Importance of 3' non-coding sequences for efficient retrovirus-mediated gene transfer in avian cells revealed by self-inactivating vectors

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Avian leukosis virus-derived vectors were constructed with an internal transcriptional promoter and various 3' non-coding sequences. Deletions were introduced into the downstream U3 long terminal repeat (LTR) to obtain self-inactivation of LTR-mediated transcription after one round of replication. However, 3' non-coding sequences appeared to determine not only self-inactivation of the vectors but also gene transfer efficiency. Further analysis revealed the influence of these sequences on both internal gene expression and RNA packaging. One construct permitted gene transfer while inactivating 5' LTR-promoted transcription.

Introduction

Retrovirus vectors provide an efficient method for the introduction of foreign genes into various cell types, where they usually ensure a high level of stable gene expression (Eglitis & Anderson, 1988). Genes of interest can be expressed either from the 5' long terminal repeat (LTR) of the retrovirus or from an internal promoter. In the latter case, however, the proximity of viral transcriptional signals can impair the activity of the internal promoter (Emerman & Temin, 1984, 1986).

An elegant way to overcome this problem is to design a vector where at least some of the viral transcription signals are lost after one round of replication. Such vectors, called self-inactivating vectors, have been previously derived from both murine leukaemia viruses and avian spleen necrosis virus (SNV) by mutating the 3' LTR; in the transfected helper cells, this mutation is not expected to impair virus production, but after one round of virus replication both LTRs are mutated and the 5' LTR-driven transcription is reduced or abolished (Cone et al., 1987; Dougherty & Temin, 1987; Soriano et al., 1991; Yu et al., 1986). This inactivation process provides an important improvement in the safety of in vivo gene transfer.

The ability of self-inactivating vectors to produce high titre recombinant viruses and to inactivate the LTR promoter relies mostly on the mutations that are created. However, the consequences of mutating the 3' non-coding viral sequences on RNA transcription, processing, translation, packaging and reverse transcription are unpredictable (Iwasaki & Temin, 1990; Yee et al., 1987; Yu et al., 1986; Olson et al., 1992).

Here we describe the construction of several avian leukosis virus-derived vectors with various modified 3' non-coding sequences. The influence of these sequences on virus replication is further analysed. DNA transfection experiments and RNA analysis revealed that the efficiency of virus-borne gene expression and viral RNA packaging are dependent on 3' non-coding sequences.

Methods

Plasmid construction

(i) OVA backbone. OVA was constructed by assembling in the polylinker of pBluescript (Stratagene) (5' to 3'): a fully active hybrid LTR (referred to as the RSV-D LTR in the text (RSV, Rous sarcoma virus), an RAV(1) leader sequence [NdeI-EcoRI fragment from pRSVneo (Gorman et al., 1982) and EcoRI-Xhol from pRAV(1) (Payne et al., 1981)], the NeoR coding sequence fused to the gag sequence (Xhol-XbaI fragment of pTXN5'; Benchaibi et al., 1989) and the simian virus 40 (SV40) promoter followed by a mutated version of the Escherichia coli lacZ gene, coding for β-galactosidase targeted to the cell nucleus (SalI-BamHI fragment from pMuMLSVSNlacoZ; Bonnetot et al., 1987). Due to the insertion of small pBluescript polylinker fragments, this structure is flanked by two SpeI restriction sites. This SpeI fragment was inserted upstream of the various LTRs described in the following section, in some cases after filling in of the protruding ends with Klenow polymerase. The last letters of OVA vectors' names denote the origin of the 3' LTR.

(ii) 3' Non-coding sequences of OVA-derived vectors. The OVA-D 3' LTR was derived from the NdeI-XhoI fragment of OVA which was cloned between the Smal and XhoI sites of pBluescript. The OVA-R-deleted 3' LTR was created by assembling the 3' part of the RAV(2) genome (from XhoI to a Clal linker inserted in the Sphi site which is
located 149 nucleotides upstream of the cap site) and the 5' part of the RAV(1) LTR (from a Clal linker inserted into the TaqI site 15 nucleotides upstream of the cap site). This deletion removes the TATA and CCAAT boxes but leaves the enhancer element intact (Aubert et al., 1991). This LTR is referred to as the RAV(1)-deleted LTR in the text. In OVA-ZZ the 3' LTR comes from the HindIII--BanHI fragment of pgJ38 (Cullen et al., 1983) carrying two copies of the RAV(0) LTR. OVA-Zdel was obtained by deleting the tandem repeats of RAV(0) LTRs by digesting with NdeI and performing a ligation at low DNA concentration. This leaves a single copy of the RAV(0) LTR deleted from nucleotide −98 to nucleotide −59 (from the cap site). OVA-Zcrip was similar to OVA-Zdel but the TATA box was deleted further by site-directed mutagenesis (Muta-Gene kit, Bio-Rad). The sequence of the mutagenic oligonucleotide was 5' ACGAAGCTATGTAGTAAGCTGTTGCCACC 3'. The deletion covers nucleotides −27 and −32 and creates a SnaBI restriction site. The structure of this LTR was confirmed by DNA sequencing.

**Cell culture.** All reagents were purchased from Gibco. Chicken embryonic fibroblasts (CEFs) were prepared from C/O SPAFAS 10-day-old embryos. CEF, QT6 (Moscovici et al., 1977) and Isolde helper cells (Cosset et al., 1990) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, 1% chicken serum, 2% tryptose phosphate broth, penicillin (100 units/ml), and streptomycin (0.05%). Medium was changed every 3 days. Phleomycin (50 μg/ml) and hygromycin (50 μg/ml) were added to maintain the virus production of the Isolde cells.

**DNA transfection, virus production and titration.** Plasmid DNA purified by CsCl banding (Maniatis et al., 1982) was used to transfect Isolde helper cells using either calcium phosphate precipitation (Maniatis et al., 1982), polybrene (Kawai & Nishizawa, 1984), DOTAP (Boehringer-Mannheim) or electroporation (10 μg/ml of DNA in DMEM, 250 μl, 350 V with a Bio-Rad Gene-Pulser). Cells were selected with G418 (500 μg/ml, Gibco), starting 24 h after transfection, for at least 10 days. Virus supernatants were harvested from subconfluent cells 16 h after medium change, and centrifuged (4000 g, 5 min) to remove cell debris. For titration, 50 to 500 μl of helper cell supernatant was spread on QT6 cells or CEFs (5 × 10⁵ cells per 25 cm² dish). Cells were selected with G418 24 h after infection, or stained for β-galactosidase activity 48 h after infection (Sanes et al., 1986). To ensure that the virus preparations were free of replication-competent virus, infected cells resistant to G418 were grown for another week and assayed in situ for the presence of replicating virus by using an anti-p27 antibody (Savatier et al., 1989).

**RNA and DNA analysis.** Virion RNA was extracted from centrifuged supernatant (45 min at 35000 r.p.m. in an SW4) Beckman rotor). Cellular RNA and DNA were purified after proteinase K digestion and phenol--CHCl₃ extraction as described (Maniatis et al., 1982). Southern blots (cellular DNA), Northern blots (cellular RNA) and slot blots (virion RNA) were hybridized with an in vitro synthesized RNA probe labelled with ³²P using standard techniques. Quantification was performed by cutting out nylon membrane pieces, immersing in a liquid scintillation cocktail (Beckman) and measuring radioactivity.

**Results**

**Construction of a highly efficient vector derived from RSV with an internal SV40 promoter**

As a first step toward the construction of a self-inactivating vector, we needed to construct an efficient vector with an internal promoter, in which the 3' LTR could easily be replaced. The structure of OVA-D is summarized in Fig. 1(a). In this construct the two LTRs are identical. The U3 promoter is from the SR-D strain of RSV. The Neo<sup>k</sup> coding sequence is fused to the first residues of the gag gene, and followed by a mutated version of the E. coli lacZ gene driven by the SV40

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**Fig. 1. Structure of OVA-D: an RSV-derived vector with a functional internal promoter.** (a) OVA-D has been designed to produce two types of RNA: a 6 kb transcript initiated at the 5' LTR which can encode the Neo<sup>k</sup> product or be encapsidated into virus particles, and a 4 kb transcript initiated at the internal SV40 virus promoter which encodes a mutated version of E. coli β-galactosidase targeted to the cell nucleus. (b) Northern blotting of cellular RNA extracted from Isolde cells stably transfected with OVA-D and CEFs infected with OVA-D. Size markers are indicated on the right. The blot was hybridized to a lacZ antisense RNA probe obtained after in vitro transcription of the Clal--BanHI fragment of OVA-D cloned into pBluescript. Brackets indicate the areas cut out for scintillation counting. After correction for background hybridization, we calculate that 4 kb transcripts represent 30% of the hybridized RNA.
Table 1. Titrations of supernatants harvested from transfected Isolde cells

<table>
<thead>
<tr>
<th>Construct</th>
<th>3' LTR</th>
<th>lacZ titre</th>
<th>NeoR titre</th>
<th>SI index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVA-D</td>
<td>RSV-D</td>
<td>1.0 x 10^4</td>
<td>1.0 x 10^4</td>
<td>1</td>
</tr>
<tr>
<td>OVA-R</td>
<td>RAV(1)</td>
<td>1.5 x 10^5</td>
<td>1.0 x 10^4</td>
<td>15</td>
</tr>
<tr>
<td>OVA-ZZ</td>
<td>RAV(0)</td>
<td>1.0 x 10^4</td>
<td>1.0 x 10^4</td>
<td>1</td>
</tr>
<tr>
<td>OVA-Zdel</td>
<td>RAV(0)</td>
<td>2.5 x 10^2</td>
<td>1.0 x 10^2</td>
<td>2.5</td>
</tr>
<tr>
<td>OVA-Zcrip</td>
<td>RAV(0)</td>
<td>1.0 x 10^2</td>
<td>5.0 x 10^1</td>
<td>2</td>
</tr>
</tbody>
</table>

* Self-inactivation index: lacZ titre/NeoR titre.

promoter (SVnlslacZ gene). This construct is expected to produce two RNA species: a 6.0 kb transcript initiated at the 5' LTR and covering the entire OVA-D genome, coding for the NeoR gene product, and a 4.0 kb RNA initiated at the internal SV40 promoter and coding for β-galactosidase targeted to the cell nucleus.

OVA-D was transfected into Isolde helper cells which were subsequently subjected to G418 selection. The recombinant virus was then harvested from the supernatant of pooled resistant cells and titrated on both CEF and QT6 cells either by selecting with G418 (giving what will be referred to as the NeoR titre) or by using an in situ assay for β-galactosidase activity (giving the lacZ titre). We obtained an average titre of 10^4 G418 r.f.u./ml and 10^4 lac+ f.u./ml. These titres are close to that obtained in Isolde cells with NLD, a similar construct which does not have an internal promoter (Cosset et al., 1991).

To confirm that avian leukosis virus-derived vectors can accommodate a functional internal promoter, Northern blots were run with RNAs extracted from the transfected Isolde cells and from CEFs infected with OVA-D and selected with G418 (Fig. 1b). The sizes of the two RNA species detected with a lacZ probe were consistent with the expected pattern of expression. Pieces of the hybridized nylon membrane were then cut out for approximate RNA quantification. About 30% of the transcripts appeared to initiate at the internal SV40 promoter. This is consistent with transient expression experiments showing that in QT6 and CEFs the RSV LTR promoter is more active than the SV40 promoter (data not shown). It appears therefore that avian retrovirus vectors, like murine retrovirus vectors (Cone et al., 1987; Morgenstern & Land, 1990), can accommodate an internal transcription promoter.

Construction of vectors with various 3' non-coding sequences

To study the possibility of deriving self-inactivating vectors from OVA-D we designed a series of vectors differing from our first construct only in their 3' end (Fig. 2). These vectors were expected to behave similarly when transfected into Isolde cells. After one round of replication, however, the U3 sequences of both LTRs are expected to derive from the U3 sequence originally located into the 3' LTR. Therefore, if the transcription promoter located in this region is inactive, the NeoR expression should be altered in the infected cells. As it is driven by the internal SV40 promoter, the nlslacZ expression should not be affected. Thus, for each vector, the lacZ titre provides us with an estimate of the gene transfer efficiency, and we can define the ratio between the lacZ titre and the NeoR titre as a 'self-inactivation index'.

Fig. 2. Structure of the OVA-derived retrovirus vectors. See Methods for the details of constructions. The vectors differ only in their 3' non-coding sequences. White triangles indicate the position of the deletions in the LTR. Angled arrows indicate the position of the SV40 transcription promoter. The presence or absence of enhancer, CCAAT and TATA elements in the 3' LTR is indicated. As discussed in the text, the absence of an enhancer element in the RAV(0) LTR is questionable.

Four putative self-inactivating vectors were constructed (Fig. 2). In OVA-R the 3' LTR derives from the RAV(1) virus. A large deletion covers the CCAAT and TATA boxes but leaves the enhancer element intact. OVA-ZZ possesses an intact RAV(0) LTR (for convenience we kept the LTR tandem repeat present in the original clone). The RAV(0) LTR is present in an endogenous provirus giving rise to relatively high-titre
Table 2. Clonal variations of virus titre after OVA-Zdel and OVA-Zcrip transfection

<table>
<thead>
<tr>
<th>Clone</th>
<th>lacZ titre</th>
<th>NeoR titre</th>
<th>SI index</th>
</tr>
</thead>
<tbody>
<tr>
<td>delA</td>
<td>$1.5 \times 10^4$</td>
<td>$2.0 \times 10^3$</td>
<td>7.3</td>
</tr>
<tr>
<td>delB</td>
<td>$1.6 \times 10^4$</td>
<td>$2.5 \times 10^2$</td>
<td>6.4</td>
</tr>
<tr>
<td>delC</td>
<td>$1.2 \times 10^4$</td>
<td>&lt; 20</td>
<td>&gt; 60</td>
</tr>
<tr>
<td>criA</td>
<td>$2.0 \times 10^4$</td>
<td>&lt; 10</td>
<td>&gt; 20</td>
</tr>
</tbody>
</table>

Recombinant virus titre and self-inactivation is strongly influenced by 3' non-coding sequences

Table 1 summarizes the various titration assays performed with the supernatants obtained from pools of Isolde cells transfected with OVA-D, OVA-R, OVA-ZZ, OVA-Zdel or OVA-Zcrip and further selected with G418 to ensure stable expression of the vector DNA. The gene transfer efficiency was drastically reduced with OVA-R. However, the large deletion that covers both the CCAAT and TATA boxes led to a partial self-inactivation. More surprisingly, OVA-ZZ which carries a natural 3' LTR had low efficiency. The lack of self-inactivation could be explained by the recently discovered cryptic enhancer element within the RAV(0) U3 sequence, which seems to be activated when moved into an RSV context (Conklin, 1991). Deleting the 3' CCAAT box in OVA-Zdel resulted in an unexpected improvement in gene transfer efficiency and to a partial self-inactivation. OVA-Zcrip gave lower titres than OVA-Zdel.

We isolated 27 clones of helper cells transfected with OVA-Zdel or OVA-Zcrip and titrated their supernatants for nlslacZ and NeoR expression. Thirteen of these clones did not give any infectious virus (<20 lac+ f.u. or G418 r.f.u./ml). This high rate of non-producer clones has been observed with other vectors and might be due to rearrangement occurring in the DNA molecules that are stably transfected into Isolde cells (Cosset et al., 1991). The results obtained with the four clones that gave the highest lacZ titre are reported in Table 2. The self-inactivation index varied widely from one clone to another and in some cases exceeded 60. Therefore the two parameters are not only dependent on the type of vector that is used but also on the producer cell clone.

3' Sequences influence internal gene transient expression

To gain further insight into the factors that determine virus production in helper cells we transfected Isolde or QT6 cells with the OVA plasmids and assayed 48 h later for SVnlslacZ transient expression by in situ staining (Table 3). In these conditions OVA-D, OVA-R and OVA-ZZ gave similar results. The deletions within U3 in OVA-Zdel and OVA-Zcrip strongly reduced the SVnlslacZ transient expression. These results support the assumption that the modest virus titre obtained with OVA-Zdel is linked to a defect in SVnlslacZ expression and that the deleted part of the RAV(0) U3 element carries an important signal for viral gene expression. By contrast, the low virus titre obtained with OVA-ZZ does not correlate with the transient nlslacZ expression, indicating that a post-transcriptional step of virus

Table 3. Transient assay for nlslacZ expression

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>3' LTR</th>
<th>Calcium phosphate precipitation</th>
<th>Polybrene</th>
<th>Electroporation</th>
<th>Average*</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVA-D</td>
<td>RSV-D</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>OVA-R</td>
<td>RAV(1) deleted</td>
<td>ND†</td>
<td>ND</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>OVA-ZZ</td>
<td>RAV(0)</td>
<td>100</td>
<td>85</td>
<td>ND</td>
<td>90</td>
</tr>
<tr>
<td>OVA-Zdel</td>
<td>RAV(0) deleted</td>
<td>7</td>
<td>14</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>OVA-Zcrip</td>
<td>RAV(0) deleted</td>
<td>ND</td>
<td>ND</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>pA0nlslacZ</td>
<td>poly(A) MoMuLV‡</td>
<td>100</td>
<td>90</td>
<td>100</td>
<td>95</td>
</tr>
</tbody>
</table>

* 'Average': ratio to the number of lac+ cells obtained with OVA-D.
† ND, Not determined.
‡ MoMuLV, Moloney murine leukaemia virus.
Clonal variation of nlslacZ expression in infected cells

Clones of infected cells resistant to G418 (G418R clones) were stained in situ for β-galactosidase activity. Although it was performed on a limited number of clones, this analysis led to two interesting observations (Fig. 3).

Some G418R clones contained no lac+ cells. This could result from a mutation occurring in the SVnlslacZ gene. However, as G418R/lac− helper cells can produce lac+ virus (like clone delB in Table 2), it is more likely that G418R/lac− cells actually express SVnlslacZ, but below the in situ detection threshold level. Therefore this assay probably leads to an underestimate of the actual gene transfer efficiency and self-inactivation index. As cells within a given G418R clone stain with approximately the same intensity, it is likely that chromosomal flanking sequences can greatly influence the SVnlslacZ gene expression.

The relative ratio of G418R/lac− to G418R/lac+ clones is dependent on the vector used to infect the cells. As expected from transient assays, this ratio is similar for OVA-D and OVA-ZZ and lower than that obtained with OVA-Zdel.

3' Non-coding sequences influence the efficiency of RNA packaging

A low virus titre can result either from a reduced production of virus particles carrying the vector RNA by the helper cells or from a low level of viral gene expression in infected cells. To identify the limiting step in the present case, we analysed retrovirus RNA in helper cells stably transfected by OVA-D, OVA-ZZ and OVA-Zdel (clone delA), and in the virus particles they produce (Table 4). Slot blot analysis showed that the lacZ titre is related to the amount of retrovirus RNA packaged into the virus particles but not to the steady-state level of vector RNA present in the cells. In fact, although OVA-D gave a much higher titre, its RNA steady-state level was not higher within producer cells selected with G418. Quantitative analysis revealed that OVA-ZZ and OVA-Zdel transcripts initiated at the 5' LTR are under-represented in the virus particles. RNA packaging appears therefore to be a limiting step in virus production for OVA-ZZ and OVA-Zdel. Whether this limited reduction in packaging efficiency can entail a significant drop in virus titre remains to be determined.

Expression of NeoR gene in cells infected by OVA-Zdel results from rearrangements of the provirus structure

Several mechanisms can be proposed to explain how cells infected with our self-inactivating vectors sometimes express the NeoR gene. The mutation introduced in the 3' LTR might not fully inactivate the viral transcription promoter. After the transfer of the mutated promoter to the 5' LTR in infected cells, a low level of 5' LTR-mediated transcription might occur and sometimes be enhanced by flanking cellular sequences. Alternatively, readthrough transcription from a flanking cellular promoter might be sufficient to ensure NeoR gene expression. Finally our vectors might recombine at high frequency in helper cells or during their reverse transcription and recover a fully active 5' LTR promoter.

To distinguish between these possibilities, we compared the proviral structure of OVA-Zdel and OVA-D in pools of infected cells expressing the NeoR gene.

Table 4. Quantification of RNA packaging

<table>
<thead>
<tr>
<th>Construct</th>
<th>6 kb RNA in cells (%)</th>
<th>6 kb RNA in virions</th>
<th>Ratio (virion/cell)</th>
<th>Relative ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVA-D</td>
<td>100</td>
<td>9.5</td>
<td>9.5</td>
<td>100</td>
</tr>
<tr>
<td>OVA-ZZ</td>
<td>50</td>
<td>1.9</td>
<td>3.8</td>
<td>40</td>
</tr>
<tr>
<td>OVA-Zdel</td>
<td>110</td>
<td>3.1</td>
<td>2.7</td>
<td>30</td>
</tr>
</tbody>
</table>

* OVA-D was used as a 100% reference for cellular RNA and packaging ratio.
Fig. 4. Structure of the OVA-Zdel provirus 5' LTR in infected cells expressing the NeoR gene. (a) DNA was extracted from pools of cells infected with OVA-Zdel or OVA-D and subjected to G418 selection, and from Isolde cells transfected with OVA-Zdel. After restriction with either AccI or EcoRI and Southern blotting, hybridization was performed with a RNA probe specific for NeoR sequences. (b) Predicted structure of the integrated proviruses. In infected cells, OVA-D should yield a 2.2 kb EcoRI fragment. The size of the AccI fragment depends on flanking cellular sequences. Cells transfected with OVA-Zdel should yield the same 2.2 kb EcoRI fragment and a 2.5 kb AccI fragment (from the plasmid polylinker to the 3' end of the NeoR gene). After one round of replication, however, an AccI site should replace the EcoRI site in the 5' LTR. Therefore one expects in infected cells a 2.2 kb AccI fragment and an EcoRI fragment, the size of which is determined by flanking cellular sequences. As a large number of G418-resistant clones were pooled, no EcoRI band should be seen on the autoradiogram. The results rather suggest that, in OVA-Zdel proviruses that express NeoR, the RSV sequences are maintained in the 5' LTR.

Southern blot analysis shown in Fig. 4 reveals the presence of an EcoRI site in the 5' LTR of the integrated proviruses and the absence of an AccI site. These features strongly suggest that the U3 sequence of the OVA-Zdel proviruses that express NeoR does not derive from RAV(0) but derives from RSV-D. In fact a Northern blot experiment failed to distinguish between the steady-state levels of both LTR and SV40 transcripts in G418-resistant cells infected by either OVA-Zdel or OVA-D. The fact that the largest messenger is of definite size also argues against a possible readthrough transcription from cellular flanking sequences (data not shown). Thus it is very likely that OVA-Zdel can recombine at a high frequency to keep a 5' RSV promoter after reverse transcription. The fact that the self-inactivation index is subject to clonal variations among producer cell clones (see above) suggests that these recombination events are favoured by the highly unpredictable mode of DNA integration that is characteristic of DNA transfection. Using electroporation, as done in a similar situation (Soriano et al., 1991), did not provide any significant improvement.

Discussion

The host-range restriction of ALV vectors offers a safe alternative to SNV-derived vectors which can infect human cells (Koo et al., 1991). We have constructed two types of vector from avian leukemia viruses that could be useful for gene transfer experiments.

OVA-D is a highly efficient vector with an SV40 internal promoter. Our Northern blot experiments are consistent with a proper functioning of the internal promoter. However, as we did not precisely map the transcription initiation of the lacZ mRNA, we cannot completely rule out the possibility that lacZ expression results from an unpredicted splicing of the LTR-driven transcript. Two observations made with OVA-D-related vectors argue against this last hypothesis. First, when self-inactivation is achieved with OVA-Zdel the lacZ expression occurs when the LTR-driven NeoR expression is abolished. Second, when we replaced the SV40 promoter by the human adenovirus E2 promoter, the lacZ expression became highly inducible by Ela, a trans-activator specific for the E2 promoter (data not shown).

OVA-Zdel is a vector with a crippled 3' LTR and which has the ability to self-inactivate after replication. The random process of integration of the vector genome in the transfected helper cells seems however to enable a high rate of recombination of the viral genome, impairing the self-inactivation process. Although we did not identify the recombination mechanism precisely, the fact that the recombination frequency depends on the producer cell clone used leads us to suppose that it is linked to the structure of the integrated DNA in the transfected cells. For example, molecules with a rescued...
5' RSV LTR might appear after readthrough transcription across large vector DNA concatemers. These large RNA molecules would then lose the mutated U3 sequence after aberrant splicing or a jump of the reverse transcriptase (Olsen et al., 1990). This problem can be circumvented by clonal screening of producer helper cells.

During our experiments we also found that 3' non-coding sequences can strongly influence not only the self-inactivation ability of retrovirus vectors but also the efficiency of retrovirus-mediated gene transfer. As the U3 deletions that we tested dramatically affected the virus titre, we are prompted to consider that LTR sequences play a major part in this phenomenon. It is known however that the non-coding sequence located upstream of the 3' LTR can also be involved (Sorge et al., 1983; Flanagan et al., 1989). It is difficult to unravel the influence of 3' non-coding sequences on RNA transcription, translation, packaging, and reverse transcription. Previous and present data suggest that several of these parameters can be affected by a 3' mutation.

Transient expression experiments have shown that a 3' LTR enhancer element can partially activate the 5' LTR transcription promoter (Norton & Coffin, 1987). If an additive effect of enhancer elements exists, it could explain why OVA-ZZ, OVA-Zdel and OVA-Zcrip yield less lacZ-expressing virus than OVA-D. However, OVA-R does not yield high titres despite the presence of a 3' enhancer. Moreover, Northern blot analysis failed to reveal any influence of 3' sequences on 5' LTR-mediated transcription and led us to suspect a translation defect. Therefore, in the present situation, the 3' LTR enhancer appears to have little or no effect on viral transcription.

Recent experiments failed to confirm that a deletion in the downstream LTR can impair the 3' end processing of SNV RNA (Iwasaki & Temin, 1990). Furthermore, it has been shown that RAV(0) LTR sequences are functional in 3' end processing (Herman & Coffin, 1986). Even if readthrough transcription were more frequent with RAV(0)-derived vectors, it would not be expected to have any significant influence on avian retrovirus replication (Swain & Coffin, 1989).

Our data show that OVA-ZZ and OVA-Zdel RNAs are slightly under-represented in virus particles, and the same is probably true for OVA-Zcrip. Therefore RNA packaging could be a limiting step in the replication of these vectors. Based on nucleotide sequence analysis, it has been proposed that the 5' leader sequence and the U3 sequence cooperate to build up an RNA secondary structure flavouring encapsidation (Darlix, 1986). As predicted by this model, a deletion in the U3 sequence of the Moloney murine leukemia virus has been shown to affect RNA packaging (Murphy & Goff, 1989). In OVA-ZZ and OVA-Zdel the RAV(1) leader sequence would not be able to cooperate with RAV(0) U3 sequences for RNA encapsidation. Indeed RAV(0) and RAV(1) leader and U3 sequences are different in the regions supposed to interact (Bizub et al., 1984; Katz et al., 1986).

It seems therefore that the limited lacZ titre obtained with OVA-Zdel might be due to the combination of minor defects in SVN-lacZ gene expression and in RNA packaging. However these considerations cannot explain why the lacZ titre obtained with OVA-ZZZ is lower than that obtained with OVA-Zdel. It is likely therefore that another parameter is affected by the U3 deletion.

In conclusion, provided that a suitable producer clone is selected, OVA-Zdel appears to be a useful vector that should increase the safety of gene transfer experiments. After replacement of the SV40 promoter by any other promoter, it could also be used to derive new vectors for promoter regulation studies, providing stable integration within the cellular genome in the absence of surrounding viral enhancers.

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