INTRODUCTION

The retroviral particle encloses a dimer of two complete viral genomic RNAs. The dimeric nature of virion RNA is absolutely conserved within the family Retroviridae, indicating that it plays a crucial role in retrovirus replication. Dimerization of the genomic RNAs may support encapsidation of the two genomes and may play additional roles in the sorting of the retroviral RNAs between different functions, such as packaging and translation (Boris-Lawrie et al., 2001).

For all retroviruses, both packaging and dimerization of the retroviral genome are controlled by an RNA region in the 5’ end of the genome. Among the murine leukemia viruses (MLVs), this region consists of four stem–loop structures (SL1–SL4), all playing a role in the packaging and dimerization of the viral genome (Fisher & Goff, 1998; Mougel et al., 1996). Notably, SL1 and SL2 contain palindromic (self-complementary) sequences and, in both cases, the central nucleotides of these self-complementary sequences are exposed on loops that are responsible for initial dimer recognition by loop–loop base pairing (Girard et al., 1995; Oroudjev et al., 1999).

The encapsidation of two distinct genomes offers retroviruses the opportunity of recombination, which is mainly the result of template switching by reverse transcriptase during first-strand synthesis, thereby allowing incorporation of genetic elements from both of the two parental strands (Hu & Temin, 1990; Stuhlmann & Berg, 1992). Although co-packaging of distinct genomes – i.e. heterozygote formation – is required for recombination, the mechanism of co-packaging is poorly understood. Co-packaging is to a large extent determined by the ability of the two distinct RNAs to recruit the same viral proteins and to some extent by complementarity between RNA-dimerization signals SL1 and SL2 (Rasmussen & Pedersen, 2004).

By definition, co-packaging of retroviral RNAs involves co-localization of the two RNAs at some step during the packaging process, possibly through RNA dimerization. Recent data published during the preparation of this manuscript show that more homozygous than heterozygous virus particles are produced when two distinct retroviral RNAs are co-expressed (Flynn & Telesnitsky, 2006). These data suggest that RNA dimers may not form randomly in the cytoplasm and that some mechanism favours the formation of homodimers, although both co-expressed RNAs carry compatible dimerization signals (Flynn & Telesnitsky, 2006; Kharytonchyk et al., 2005). Also, MLV RNA dimerization was recently shown to change the binding affinity for NC protein to retroviral RNA, indicating a mechanism by which RNA dimerization governs RNA packaging (D’Souza & Summers, 2004) and cytoplasmic transport (Basyuk et al., 2005). These observations are all in line with the notion that RNA dimerization takes place prior to encapsidation (Fu & Rein, 1993; Méric & Goff, 1989).

In this study, we test the hypothesis that co-localization of two retroviral transcriptional cassettes promotes co-packaging...
of their RNA products. We show for the first time a direct linkage between the degree of RNA co-packaging and nuclear RNA co-localization, as addressed by in situ hybridization. The data suggest that retroviral RNA dimerization among the gammaretroviruses is favoured at an early step in the replication cycle and perhaps takes place soon after transcription in the vicinity of the transcription site.

METHODS

Cell culturing, transfection and transduction. NIH3T3 cells and Ψ2 cells (Mann et al., 1983) were cultured, calcium phosphate-transfected and selected as described previously (Mikkelsen et al., 1996). Briefly, the Ψ2 cells were seeded at 5 × 10⁵ cells cm⁻² day prior to transfection in T80 culture bottles. For the one-step-co-transfected cells, the transfection mixture contained 10 μg of each vector plasmid, whereas in the two-step approach, only 10 μg was transfected at a time, starting with the neo-containing plasmid. Single and double selections were sustained for 14 days until well-defined colonies (approx. 100–150) were observed; subsequently, colonies were pooled and grown to confluence. The single-transfected cells were reseeded and transfected with the pac-containing rescue-vector constructs as outlined above. Following an additional 14 days puromycin selection, approximately 100 colonies were pooled, thereby producing the population of two-step-transfected Ψ2 cells. Finally, both one- and two-step-transfected cells were selected in HAT medium for 9 days to select for expression of the viral packaging constructs (Mann et al., 1983).

For titre experiments, the one-step- and two-step-transfected cells were seeded at a density of 5 × 10⁴ cells cm⁻² and, the succeeding day, medium was collected for transduction experiments as described previously (Mikkelsen et al., 1996).

Vector construction. All of the employed vector constructs have been described previously (Rasmussen & Pedersen, 2004). The SL1 mutant (SL1mut) of MLV-like endogenous virus (MLEV) was designed by introducing a non-palindromic sequence into the kissing-loop sequence (5'-GCGC-3'), thereby eliminating homodimerization through this sequence, and similarly for the SL1 in the Akv strain of MLV SL1mut (5'-CGGC-3'). However, the non-palindromic SL1 of Akv was designed so that it is complementary to the SL1mut of the MLEV vectors. Similarly, the SL2 mutation of MLEV (5'-ATGAT-3') is a non-palindromic sequence, but complementary to the Akv SL2mut (5'-TAGCTA-3').

In situ hybridization. Fluorescent hybridizations were carried out as described by Chartrand et al. (2000). In short, we used four distinct, Cy3-labelled probes complementary to the neomycin-coding region and five Cy5 probes complementary to sequences in the AkvMLV-derived vector. Images were acquired on a Zeiss Axiosvert 200 microscope equipped with a 60 x planapochromatic lens, a 2-axis motor and a Cool snap HQ camera from Roper Scientific. By using MetaMorph software (Molecular Devices), the images were thresholded to remove some of the background haze. Images were recorded in optical sections every 300 nm and the image stack was mounted by using MetaMorph. When scanning through the stack of optical sections, transcription sites were easily identified as large spots with high fluorescence intensity.

Sequence analysis. Well-defined colonies from transduced NIH3T3 cells were isolated and grown to confluence in six-well dishes. Genomic DNA was prepared by using DNazol (Molecular Research Center, Inc.). By use of a neo antisense primer (5'-CCAT-AAAACCGGCCAGTCTA-3') and a U3 sense primer (5'-GCTCG-CTTCAGCTTCTGTTACC-3'), a PCR band of approximately 935 bp was generated. The sequence-identity windows (SIWs) were defined as windows of ≥ 2 nt with 100 % similarity. The kissing-loop SIWs are defined as the two SIWs containing a part of the 16 nt long autocomplementary sequence (Fig. 7b).

Cellular and viral RNA preparation and analysis. On the day of transduction, the Ψ2 producer cells were lysed and total cellular RNA was isolated with TriReagent (Sigma) as specified by the manufacturer. Viral RNA preparation was carried out as described previously, adding a loading control consisting of virus-containing medium produced from Plat-E cells transfected with pMSCV-hyg (Clontech) and pelleting the virus particles by centrifugation (Rasmussen & Pedersen, 2004). Viral RNA was isolated from pelleted viruses by using TriReagent (Sigma). When using the pac-neo probe, 300 μl 'hyg-virus medium' was added and 100 μl when using the neo probe (see below). Subsequently, an RNase-protection assay (RPA) was performed by using an RPA III kit (Ambion). Probes were produced by in vitro RNA transcription using a T7 Maxiscript kit (Ambion) with [α-32P]UTP [3000 Ci mmol⁻¹ (111 TBq mmol⁻¹); Hartmann] as the radioactive source.

The pac-neo probe (338 nt) and hyg probe (181 nt) were generated by a PCR (Rasmussen & Pedersen, 2004). The β-actin probe was obtained from an Ambion RPA III kit. The gag probe was also generated by a PCR and in vitro transcription yielded a 412 nt full-length probe that protected a 381 nt fragment of the Moloney MLV gag-containing mRNAs. The undigested probes from the 'no RNase' control were easily used as molecular markers to identify the protected bands. Documentation and quantification of protected fragments were done by use of Kodak Phosphorimager screens, a Bio-Rad Personnel FX scanner and Bio-Rad Quantity One software.

For RPA analysis of viral RNA, we used the 338 nt pac-neo probe. However, due to smearing of the pac-protected band, the much weaker neo-protected band was difficult to detect. In order to circumvent this, a neo-specific probe was also made. This was generated by PCR and yielded by in vitro transcription a 324 nt probe, which protects a fragment of 291 nt of neo-containing RNAs.

RESULTS

Co-packaging assay

In order to measure co-packaging efficiency, we previously designed a two-vector rescue system as outlined in Fig. 1. In this system, transduction of a primer-binding site (PBS)-defective vector (PBSATGG) carrying the neomycin-selection gene (neo) relies strictly on co-packaging with a fully functional rescue vector. Upon co-packaging, the rescue vector donates a functional PBS for initiation of reverse transcription and, following an inter-strand first-strand transfer, the neo gene of the PBS-impaired vector is reverse-transcribed. Although PBSATGG is incapable of transcription initiation, it can still facilitate second-strand transfer or, alternatively, rescue can occur by template switching in the leader region, thereby incorporating the PBSPro of the rescue vector, which can then facilitate the second-strand transfer (Fig. 1) (Mikkelsen et al., 1996). Notably, in both rescue models, the rescue vector and the PBS-impaired vector must co-package in order to transduce the neo gene. Hence, the neomycin rescue titre is a read-out of the ability of the two vectors to (i) first co-package and (ii) go through reverse transcription involving an inter-strand
transfer. The rescue titre is therefore an indirect measure of the amount of transduction-competent heterozygous virus particles produced when two distinct vector RNAs are co-expressed.

**One- and two-step stable transfections**

To investigate the potential effect of co-localized transcription sites on RNA co-packaging, we took advantage of the previous finding that stable co-transfection of two or more selectable or non-selectable markers leads to integration as concatamers (Chen & Chasin, 1998; Perucho et al., 1980). In contrast, two-step stable transfection leads to two independent integration events (Fig. 2b) (Chen & Chasin, 1998; Perucho et al., 1980). The two different transfection approaches were employed, to generate one-step co-transfected and two-step-transfected packaging-cell populations. In the initial experiments, we used the rescue vector pAkvPro-pac, carrying the puromycin-resistance gene (pac) and the PBS-impaired vector pAkvΔTGG-neo, both derived from the Akv strain of MLV.

In the one-step approach, ψ2 packaging cells (Mann et al., 1983) were transfected by calcium phosphate precipitation, selected for both neomycin (G418) and puromycin resistance (see Methods and Fig. 2) and, subsequently, 50–100 colonies were pooled. In the two-step approach, the ψ2 cells were initially transfected by calcium phosphate precipitation with pAkvΔTGG-neo and selected with G418. The arising colonies (50–100) were pooled, reseeded, transfected with the pAkvPro-pac rescue vector and selected for puromycin resistance.

**Accumulation of RNA at the transcription sites**

Previous results have indicated that newly synthesized RNAs accumulate at the transcription sites; subsequently, they will be dispersed in the nucleus by diffusion (Shav-Tal et al., 2004). To investigate the extent of accumulation of RNA at the transcription site in stably transfected packaging cells, we performed fluorescent in situ hybridization in the two distinct populations of cells. The two cassettes differ by the sequence of the selection gene; however, the puromycin-sequence, with a very high G + C content (>70 mol%), turned out not to be a feasible target sequence for in situ probes, giving rise to high levels of unspecific hybridization. As an alternative approach, we constructed four probes against the neomycin/tr5 sequence and five probes against vector sequence contained in both vector RNAs. The vector-specific probes thereby recognized both puromycin sequence- and neomycin sequence-containing RNA transcripts. Carrying out in situ hybridizations with these probes
gave rise to very intense nuclear spots not found in the untransfected cells. Intensity and size of these spots correspond to what have previously been identified as transcription sites (Janicki et al., 2004). As expected, the most intense signal of the neo probe always co-localized with the vector-specific probe, confirming the specificity of the probes. Thus, in one-step-transfected cells, the in situ hybridizations revealed an obvious overlap of both probes, demonstrating clearly that the two transcripts were transcribed from the same locus (Fig. 3). By inspection of approximately 100 nuclei, we found only very few cells containing high-intensity spots that were only positive for the vector probe, and no cells showed high-intensity spots that were only positive for the neo probe. For the two-step-transfected cells, approximately 100 nuclei were also inspected. The in situ hybridization of these cells showed a distinct high-intensity spot that was only positive for the vector probe and one spot positive for both probes, demonstrating two integration events and accumulation of vector RNAs at two distinct loci.

In conclusion, the one-step-transfected cells showed co-localization of the two introduced vector transcripts, probably at the transcription site, whereas the two-step-transfected cells showed distinct localization of the two transcripts with no significant overlap.

**Rescue is favoured in cells with overlapping RNA accumulation**

Next, titre experiments were carried out in parallel for the two cell populations (see Methods). Interestingly, the one-step-co-transfected cells showed consistently high G418-resistance titres of $3.5 \times 10^4 \pm 2 \times 10^4$ c.f.u. ml$^{-1}$ ($n=4$), whereas the two-step-transfected cells showed low titres of $450 \pm 300$ c.f.u. ml$^{-1}$ ($n=4$) (Fig. 4). This corresponds to an approximately 80-fold titre increase in cells with overlapping vector RNA populations compared with cells with non-overlapping RNA populations.

To further test this finding, additional cell populations and vector combinations were used. The vectors were constructed by substituting the Akv leader sequence and packaging signal with that of MLEV (Rasmussen & Pedersen, 2004). These vectors thereby contain the Akv long terminal

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**Fig. 2.** (a) Construction of one-step-co-transfected packaging-cell populations, in which the two co-transfected plasmid DNAs co-integrate. (b) Workflow for construction of the two-step-transfected cell populations, leading to separate integration sites.
repeats (LTRs) required for participation in inter-strand transfer. The leader regions of MLEV and Akv show high sequence similarity of approximately 80% and contain 49 mutations dispersed throughout the leader region.

In these experiments, a total of three MLEV vectors, all carrying the impaired PBSATGG, were introduced: an MLEV wild-type (wt) construct and two with MLEV mutants with mutations in SL1 and SL2. These vectors were complemented by two Akv rescue vectors with similar point mutations in SL1 and -2 (see Methods). Subsequently, the new vectors were employed to generate an additional set of one-step- and two-step-transfected cell populations, as outlined in Fig. 2. Finally, G418-resistance titres were measured as described above.

Again, we observed the same tendency: one-step-co-transfected cells with overlapping vector RNA populations showed titres approximately 15–40-fold higher than the two-step-transfected cells with non-overlapping RNA populations (Fig. 4). This demonstrates clearly that overlapping accumulation of vector RNAs stimulates rescue titres compared with non-overlapping RNA accumulation. The introduction of modified SL1 structures modestly reduced the relative difference between one- and two-step-transfected cells; however, in the current set-up, this was not statistically significant. Notably, the absolute rescue titres of the MLEV-containing vectors were five- to 20-fold lower than those observed for the Akv-derived vectors. We have previously observed lower titres of MLEV-derived vectors, in particular when co-expressed with an Akv-derived vector that contained a stronger packaging signal (Rasmussen & Pedersen, 2004).

Cellular and viral RNA levels are not biasing titres

Although selection conditions were identical for the two cell populations, it is possible that the selection procedure of the two-step-transfected cells may affect the expression level of either one of the two vectors or packaging constructs. Even though this was not indicated by in situ hybridization results, this could bias the experiments and contribute to the reduced rescue efficiencies of two-step-transfected cells. To investigate this, cellular RNA levels were analysed by RPA for both one- and two-step-transfected cells (Fig. 5). The results showed no major variations in packaging-construct expression levels. The neo expression level varied somewhat more between the different cell populations and was slightly higher in two-step-transfected cell populations than in one-step-transfected cells. Notably, the expression levels of the pac-containing rescue vector were, for two of the vector pairs, significantly higher (threefold) in the one-step-transfected cell populations. In the two other vector pairs, pac expression was slightly higher or slightly lower in the one-step- than in the two-step-transfected cells. On the basis of these results, it does not seem likely that the relative expression level of pac and neo vectors affects the co-packaging level radically.
To address possible secondary effects on RNA packaging, we also measured viral RNA content of virions produced from one- and two-step-transfected cells. The cellular expression levels were, to some extent, reflected in the relative packaging levels, as the high cellular level of a vector RNA was found to give rise to high virion RNA content. With the exception of pAkv\textsuperscript{DTGG-neo} + pAkvPro-pac, all other vector combinations showed similar packaging ratios of PBS-impaired and rescue-vector RNAs in virus particles when comparing one- and two-step-transfected cells. For the pAkv\textsuperscript{ΔTGG-neo} + pAkvPro-pac combination, the ratio between PBS-impaired and rescue vector was 1:4 and 4:1 \((n=2)\) for one- and two-step-transfected cells, respectively. An equal excess of either rescue vector or PBS-impaired vector only affects ratios between the two homozygotic particles and not the relative amount of heterozygotic particles, as formation of heterozygotes is independent of which of the vectors is in excess and, thus, these variations in packaging level would not bias the rescue titre. Moreover, by quantification of the total amount of encapsidated RNA (pac + neo), encapsidation levels were not found to vary largely between one- and two-step-transfected cells.

Fig. 5. Cellular RNA analysis. (a, b) Representative RPAs of cellular RNA isolated from one- and two-step-transfected cells, respectively. The vector combinations and control are indicated above each lane. Notice that the neo and pac RNA controls are from RNA isolated from human cells, thus the β-actin probe is cleaved slightly differently from in murine cells. (c) Quantification of RPA analysis as a mean of two independent experiments. The results are normalized to the β-actin internal control.
However, in experiments involving MLEV-derived vectors, the total encapsidation level was reduced (Fig. 6d), which, along with lower packaging ratios of the MLEV vectors (Fig. 6c), correlates with the reduced rescue titres compared with the rescue of the Akv-derived vector (Fig. 4).

Altogether, none of the two-step-transfected cell populations showed notably low expression levels or encapsidation levels of either PBS-impaired or rescue vector. None of the modest variations in RNA packaging can explain the observed 15–80-fold difference in rescue titre between one- and two-step-transfected cells.

Furthermore, the ability of both one- and two-step-transfected producer cells to generate infectious virus particles was also confirmed by measuring the puromycin-transduction titre of the wild-type rescue vector, pAkvPropac, which gave a titre of $5.7 \times 10^5 \pm 3.3 \times 10^5$ c.f.u. ml$^{-1}$ ($n = 3$) and $2.8 \times 10^5 \pm 2 \times 10^5$ c.f.u. ml$^{-1}$ ($n = 3$) from one- and two-step-transfected cell populations, respectively.

**Fig. 6.** RNA-packaging analysis. (a) Representative RPAs of viral RNA using a pac-neo probe for quantification of the rescue-vector packaging level and a hyg probe to quantify the external control (see Methods). (b) Representative RPA of viral RNA using a neo probe and the hyg probe. (c) Quantification of results from RPAs (a) and (b). The diagram shows the relative packaging level of the neo-containing RNA (PBS-impaired vector) as a mean of two independent experiments. (d) Total RNA encapsidation levels (neo+pac) normalized to the Akv+Akv vector combination from one-step-transfected cells.
These titre results correlate with the slightly higher packaging level of rescue vector in one-step-transfected cells than in two-step-transfected cells. In conclusion, none of the above results indicate that one- and two-step-transfected producer cells would behave differently with respect to RNA expression, packaging and transduction capabilities.

Confirmation of the rescue-transfer mechanisms

To investigate the suggested rescue mechanisms (Fig. 1), resistant colonies transduced with viruses produced from both one- and two-step-transfected cell populations were isolated and the leader region and PBS of the proviruses were sequenced. As the PBS-impaired MLEV vectors and the Akv rescue vectors harbour 49 mutations spaced with SIWs, it was also possible to map recombination events between the two vectors.

In total, 41 colonies arising from the one-step-transfected cells were sequenced. Twenty-two of these contained no signs of recombination in the leader region, but contained either the PBSPro (nine of 22), PBSATGG (five of 22) or mixed sequences of PBSPro/-ATGG (eight of 22) (Fig. 7a). This proviral structure is consistent with the mechanism in which the PBSPro of the rescue vector is used for the initiation of reverse transcription followed by an intersstrand template switch (Fig. 1) (see Discussion).

The second group of proviruses (17 of 41) showed recombination between the PBS-impaired MLEV vector and the
Akv rescue vector (Fig. 7b). In all of the analysed proviruses, recombination was accurate and none of the 17 analysed proviruses contained deletions or additions at the recombination site. Moreover, in 13 of 17 colonies, recombination had taken place within the kissing-loop SIW previously shown to be a hot spot for recombination during rescue of a PBS-impaired vector with the endogenously expressed MLEV RNA (Mikkelsen et al., 1998). Notably, proviral sequences from colonies transduced with viruses from the two-step-transfected cells showed a similar distribution of leader recombination events, as three of five recombinants mapped to the kissing-loop SIWs, as opposed to 13 of 17 for the one-step-transfected cells. Likewise, for the suggested strand-transfer rescue, the sequenced proviruses generated from two-step-transfected cells segregated in roughly the same ratio between PBSPro-, PBSATGG- and mixed PBSPro/-ATGG-containing proviruses (Fig. 7a).

In addition, three proviral sequences were generated by rescue through neither leader recombination with the rescue vector nor strand-transfer mechanisms. Of these proviruses, one from both one- and two-step-transfected cells contained the leader region, PBS (PBSGln) and LTRs derived from MLEV, indicating that these two proviruses were generated through recombinatorial rescue by the endogenously expressed MLEV RNA. The remaining provirus, isolated from cells transduced with viruses from one-step-transfected cells, showed an aberrant structure, carrying a deletion of 409 nt spanning almost the entire MLEV 5’ untranslated region. As the provirus also contained the PBSATGG sequence, it is probably generated by a strand-transfer mechanism in which an internal template shift during first-strand synthesis results in the deletion.

In summary, with the exception of two proviruses, all proviral sequences are consistent with the suggested rescue mechanisms, as shown in Fig. 1, and there are no signs of abnormal transfer that can explain the observed differences in rescue efficiency of one- and two-step-transfected cells.

DISCUSSION

In this report, we address the question of whether co-packaging of two genetically distinct retroviral RNAs is affected by the physical position of their transcriptional cassettes and resulting overlapping of RNA accumulation. We have previously established a two-vector rescue system in which transduction efficiency is an indirect measure of co-packaging frequency of the two co-expressed vector RNAs (Rasmussen & Pedersen, 2004).

By constructing two different producer-cell types by either one- or two-step transfection, we demonstrated by in situ hybridization that this leads to strong overlapping RNA accumulation, most likely in the vicinity of the transcription site. Subsequent titre measurements showed that rescue was indeed favoured (15–80-fold) when expressed from cells with overlapping RNA accumulation.

One possible concern for our experiments was that recombination at the DNA level during transfection could produce stably integrated proviruses, in which the rescue vector donates a functional PBS to the PBS-impaired vector, thereby generating a neo-containing vector capable of transducing independently of co-packaging with the rescue vector. However, sequencing of proviral structures supported the suggested rescue mechanisms in the following ways: (i) vectors show the same recombination prevalence in the kissing-loop region as rescue with the endogenous MLEV, indicating strongly that recombination takes place during reverse transcription and not at the DNA level; (ii) none of the recombinant proviruses contained deletions at the recombination site, which has otherwise often been reported for DNA recombinants (Hamada et al., 1993; Nicolás & Young, 1994); (iii) sequence analysis showed that a significant fraction of the integrated proviruses were composed of a mixed population of PBSPro/-ATGG-containing proviruses (Fig. 7). This is clear evidence of a PBSATGG-mediated second-strand transfer (Fig. 1), as this leaves a proviral intermediate with a bulge mismatch in the PBS region. If cell division takes place prior to mismatch repair, this generates two cells harbouring PBSPro and PBSATGG, respectively. Expansion of the two cells would give rise to a colony consisting of a mixed population of cells harbouring the PBSATGG- or the PBSPro-containing provirus. Mixed populations were not due to cross-contamination between colonies, as mixed populations were never observed in colonies containing proviruses with recombinations in the leader region. Furthermore, clones containing mixed PBSPro- and PBSATGG-containing proviruses occurred at a similar frequency among clones arising from one- and two-step-transfected packaging cells.

For clones containing ‘pure’ populations of either PBSPro- or PBSATGG-harbouring proviruses, we assume that mismatch repair occurred prior to cell division; alternatively, only one of the two daughter cells proliferated successfully. However, in principle, we cannot reject the possibility that some read-through transcripts of adjacent co-integrated proviruses generated bicistronic RNAs containing both the neo and pac genes and a complete set of cis elements. Such transcripts, containing more than two LTRs, may be reverse-transcribed independently of co-packaging with a rescue vector and give rise to neo-containing proviruses with ‘unmixed/pure’ PBSATGG sequence. If this was a major mechanism for rescue, we would expect to find a high percentage of transduced proviruses from one-step-transfected cells to contain pure PBSATGG sequence. However, the one- and two-step-transfected cells show similar frequencies of pure PBS and mixed PBS sequences and, moreover, as only five of 41 clones contained unmixed PBSATGG, read-through transfer could, at the most, constitute only a few per cent of the total transduction event and could not explain the observed 15–80-fold difference in rescue titre between the two types of producer cells.
In conclusion, the sequence analysis of transduced clones supports the rescue mechanism in Fig. 1, as well as the notion that an increased number of heterozygotic virus particles are produced from the cells with overlapping RNA accumulation. Furthermore, RNA analysis showed similar RNA-packaging efficiency and similar expression levels of both packaging and vector constructs. Moreover, titration measurements of the rescue vector gave rise to similar transduction titres, all confirming similar capabilities of both types of producer cells to produce infectious virus particles.

These results encourage us to suggest a model in which two RNAs transcribed from nearby loci, due to high a high local concentration, are more likely to collide and dimerize than are RNAs produced from spatially distant loci. This mechanism is supported by previous studies showing that MLV RNA dimerization is a concentration-dependent reaction in vitro (De Tapia et al., 1998; Girard et al., 1996; Oroudjev et al., 1999). We can only speculate that RNA dimerization might function in order to recruit or prevent recruitment of certain heterogeneous nuclear ribonucleoprotein (hnRNP) constituents and, in this way, sort monomeric and dimeric RNAs between splicing and not splicing, different export pathways and association with the translation and packaging machinery. Alternatively, RNA co-localization at the transcription site may favour the RNAs to enter the same hnRNP complexes, nuclear-export routes and cytoplasmic-transport pathways, eventually leading to co-encapsidation and dimerization.

During the preparation of this work, a number of studies have addressed the relationship between localization of RNA-transcription sites and RNA co-encapsulation. Our results and model support and complement these studies by demonstrating that vector RNA accumulates at the transcription sites, leading to increased co-packaging. Compared with the recent study by Kharytonchyk et al. (2005), we observed a more significant effect on the relative increase in heterozygotic virus particles by co-expressing RNAs from one locus (15–80-fold increase compared with fourfold increase). This may relate to technical differences in transfection methods, packaging cells, rescue system, etc. Moreover, if, as indicated by this study, the efficiency of dimerization at the transcription site is dependent on local RNA concentration, the bimolecular reaction of RNA heterodimerization may be very dependent on both the absolute and relative transcription levels of the two constructs, which again may be affected strongly by the different use of transfection and selection conditions.

A very recent study also suggests a similar model. However, the effect of expressing from two distinct nuclear sites was carried out by transient co-transfections of two vector-containing plasmids (Flynn & Telesnitsky, 2006). As transient transfection introduces thousands of plasmids into the mammalian cell, multiple and overlapping transcription sites probably exist, although transcribed from separate plasmids; therefore, the effect on separating the transcription site may be limited, resulting in a less pronounced effect on the relative production of heterozygotic virus particles. With vectors identical to those used here, by transient transfection, we have previously obtained rescue titres 10–15-fold higher than those obtained in the current study, where expression is from stable, separate integration sites. We think that this possibly reflects partly overlapping RNA accumulation in transiently transfected cells. Flynn & Telesnitsky (2006), on the basis of in vitro dimerization experiments during in vitro transcription, hypothesized that overproduction of homozygotic virus particles is partly due to co-transcriptional RNA dimerization; however, the observation that formation of heterozygotic virus particles is favoured by co-integration of transcriptional cassettes would argue that RNA dimerization under these conditions succeeds the release of RNA by the polymerase. Alternatively, the binding of DNA may allow interaction between co-transcribed RNAs from different transcriptional cassettes. We have not addressed the absolute amount of heterodimers relative to homodimers in our experiments; however, it would be interesting to see whether homodimers are still favoured over heterodimers when co-integrated.

Dimerization of retroviral RNA prior to its packaging clearly facilitates assembly of retroviral particles containing two copies of retroviral genomes, and we can only speculate on additional functions of nuclear RNA dimerization. However, in simple retroviruses, cis-acting RNA elements must support a number of functions that are otherwise facilitated by accessory proteins in complex viruses and by proteins recruited through splicing of the cellular RNAs (Tange et al., 2004). Therefore, the full-length RNA of simple retroviruses may control the extent of splicing, nuclear export and recruitment of translation machinery, as well as sorting between translation and packaging. Previous results have also shown that unspliced MLV RNAs divide into two non-equilibrated pools with different half-lives, one for translation and one for packaging (Levin & Rosenak, 1976). On the basis of our results, we propose that RNA dimerization might function in order to favour or prevent the recruitment of certain hnRNP constituents, which would thereby decorate monomeric and dimeric RNA for different destinations. Interestingly, MLV RNA dimerization has recently been shown to affect protein binding, as dimeric MLV RNA spanning the packaging signal results in a higher binding affinity of the NC protein, possibly increasing packaging specificity (D’Souza & Summers, 2004). Possibly reflecting such mechanisms, we have observed previously that deletion of the SL1 kissing-loop dimerization signal leads to a five- to 10-fold increase in splicing (Aagaard et al., 2004).

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