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Specific packaging of spliced retroviral vector transcripts lacking the Ψ-region

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Abstract

The effect of a cryptic splice acceptor (cSA) site located at the end of the extended packaging signal in murine leukemia virus (MLV)-based vectors was investigated. Although this cSA is also present in wild type MLV, it was found to result in a smaller transcript in which the packaging signal (Ψ) had been removed by splicing only in MLV-derived vectors. Splicing occurs both in packaging cells producing the MLV-vectors as well as in the infected target cells. Transcripts lacking the Ψ-sequence (Ψ(−)) are packaged relatively efficiently into virus particles, even in the presence of wild type Ψ(+)-vector transcripts. The Ψ(−)-viral RNA is reverse transcribed in vector transduced cells as is any other retroviral genome. The titer obtained from the Ψ(−)-vector was only 1000-fold reduced in comparison to the same Ψ(+)-vector. These results suggest that Ψ(−)-transcripts may be packaged more frequently than previously supposed and that splicing patterns should be carefully analysed on an individual basis for retroviral vectors used in gene therapy.

Keywords: Gene therapy; Retroviral vector; Splicing; Packaging signal; Titer

Retroviral vectors based on murine leukemia virus (MLV) are the most commonly used gene delivery vehicles in gene therapy clinical trials, being employed in almost 70% of approved protocols [1,2]. To date, however, many of the biochemical and genetic properties of MLV are still not completely understood. Although production of replication defective vectors for gene therapy without the use of helper virus is possible due to the availability of retroviral packaging cell lines [3,4], generation of replication competent virus by recombination or packaging of retroviral genes not included in the vector is still possible and therefore a safety issue.

Packaging of retroviral genomes into virus particles is dependent upon the specific interaction of certain regions on the viral genomic RNA with the nucleocapsid protein domain of the Gag precursor protein [5]. At least for MLV, a distinct region located downstream of the *env* splice donor (SD) site and just upstream from the initiation codon of $Pr65^{gag}$ is a requirement for packaging [6]. This region, the packaging signal or psi (Ψ), spans a sequence of about 350 nucleotides (nt) between MLV RNA position 215 and 565. The first 135 nt of this sequence are described as being absolutely essential for packaging MLV RNA into virions [7,8]. Viral titers are not only dependent on the presence but also on the position of the packaging sequence [7].

Most MLV-based retroviral vectors which are widely used for somatic gene transfer, such as the LN-vector family [9], contain the extended packaging or Ψ(+)-signal [10], which is composed of the 350 basepair (bp) sequence described above and an additional facultative 472 bp stretch derived from the *gag* coding sequence between MLV RNA position 565 and 1036. The presence of these extra sequences encoding part of the viral *gag* gene in MLV-based vectors results in the generation of 10–40-fold higher virus titers [11,12]. Introduction of this 822 (350 + 471) nucleotide stretch of the MLV genome, starting just downstream of the *env* SD and

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extending into *gag*, into a heterologous DNA sequence is sufficient to direct efficient packaging of the respective transcript [12,13]. Experiments using in vitro transcribed RNA containing the Ψ-signal suggest a four stem-loop structure between the *env* SD and the *gag* initiation codon as being important [14,15]. A reduced encapsidation efficiency was observed after deletion of individual motifs, but deletion of all four stem-loops in one single mutant results in almost complete loss of encapsidation of viral RNA [14].

Despite the presence of the *env* SD, retroviral vector transcripts are thought not to be spliced due to a lack of a downstream splice acceptor (SA) sequence in most vector constructs. However, splicing has been shown to improve gene expression by facilitating the nuclear export of messenger RNA [16]. Yu and colleagues [17], for example, have shown that even the introduction of heterologous SA sequences into retroviral vectors significantly increases expression of the transferred gene. Therefore, in order to analyse the effect of splicing on gene expression and titers of retroviral vectors, we introduced an artificial splice acceptor (aSA) sequence identical to the original MLV *env* SA 5′ of an enhanced green fluorescent protein (eGFP) encoding reporter gene. RT-PCR analysis of obtained transcripts, however, revealed preferential usage of a cryptic splice acceptor (cSA) sequence four nucleotides from the end of the extended packaging signal rather than the introduced artificial SA.

Here, we report the characterization of this cSA sequence which is not functional in wild type MLV but active in the commonly used LN-retroviral vector family. Splicing from the *env* SD to the cSA caused the removal of the packaging signal in a significant percentage of retroviral vector transcripts. We show that these spliced Ψ-lacking transcripts are packaged into viral particles without co-packaging of Ψ(+)-full length genomic RNA. Titers of Ψ(−)-vector RNA viruses were only about 1000-fold reduced compared to Ψ(+)-viral vectors. These results indicate that packaging of Ψ-lacking transcripts into retroviral particles might occur more frequently than previously thought and should be taken into account especially in gene therapy approaches.

Materials and methods

Plasmid construction. To generate plasmid pKP1, a 60 bp double-stranded oligo-nucleotide linker was synthesized (5′-AATTGA GATCTTCCCTGACCCTGACATGACAAGAGTACGCGTCAGC CCCTCTCTCCA**AGG**TCGA-3′) carrying an aSA (bold) sequence and an *Mfe*I and *Sal*I site (underlined) at the 5′ and 3′ end, respectively. This linker was inserted into the 6747 bp *Eco*RI/*Sal*I-fragment of pLXSNeGFP, an MLV-based retroviral vector containing an eGFP reporter gene and an SV40 promoter/neomycin resistance gene expression cassette [18]. Plasmid pKP2 was generated by ligating the 5782 bp *Apo*I-fragment of plasmid pKP1 to the 750 bp *Eco*RI-fragment of plasmid pGTLTRΨ. This plasmid was obtained from the PCR-cloning vector pGEM-T Easy (Promega) by inserting a 732 bp PCR-fragment amplified from plasmid pLXSNeGFP using the oligonucleotide *LTR* (5′-TTCTGCCT CTTAGACCACTC-3′) as forward and *SD* (5′-AGCTTACCTCACGGTGTGGGGTCGGTGGTC CCTGG-3′) as reverse primer. To generate plasmid pKP3, RT-PCR using a sense primer (5′-TGCATCCGAATCGTGGTCTC-3′) complementary to the R-region of vector LXSNeGFP and a reverse primer (5′-TCGCCGGACACGCTGAAC TT-3′) complementary to the eGFP-encoding region was performed with viral RNA isolated from cell culture supernatants of pKP2-transfected packaging cells. An amplified fragment corresponding to the spliced KP2 message was digested with *Dra*III and *Mlu*I (pos. 160 and 752 in unspliced KP2 vector mRNA), producing a 56 bp fragment containing the fused SD/SA sequence that was ligated to the 5933 bp *Dra*III/*Mlu*I-fragment of pKP2.

To generate plasmids pSDmut and pSDSAmut, the GeneEditor in vitro site-directed mutagenesis system (Promega) was used as recommended by the manufacturer. Briefly, following alkaline denaturation of plasmid pLXSNeGFP to obtain single-stranded DNA, an annealing reaction including the appropriate phosphorylated mutagenic oligonucleotide (5′-CCACCACCGGGATTTAAGCTGGCCAGC-3′ for SD mutation and 5′-CACTCCTTCTCTTCGCGCCGGAATTCTG-3′ for the cSA mutation) and the bottom strand selection oligonucleotide (Promega) was performed. After mutant strand synthesis and ligation, 1.5 μl of the mutagenesis reaction was transformed into BMH 71-18 *mut*S competent cells (Promega), which were then grown overnight in 4 ml LB containing 50 μl of the GeneEditor antibiotic selection mix. Plasmid DNA was isolated and re-transformed into JM109 competent cells (Promega). Hundered microlitres of bacteria was plated out and selected on ampicillin overnight. DNA of single colonies was extracted and analysed by restriction enzyme digests and sequence analysis. In general, cloning junctions of all plasmids used were sequenced to confirm their structural integrity.

Cell culture, transfection, and infection. Human 2GP19Talf amphotropic retroviral packaging cells (293 human embryonic kidney cells [19], ATCC CRL-1573) stably transfected with pGagPolGpt [20] and pALF [21], murine PA317 packaging cells [3], HeLa cells [22] (ATCC CCL-2), and NIH3T3 cells [23] (ATCC CRL-1658) were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 5% fetal calf serum (Invitrogen) under an atmosphere of 95% humidity and 5% CO_2. Culture medium used for G418-selection additionally contained 400 μg/ml geneticin (Invitrogen).

Twenty-four hours prior to transfection, 5×10^5 packaging cells per well were seeded into 6-well plates. Two to four micrograms of recombinant plasmid DNA was introduced into the cells by calcium phosphate coprecipitation as recommended by the supplier (Amersham Pharmacia Biotech). In order to generate stable virus producing populations, cells were washed twice with PBS, trypsinized and expanded into one T80 flask 18 h post transfection. Twenty-four hours later, normal culture medium was replaced by selection medium.

For infections, 1 ml of culture supernatant from 1×10^6 virus producing cells was filtered through a 0.45 μm filter (Sarstedt) and used to transduce 4×10^5 HeLa or NIH3T3 cells. These target cells were incubated for 6 h with the virus containing supernatant in the presence of 8 μg/ml polybrene (Sigma), then 3 ml of fresh medium was added and the cells incubated overnight.

Titer analysis. To obtain single cell clones, cells were trypsinized 24 h post infection, diluted and subjected to G418-selection. Two weeks later, G418-resistant colonies were counted. For further experiments, single colonies were isolated and expanded. Viral titers were either determined by number of G418-resistant colonies or by number of eGFP-positive cells measured 48 h after infection via fluorescence activated cell sorting (FACS Calibur, Becton Dickinson).

PCR and RT-PCR analysis. DNA was prepared using the QIAamp Tissue Kit (Qiagen), cellular RNA was extracted using the RNeasy Kit (Qiagen) as recommended by the manufacturer. For the preparation of

viral RNA, 14 ml of 24 h conditioned medium from a T175 flask of confluent virus producing cells was filtered through a 0.45 μm filter (Sarstedt) and centrifuged at 36,000*g* for 1 h at 4 °C. The virus pellet was resuspended in 300 μl lysis buffer (1% Triton × 100, 0.5% sodium deoxycholate, 0.1% SDS), mixed with 10 μg yeast tRNA (Sigma), purified by phenol/chloroform extraction and precipitated with ethanol. The RNA pellet was resuspended in 5 μl of diethylpyrocarbonate (DEPC, Sigma) treated H_2O.

All PCR and RT-PCR analyses were performed using the same primer pair. The sense primer (5′-TGCATCCGAATCGTGGTCTC-3′) is complementary to the R-region of vector LXSNeGFP, the reverse primer (5′-TCGCCGGACACGCTGAACTT-3′) hybridizes to the eGFP-coding region. RT-PCR analyses were performed using the Titan one tube RT-PCR system (Roche Diagnostics) as follows: reverse transcription for 30 min at 50 °C followed by a denaturing step at 94 °C for 2 min. PCR was performed according to these parameters: 30 s at 94 °C, 30 s at 57 °C, and 45 s at 68 °C for 10 cycles and 30 s at 94 °C, 30 s at 57 °C, and 45 s at 68 °C (+5 s cycle elongation) for 25 cycles. The amplified DNA fragments were analysed by agarose gel electrophoresis.

Southern blot analysis. Fifteen to twenty micrograms of genomic DNA was digested overnight with *Sac*I and then subjected to agarose gel electrophoresis. Subsequently, the DNA was depurinated in 0.25 M HCl, denatured in 0.5 M NaOH and transferred to a Zeta-Probe GT membrane (Bio-Rad) via capillary transfer with 10× SSC (1.5 M NaCl, 0.15 M Na-citrate). The membrane was prehybridized at 65°C in 0.25 M Na_2HPO_4/7% SDS, pH 7.2 for 10 min. For hybridization, 10^6 Cerenkov cpm per ml of a ^{32}P-labelled eGFP-gene-specific probe (specific radioactivity: 10^8–10^9 Cerenkov cpm per μg of DNA) was added and the membrane was incubated at 65 °C overnight. Subsequently, the membrane was rinsed for 20 min in 20 mM Na_2HPO_4/5% SDS, pH 7.2 and for another 20 min in 20 mM Na_2HPO_4/1% SDS, pH 7.2, both at 65 °C, and finally analysed using a phosphorimager system (Storm 860, Amersham Pharmacia Biotech).

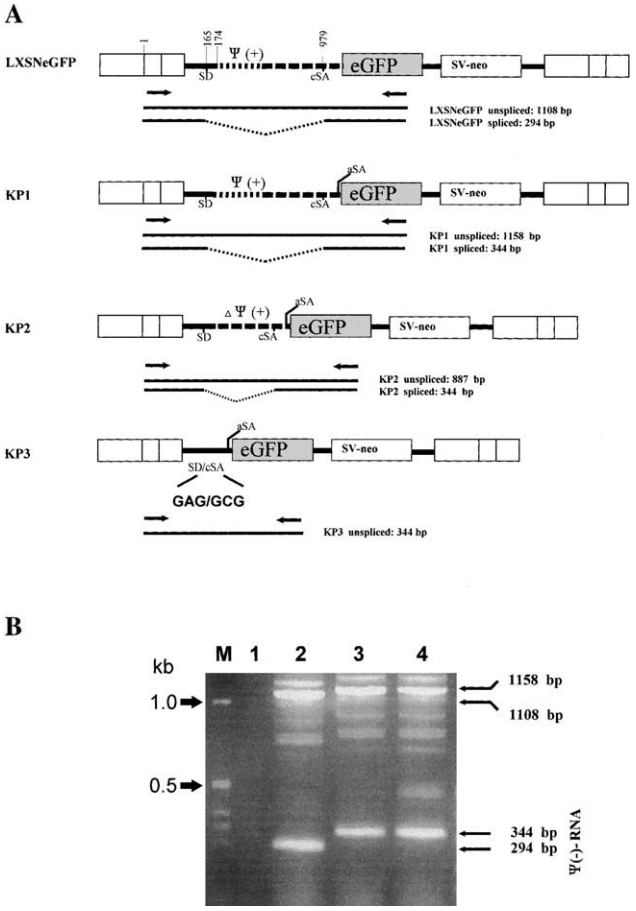

Fig. 1. (A) Schematic proviral structure of vectors KP1, KP2, and KP3. In all of the LXSNeGFP-derived vectors, a cSA at LXSNeGFP RNA position 979 is present in addition to a newly introduced artificial SA. Vector KP1 contains the extended packaging sequence Ψ(+) (806 bp from MLV nt 174 to 978 of LXSNeGFP RNA). In KP2, the first 271 bp of Ψ(+) are deleted including the 135 bp fragment known to be essential for packaging. In KP3 all packaging sequences were deleted by fusing the *env* SD sequence directly to the cSA, mimicking a spliced message. SD = splice donor; aSA = artificial splice acceptor; cSA = cryptic splice acceptor; Ψ(+) = extended packaging sequence; eGFP = enhanced green fluorescent protein gene; SV-neo = SV40-neomycin resistance gene expression cassette; numbering is according to LXSN RNA sequence (GB Accession number M28248) [9]. (B) RT-PCR analysis of retroviral vector-derived spliced transcripts. RNA isolated from murine packaging cells transfected with pLXSNeGFP (lane 2) or pKP1 (lane 3) or from HeLa cells infected with KP1 virus particles (lane 4) was analysed by RT-PCR (positions of the respective primers are indicated in Fig. 1A). In all samples a larger fragment of about 1.1 kb, corresponding to an unspliced transcript, as well as a small 0.3 kb fragment was amplified. The presence of the 294 bp fragment in lane 2 and a 344 bp fragment in lanes 3 and 4 indicates transcripts generated by splicing from the *env* SD to the cSA. As a control, RNA analysis of non-transduced HeLa cells is shown in lane 1. One microgram of a molecular weight DNA standard (1 kb DNA ladder, Invitrogen/Life Technologies) was applied in lane M.

Results

Characterization of a cryptic splice acceptor adjacent to the 3′-end of Ψ(+)

Most MLV-based retroviral vectors contain an SD site, which is utilized in the original replication competent virus for production of the Env-encoding transcript [24]. This SD is located between the 5′-LTR and the extended packaging signal [24]. Retroviral vectors, however, usually lack the viral *env* splice acceptor (SA) sequence and thus do not facilitate splicing. It has previously been reported that vectors which carry a heterologous intron and are able to splice show improved gene expression [17,25].

In order to investigate the effects of splicing on expression of transferred genes and titers of retroviral vectors, we introduced an artificial splice acceptor (aSA) into the MLV-based eGFP-encoding retroviral vector pLXSNeGFP [18]. An oligonucleotide containing the branchpoint and the splice acceptor sequence identical to the MLV *env* SA [24] was inserted 5′ of the eGFP reporter gene giving rise to the plasmid pKP1 (Fig. 1A). Human (2GP19Talf) and murine (PA317) packaging cells were transfected with pKP1 and transcribed RNA was analysed for spliced products by applying RT-PCR using primers specific for the R-region and the eGFP gene, respectively (Fig. 1A). Surprisingly, the results of this analysis revealed that a cSA site, located upstream of the aSA, i.e., 815 bp downstream of the *env* SD in vector LXSNeGFP (Fig. 1A), is preferentially used.

RT-PCR of RNA isolated from pKP1-transfected packaging cells shows two major products: the larger one with a calculated length of 1158 bp corresponds to an unspliced transcript (Fig. 1B, lane 3, upper band); the smaller product appeared to be about 50 bp larger (344 bp, lane 3) than calculated for a product derived from a transcript spliced to the artificial SA. In addition, analysis of RNA isolated from pLXSNeGFP-transfected packaging cells not only revealed the expected product corresponding to the unspliced message (Fig. 1B, lane 2, 1108 bp) but also a small band of 294 bp in length, demonstrating that the cSA is also active in this context. Finally, an identical splicing pattern was detected with RNA isolated from HeLa cells which had been transduced with recombinant KP1 virus (Fig. 1B, lane 4). RNA from mock infected cells did not produce any signals (Fig. 1B, lane 1). Usage of the cSA instead of the newly introduced aSA was demonstrated by DNA sequence analysis of isolated RT-PCR products (data not shown). Although the cSA is present in the wild type MLV sequence (nt position 1032 of MLV RNA [24]), RT-PCR analysis of MLV-infected NIH3T3 cells did not yield any corresponding product (data not shown). Alignment of database sequences revealed that this cSA site is present in retroviral vectors which carry the extended packaging signal [9–11,25]. As demonstrated for pLXSNeGFP and pKP1, splicing to this cSA site does occur and would, due to its position, lead to viral vector genomes lacking the Ψ-signal.

Ψ(−)-viral vector RNA is packaged

To analyse whether RNA with a shortened or even no packaging signal can be packaged, the vectors KP2 and KP3 were constructed (Fig. 1A). Plasmid pKP2 is missing the first 271 bp of Ψ(+), including the 135 bp described as being essential for packaging into virions [10]. The remaining packaging sequences are still flanked by the *env* SD and the cSA, respectively. In plasmid pKP3, the SD and the cSA sequences have been fused. Thus, the viral RNA derived from this construct mimics a spliced message and therefore contains no Ψ-signal sequences at all (Fig. 1A).

Packaging cell lines PA317 and 2GP19Talf were transfected with plasmids pKP1, pKP2, and pKP3, respectively. After G418-selection stable transfected populations were obtained. Subsequently, RNA was extracted from virus producing cells as well as from purified viral particles and RT-PCR analyses were performed using primers flanking the packaging signal (Fig. 1A). PCR products derived from cellular RNA corresponded both to full-length and spliced transcripts in pKP1- and pKP2-transfected cell samples with clearly higher levels of unspliced compared to spliced messages (Fig. 2A, lane 1: 1158 bp band compared to 344 bp band; lane 2: 887 bp product compared to 344 bp band).

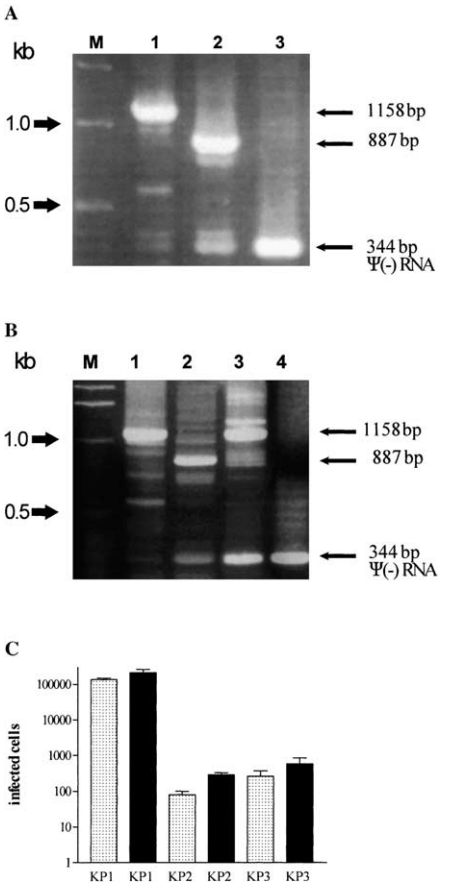

Fig. 2. (A) RT-PCR analysis of transcripts derived from KP1-, KP2-, and KP3-retroviral vectors. RNA isolated from transfected human 2GP19Talf virus producer cells was subjected to RT-PCR. Lane 1 shows the products generated from RNA of pKP1-transfected cells. A 1158 bp and a 344 bp fragments were amplified, corresponding to the unspliced and spliced Ψ(−)-RNA transcripts, respectively. In lane 2, the pKP2-derived full-length RT-PCR product is only 887 bp in length due to the 271 bp deletion in the Ψ region (compare Fig. 1A). Again, a 344 bp fragment corresponding to the respective spliced Ψ(−)-transcript is detected. In packaging cells, carrying the vector pKP3 lacking any intervening sequences, a single transcript giving rise to a 344 bp fragment is detected (lane 3). Lane M shows a molecular weight DNA standard (1 kb DNA ladder, Invitrogen). (B) RT-PCR analysis of RNA isolated from KP1-, KP2-, and KP3-virions. Virus particles were purified from cell culture supernatants of stably transfected 2GP19Talf-packaging cells, RNA was isolated and again subjected to RT-PCR. The result for KP1-virions is shown in lane 1. The strong 1158 bp band represents unspliced KP1-RNA. A faint 344 bp band is visible, indicating that spliced Ψ(−)-RNA is also packaged into virus particles. RNA derived from KP2-virions (lane 2) gave rise to a 887 bp fragment corresponding to the full-length RNA and a 344 bp fragment representing the spliced transcript. RT-PCR of RNA isolated from KP3-viral particles resulted in a single product, indicating that also transcripts completely lacking the Ψ-sequence are packaged (lane 4). As a positive control, RT-PCR was applied to RNA extracted from packaging cells stably transfected with pKP1 (lane 3). A molecular weight DNA standard (1 kb DNA ladder, Invitrogen) is shown in lane M. (C) Titer analyses. 4×10^5 HeLa cells were infected with the virus containing cell culture supernatants of pKP1-, pKP2-, or pKP3-transfected 2GP19Talf packaging cells. Dotted bars reflect the number of infected HeLa cells determined via FACS analysis, black bars indicate the number of colonies obtained after two weeks of G418-selection. All experiments were performed at least three times.

As expected, only the short 344 bp band mimicking the spliced message was detected in KP3 producer cells (Fig. 2A, lane 3).

Surprisingly, when packaged viral RNA was investigated, shorter spliced messages were also detected in addition to unspliced full length transcripts in virions derived from pKP1- (Fig. 2B, lane 1) and pKP2-transfected packaging cells (Fig. 2B, lane 2). Most interestingly, the KP3 viral vector genome which does not contain any Ψ-signal sequences also appeared to be effectively packaged (Fig. 2B, lane 4). DNA sequence analysis of the obtained RT-PCR products in each case confirmed that the corresponding viral genome was indeed lacking the packaging signal (data not shown).

To further analyse whether virions carrying Ψ(−)-viral genomes are infectious, culture supernatants of human 2GP19Talf packaging cells stably producing KP1-, KP2-, or KP3-virus particles were used to infect HeLa or NIH3T3 cells. Forty-eight hours post infection viral titers were determined by measuring eGFP expression in infected cells using FACS analysis. Viral titers were obtained in the range of $1.4–2.0 \times 10^5$ (50% infected cells) for KP1-transduced HeLa and NIH3T3 cells, respectively (Fig. 2C, KP1 dotted bar). Transduction experiments using KP2 and KP3 viruses, both lacking the essential packaging sequences, still yielded titers of $0.8–2.9 \times 10^2$ (Fig. 2C, KP2 and KP3, dotted bars). Using G418-selection of infected cells for titer analysis, similar results were obtained (Fig. 2C, black bars).

Finally, Southern blot analyses were performed to investigate whether both full length viral KP2 RNA and viral RNA lacking the complete packaging signal underwent correct reverse transcription and integration. Chromosomal DNA from KP2- and KP3-infected HeLa cells was digested with *Sac*I which cuts in the LTR of the retroviral vector, separated by agarose gel electrophoresis, blotted and hybridized to an eGFP-gene specific probe (Fig. 3A). In one of the three analysed KP2-infected HeLa cell clones a complete, integrated full length vector DNA (3412 bp) was detected (Fig. 3A, lane 2). DNA obtained from the two other KP2 cell clones gave rise to a discrete smaller band, the mobility of which is in accordance with a reverse transcribed spliced message (Fig. 3A, lanes 1 and 3, 2869 bp). Southern blot analysis

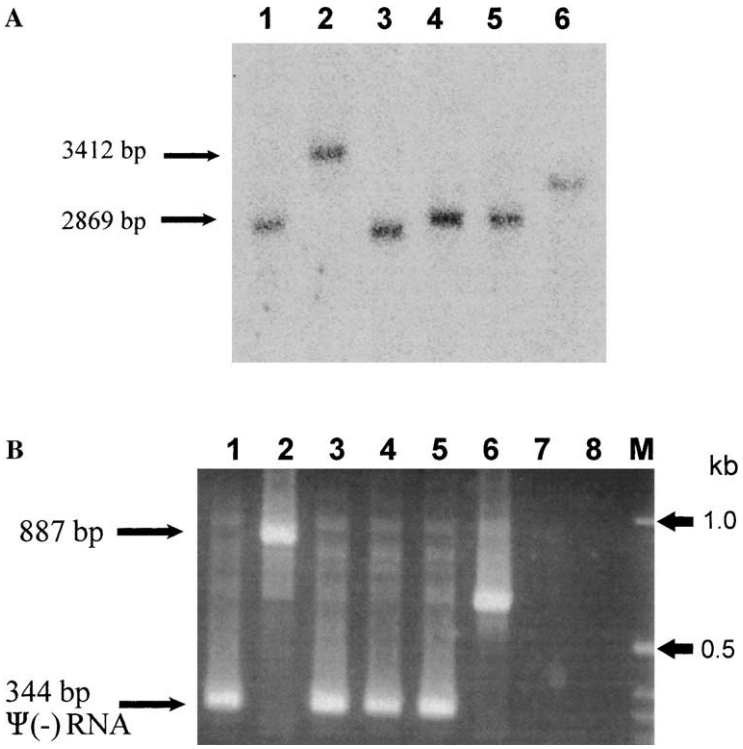

Fig. 3. (A) Southern blot analysis of retroviral vector-transduced cells. Twenty micrograms of chromosomal DNA isolated from distinct KP2- or KP3-infected HeLa cell clones was digested overnight with *Sac*I, separated by agarose gel electrophoresis, transferred to a nylon membrane and finally hybridized to an eGFP-gene-specific probe. DNA applied in lanes 1, 2, and 3 was obtained from HeLa cell clones infected with KP2-viruses, lanes 4, 5, and 6 contain DNA extracted from KP3-infected cell clones. The sizes of the expected fragments that correspond to integrated proviruses derived either from unspliced or spliced genomic RNA are indicated by arrows. (B) PCR analysis of chromosomal DNA of transduced HeLa cell clones. Fifty nanograms of chromosomal DNA isolated from KP2 (lanes 1, 2, and 3) or KP3 (lanes 4, 5, and 6)-infected HeLa cell clones are shown in Fig. 3A were further analysed by PCR using primers binding to the R-region and to the eGFP-encoding region of the retroviral vectors (Fig. 1A), respectively. As a control, PCR was performed with DNA isolated from non-transduced HeLa cells (lane 7). Neither this nor the mock reaction (lane 8) reveals any unspecific signals. A molecular weight DNA standard (1 kb DNA ladder, Invitrogen) is shown in lane M.

of three KP3-infected HeLa cell clones showed the correct size for a complete integrated vector in two cases (Fig. 3A, lanes 4 and 5), whereas a third clone (KP3/3, Fig. 3A, lane 6) gave rise to a fragment about 300 bp larger than expected. PCR analyses with genomic DNA of these KP2- and KP3-transduced HeLa cell clones using primers binding upstream and downstream of the SD and the cSA site, respectively, confirmed the integrity of this region (Fig. 3B, lanes 1-6). DNA from cell clones carrying a spliced $\Psi(-)$-vector genome gave rise to a 344 bp product (lanes 1, 3, 4, and 5), PCR with DNA containing a full length KP2 integrate showed a product of 887 bp in length (lane 2). Interestingly, in accordance with the Southern blot data, a larger fragment was also obtained in the PCR analysis with DNA isolated from clone KP3/3 (Fig. 3B, lane 6). DNA sequence analysis of this PCR fragment revealed that a 300 bp fragment of a previously described human sequence (accession number NCBI HS402G11) was recombined into the provirus exactly after the 5′-LTR. This fragment is part of a nearly 180 kb sequence containing various protein kinase encoding genes as well as ESTs, GSSs, and 15 putative CpG islands. The 300 bp fragment inserted in clone KP3/3 was not found to belong to any of the described genes.

Splicing improves retroviral vector titers

Splicing is generally associated with an improved transport of mRNA-molecules from the nucleus into the cytoplasm. However, removal of the packaging signal was shown to have adverse effects on retroviral vector titers. To investigate, whether inhibition of splicing while conserving the packaging signal would further affect retroviral vector titers, we mutated either the *env* SD or both, SD and the cSA site in vector pLXSNeGFP, respectively (Fig. 4A). The resulting plasmid pSDmut carries two G to T mutations in the AG/GT consensus sequence [exon-GGA(G → T)/(G → T)TAA-intron] in the splice donor, while the plasmid pSDSAmut carries, in addition to the mentioned SD mutation, an intron-CT(A → T)(G → C)/GCGC-exon cSA mutation. After stable transfection of 2GP19Talf packaging cells with the generated plasmids, culture supernatants were used to transduce HeLa cells and virus titers were evaluated by FACS analyses. The titer obtained with vector SDSAmut, where both the SD and cSA sites are mutated, is slightly reduced compared to wild type LXSNeGFP virus. In contrast, titers obtained with SDmut were found to be nearly three times higher (Fig. 4B) compared to LXSNeGFP, suggesting a positive effect of a 100% retained packaging signal. However, DNA sequence analysis following RT-PCR amplification of the transcribed SDmut RNA in packaging cells revealed that instead of the mutated *env* SD a cryptic splice donor site at nt position 752 of vector LXSNeGFP was activated

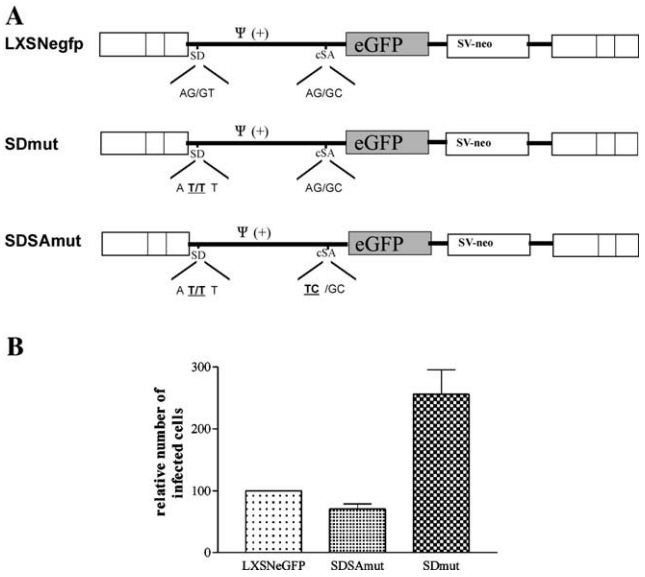

Fig. 4. (A) Schematic proviral structure of vectors SDmut and SDSAmut. Both vectors are based on LXSNeGFP. In vector SDmut, the *env* SD was mutated from AG/GT to AT/TT by site-directed mutagenesis. In vector SDSAmut, in addition the cSA site was also mutated. For this purpose, the sequence was changed from AG/GC to TC/GC. SD indicates the splice donor; cSA the cryptic splice acceptor; $\Psi(+)$ the extended packaging sequence; eGFP indicates the enhanced green fluorescent protein-encoding gene and SV-neo the SV40-neomycin resistance gene expression cassette. (B) Titer analysis of splicing defective retroviral vectors. Virus containing cell culture supernatants of pSDmut- and pSDSAmut-transfected human 2GP19Talf packaging cells was used to infect 4×10^5 HeLa cells. The number of infected cells was determined via FACS analysis and compared to the number of HeLa cells infected with wild type LXSNeGFP (set to 100).

and enabled splicing to the non-mutated cSA. In pSDSAmut-transfected cells activation of this cryptic SD was not observed. Thus, both spliced and unspliced transcripts of pSDmut carry at least the first 580 bp of $\Psi(+)$ which would in conclusion result in more efficient packaging finally contributing to higher viral titers.

Discussion

MLV-based retroviral vectors such as those of the LN- and MFG-series [26] carry a 471 bp *gag*-coding sequence after the packaging sequence Ψ, resulting in the extended packaging signal $\Psi(+)$. A previously noted, though not characterized, cSA site (cSA) [11] is located at the end of $\Psi(+)$ at MLV RNA position 1036 (Fig. 1B). Thus, in these retroviral vectors the $\Psi(+)$-region is flanked by splice recognition sites, possibly leading to the generation of spliced transcripts carrying no packaging signal. In the present study, RT-PCR of RNA isolated from retroviral vector-transfected human and murine packaging cells as well as from infected HeLa and NIH3T3 cells (data not shown) followed by DNA sequence analyses demonstrated that splicing to

this cSA does indeed occur both in producer and in transduced target cells. Interestingly, we could further show that this cSA is not functional in wild type MLV (data not shown). Subsequent RT-PCR analysis of genomic viral vector RNA also detected spliced transcripts in virus particles, demonstrating that these are packaged relatively efficiently despite lacking the packaging signal, although it is difficult to make a quantitative assessment of the actual amount of spliced transcripts packaged. Previous studies have suggested that Ψ(−)-transcripts are usually excluded from packaging into MLV particles [6,7,14,27]. In the case of vector KP1, which is similar to the original LXSNeGFP, the presence of Ψ(−)-RNA in virus particles might be explained by co-packaging with RNA carrying the packaging sequence (Fig. 2B, lane 1), although it is difficult to understand why Ψ(−)-RNA should be packaged at all in a system where there is an excess of Ψ(+)-RNA. Moreover, even retroviral vectors in which the first 280 bp of the packaging signal have been deleted (KP2, Fig. 1A) facilitate packaging of unspliced and spliced Ψ(−)-RNA into virus particles (Fig. 2B, lane 2). Finally, the vector construct mimicking a completely spliced transcript and thus entirely lacking the packaging signal (KP3, Fig. 1A) is efficiently packaged into infectious viral particles (Fig. 2B, lane 4). Although the mechanism is not clear yet, it seems to be likely that other regions of the retroviral genome may contribute to the recognition of viral RNA to be packaged (for review see [28]). These regions are suggested to form secondary structures that represent cryptic or facultative packaging signals. However, heterologous sequences with low sequence homology to the retroviral packaging signals may also form secondary structures mimicking structural motifs required for packaging [14]. This hypothesis is strengthened by data indicating that the substitution of the spleen necrosis virus packaging signal with MLV Ψ-sequences does not impair viral replication [29]. Thus, it seems likely that the encapsidation sequences of both viruses are recognized largely by their secondary and tertiary structures.

Viral RNA represents only a small fraction of total RNA in an infected cell (usually $< 1\%$), but is specifically packaged into virions [30]. The specificity has been shown to be mediated by the 350 nt packaging signal Ψ at the 5′-end of the MLV-genome. Non-specific packaging of heterologous endogenous retroviral transcripts or MLV-sequences lacking the Ψ-signal was thought to be a rare event, estimated to occur for around 1 in 17,000 sequences in a human packaging cell system [31]. Retroviral vectors such as the IUCT-vector [32] which, after one round of reverse transcription and integration into target cells are designed to produce only RNA transcripts not capable of being packaged, still allow the rescue of infectious viral particles from target cells at titers 10,000-fold lower than wild type vectors [32]. However, the authors argue that the low titer of rescuable virus produced may be due to low levels of unspliced RNA being packaged rather than packaging of spliced Ψ(−)-transcripts. In contrast, the data obtained in the present study demonstrate that a retroviral vector lacking the entire packaging signal gives rise to infectious viral particles with only a 1000-fold reduction in virus titer, even though co-packaging with Ψ(+)-RNA is not possible. In a study designed to elucidate the function of the MLV dimer linkage sequence (DLS), Tchenio and Heidmann [33] deleted the first 350 nts of the packaging signal and observed a similar reduction in titer. Thus our data confirm and extend this observation.

We have demostrated that Ψ(−)-retroviral transcripts are considerably more efficiently packaged than previously thought, even in the presence of Ψ(+)-transcripts. This finding has obvious implications for the inadvertent transfer of Ψ(−)-RNAs—such as RNA messages of packaging cells coding for viral proteins—especially in gene therapy approaches.

Although it is well documented that splicing increases gene expression levels in retroviral vector transduced cells [25,26,34], the effect on titer is less well established. Since we have shown that even in the wild type vector context the Ψ-region is spliced out in a percentage of proviral transcripts, we investigated whether retroviral titers could be improved by preventing this splicing event. Two constructs based on plasmid pLXSNeGFP with mutated splice sites, pSDmut and pSDSAmut, were investigated. In contrast to pSDmut, where only the *env* SD site was mutated, both the SD and the cSA were destroyed in pSDSAmut via site-directed mutagenesis. FACS analysis of infected HeLa cells showed that the viral titer of the SD/SA mutated vector is slightly lower compared to that obtained with the wild type vector. Analysis of RNA from cells transduced with the SD/SA mutated vector did not detect any spliced product. Interestingly, however, viral particles derived from pSDmut which lacks the *env* SD but still has a functional cSA, were able to infect up to three times more cells compared to the basic construct. This is probably due to the fact that a cryptic SD at RNA position 752 of vector LXSNeGFP, which is able to promote splicing to the cSA, is activated when the original *env* SD is deleted. The RNA that results from this splicing event carries a 220 bp smaller packaging sequence but maintains the first 580 bp of Ψ(+). Assuming that spliced RNA is exported from the nucleus more efficiently than unspliced RNA, more SDmut-specific RNA molecules containing the Ψ-signal would be available for packaging in the cytoplasm, thus contributing to higher titers.

Our results suggest that, to increase viral titer, it will be important to thoroughly investigate the potential splicing patterns for each retroviral vector used. Similarly, our results demonstrating packaging of spliced Ψ(−)-RNA, even in the presence of Ψ(+)-genomic RNA, underline the requirement for careful screening of viral

supernatants for co-transfer of Ψ(−)-RNAs encoding viral gene products emanating from packaging cells.

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