching can lead to the generation of PBS-repaired Akv–VL30 chimaeric proviruses. This example of complex genetic interactions during reverse transcription illustrates how recombination between unrelated retrovirus species harbouring distinct PBS, Ψ elements and R regions may have contributed to retrovirus evolution. In consideration of the lack of sequence similarity between Akv and VL30, any two copackaged retrovirus-derived sequences will probably be able to interact genetically and, at present, there remains an open question of whether a specific dimerization process is required for this interaction. Based on these observations, it seems unlikely that specific alterations intro-
duced into a retrovirus vector are capable of rendering the vector nonrecombinogenic without disturbing crucial cis-acting functions required for efficient vector transduction.

The possibility exists that development and outgrowth of novel replication-competent retroviruses occur through recombination between viruses that replicate or are present endogenously in different animal species. Creation of such chimaeric viruses requires co-encapsidation of heterologous viral RNAs and subsequent recombination during reverse transcription. We believe that forced recombinational rescue of PBS-impaired retrovirus vectors constitutes a powerful tool to investigate whether retroviruses of different species interact genetically and hold the potential to form novel biologically active retroviruses. Forced recombination assays may thus be utilized to study rare genetic interactions between heterologous RNAs as well as to detect unknown ERV sequences (e.g. in human cells) that harbour functional cis-elements, some of which may constitute important sequence patches in repair of retrovirus vectors.

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