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Improvement of Avian Leukosis Virus (ALV)-Based Retrovirus Vectors by Using Different *cis*-Acting Sequences from ALVs

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Production and expression of double-expression vectors which transduce both Neo^r and *lacZ* genes and are based on the structure of avian leukosis virus were enhanced by using *cis*-acting sequences (long terminal repeats and noncoding sequences) from Rous-associated virus-1 and Rous-associated virus-2 rather than those of avian erythroblastosis virus previously used in our constructs. Polyclonal producer cells obtained after transfection of these vectors into the Isolde packaging cell line gave rise to titers as high as 3×10^5 *lacZ* CFU/ml, whereas it was possible to isolate clones of producer cells giving rise to titers of more than 10^6 resistance focus-forming units per ml.

Retrovirus vectors are now widely used for many studies both in vitro and in vivo (22, 23, 27, 43). The most suitable replication-defective vectors for many experiments can be classified into four groups. The first group is represented by double-expression vectors carrying two genes, both under control of the 5′ long terminal repeat (LTR) (6, 11, 25, 41). The second group corresponds to vectors with internal promoters, the inserted genes being driven by internal promoters (24, 31, 32, 35, 45, 47). The third group is related to self-inactivating vectors which also allow gene expression from an internal transcriptional unit because of inactivation of the LTRs consequent to being integrated into target cells (20, 28, 49, 50). Finally, the fourth group corresponds to self-disintegrating vectors, which cause integrations of a disorganized proviral structure after one round of the virus cycle because of the presence of an internal retroviral attachment sequence, allow specific expression of the transferred gene from an internal promoter, and cause the inability to obtain further virus production (12).

A common characteristic of all these vectors is that, after transfection into packaging cells, the 5′ LTR controls the level of production of genomic RNAs to be packaged into virions. Thus, the activities of both the 5′ LTR and the other *cis*-acting elements must be optimized to generate the highest titers of vector viruses from producer cells (1, 7). Another critical requirement of a retrovirus-mediated gene transfer system that uses replication-defective vectors is related to the efficiency of the packaging cell line. At least three criteria have to be considered to design an efficient packaging cell line: high production of the viral structural proteins, absence of release of replication-competent virions, and stability of both expression of retrovirus genes and helper-free characteristics. Previously, we described the generation of such an efficient packaging cell line (referred as Isolde) which allowed production of avian leukosis virus (ALV)-based vectors with titers higher than 10^5 resistance focus-forming units (RFFU) per ml (13, 14).

A set of vectors based on the structure of a defective ALV and corresponding to avian erythroblastosis virus (AEV) was generated by removing the viral oncogenes v-*erbA* and v-*erbB* and replacing them with the genes to be transferred (6). In this report, we present new ALV-based vectors that give titers higher than those obtained previously by testing *cis*-acting elements from different retroviral origins. Ten times more infectious particles were produced with vector constructions containing *cis*-acting elements from Rous-associated virus-1 (RAV-1) or RAV-2 than with those bearing *cis*-acting sequences from AEV. Moreover, in infected cells, these new vectors could give rise to enhanced expression of the *lacZ* gene, which was inserted into the vectors. Our results also provide evidence that stable production of helper-free vector viruses could be obtained from our Isolde packaging cell line during a long period of culture (more than 1 year) without a significant decrease of vector titers and without formation of replication-competent viruses.

Vector constructions. In avian retroviruses, several *cis*-acting elements have been located in the coding sequences (Fig. 1), including splice donor sequences (48) and dimer linkage sequences (8, 36) in the beginning of the *gag* gene, enhancers (3, 10), and sequences responsible for balanced genomic versus subgenomic mRNA (2, 44) within the *gag* genes and other *cis*-acting sequences (like splice acceptor sequences at the end of the *pol* gene). In our vectors, we chose to retain donor and dimer linkage sequences upstream of the inserted genes. Therefore, these genes were translated as fusion proteins because of the presence of the 5′ residue of the *gag* gene (extending from the initiator codon to the *Xho*I restriction site). The *gag* initiator codon in the same reading frame as the inserted gene was used (6) (Fig. 1). The other *cis*-acting sequences included in our vectors were the LTRs, the leader sequence, the 3′ end of the *env* gene (about 500 bp), and the 3′ noncoding region located between the end of the *env* gene and the 3′ LTR.

Starting with our basic vector construction, called NL53 and containing *cis*-acting elements derived from AEV (30a, 34), we generated a set of double-expression vectors containing the Neo^r gene expressed from genomic RNA and the bacterial *lacZ* gene expressed from subgenomic RNA. The vectors NLA, NLB, and NLD contained the *cis*-acting elements derived respectively from RAV-1, RAV-2, and a Schmidt-Rupin-Rous sarcoma virus subgroup D (SR-RSV-

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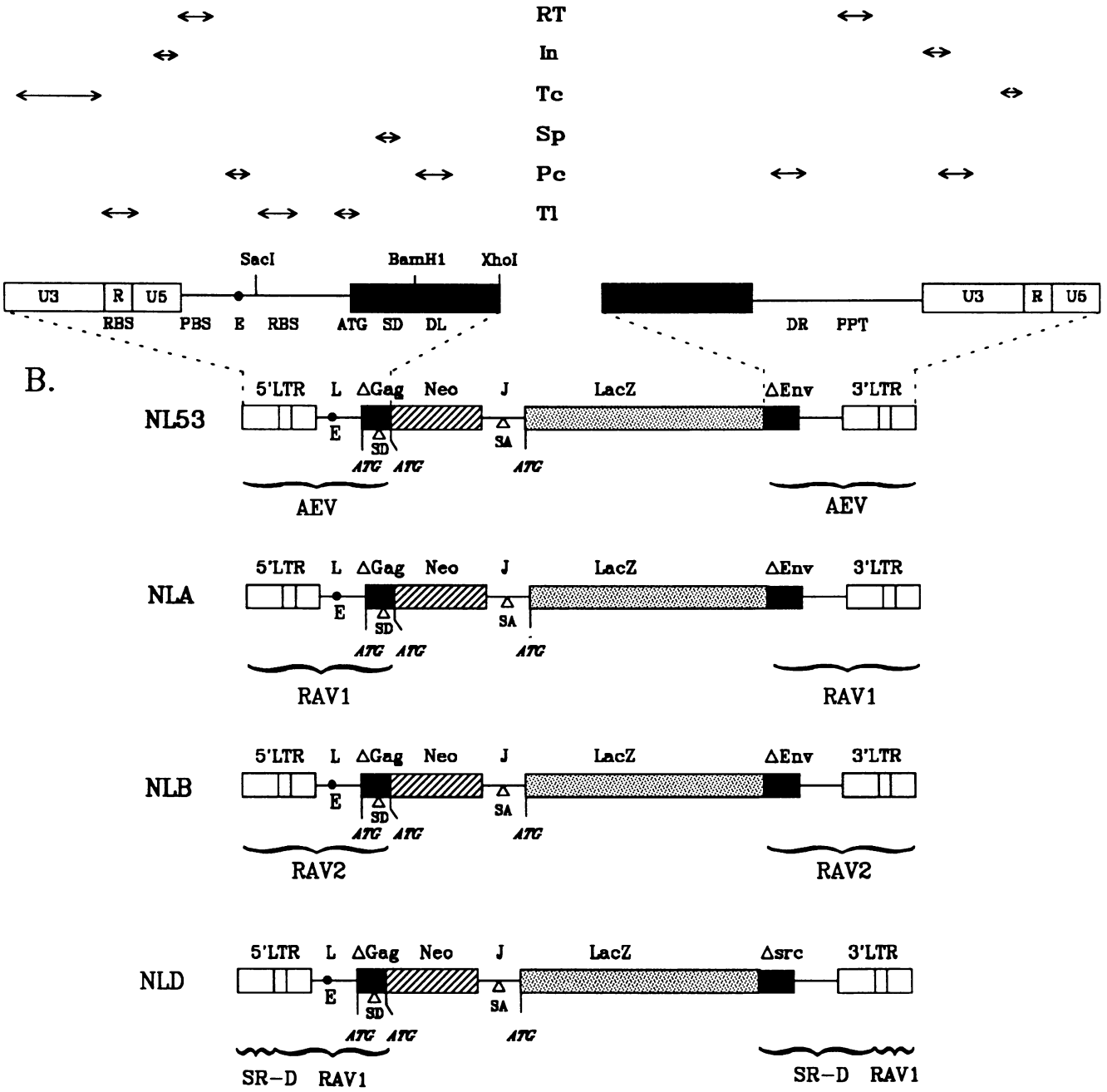

FIG. 1. Structure of NL retrovirus vectors. (A) The locations in the terminal regions of retroviruses of *cis*-acting sequences known to be involved in the different steps of retrovirus replication are indicated by horizontal arrows. RT, reverse transcription; In, integration; Tc, transcription; Sp, splicing; Pc, packaging; Tl, translation; RBS, ribosome-binding site; PBS, primer-binding site; E, encapsidation sequence; ATG, *gag* gene initiator codon; SD, splice donor sequence; DL, dimer linkage sequence; DR, direct repeat sequence; PPT, polypurine track. Positions of some restriction sites are also indicated. (B) Structures corresponding to retrovirus vectors NL53, NLB, NLA, and NLD. The same *neo-J-lacZ* fragment was inserted into the same location (*Xho*I site located in the *gag* gene) within the genomes of AEV (for NL53), RAV-2 (for NLB), and RAV-1 (for NLA). For the NLD vector, a chimeric terminal region was constructed (see text). For every vector, the splice acceptor sequence (SA) contained within the junction fragment (J) originated from AEV. L, leader region.

TABLE 1. Titers obtained with NL retrovirus vectors transfected into Isolde packaging cell line

Vector[a]	Titers				
	Neo^r (RFFU/ml[b])	*lacZ* (CFU/ml[c])			
		Initial	1 mo	4 mo	5 mo
NL53	2×10^4	3×10^4	2×10^4	1.5×10^4	1×10^4
NLA	2×10^5	2×10^5	2×10^5	1.4×10^5	1×10^5
NLB	2×10^5	3×10^5	1×10^5	2×10^5	2×10^5
NLD	2×10^4	5×10^4	ND	ND	ND

[a] Vector structures are depicted in Fig. 1. NL vectors were introduced into Isolde packaging cells by lipofection according to the standard procedure recommended by the supplier (Lipofectin, GIbco-BRL).

[b] RFFU are per milliliter of supernatant collected from pools of stable producer Neo$^+$clones of Isolde cells.

[c] Viral supernatants were harvested after different growth periods (up to 5 months) of NL vector producer cells. ND, not determined. Fewer than 1 helper virus was detected at each titration per 5 ml of viral supernatants, as previously described (38).

TABLE 2. Titers obtained with NLA retrovirus vector from clonal producer cells

Isolde-NLA clone assayed	Titer[a] (RFFU/ml)
29	8×10^5
40	1.2×10^5
43	1.5×10^6
52	6×10^5
56	4×10^5
69	2×10^6
82	1.5×10^6

[a] Titers were determined per milliliter of supernatant collected from clonal producer cells. Fewer than 1 helper virus was detected at each titration per 5 ml of viral supernatants.

D)/RAV-1 chimeric fragment (see schematic diagram of the constructions in Fig. 1). In this last vector, sequences R-U5, of the leader, and the 5′ end of *gag* originated from RAV-1, whereas the 3′ noncoding region and U3 were from SR-RSV-D. It should be noted that the four NL vectors tested in this work shared the same Neo^r gene-*lacZ* gene inserted within the viral genome. Therefore, differences observed in viral expression should be correlated with discrepancies between the activities of the *cis*-acting sequences of ALVs.

Production of helper-free viral stocks. Plasmid DNAs carrying the virus genomes of the four NL retroviral vectors were introduced by transfection into the Isolde packaging cell line. After G418 selection, polyclonal cultures were established for every plasmid transfection. In order to test an average value of NL vector production, pools of more than 200 Neo$^+$ Isolde colonies were grown for each vector. Supernatants resulting from Isolde transfections were then diluted and added to QT6 cells (33), which were subsequently either selected with G418 to test transmission of the Neo$^+$ phenotype and expressed in RFFU per milliliter or stained by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as previously described (13) to check β-galactosidase (β-Gal) phenotypes (in *lacZ* CFU per milliliter). Results are reported in Table 1. Supernatants display different levels of production according to the NL vector tested. Titers obtained with the NLA and NLB vectors and expressed as transmission of the Neo$^+$ phenotype (2×10^5 RFFU/ml) or β-Gal$^+$ phenotype (3×10^5 *lacZ* CFU/ml) were 10 times higher than those obtained with the NL53 vector (2×10^4 *lacZ* CFU or RFFU/ml). With vector NLD, an intermediate level of production of infectious particles was obtained (5×10^4 *lacZ* CFU/ml). Each viral supernatant was also tested for replication-competent viruses. No wild-type viruses (fewer than one per 5 ml of supernatant tested) could be detected by a sensitive assay performed as previously described (38).

Although transfections of the NL vectors were carried out in the Isolde cell line which had been grown in culture for 12 months under appropriate selective conditions (50 μg of hygromycin and 50 μg of phleomycin per ml to maintain expression of the two transcomplementing vectors introduced into Isolde cells that express the viral genes linked to selectable markers), no evidence of significant decrease in the production of vector NL53 was observed compared with results obtained 1 year before (5×10^4 *lacZ* CFU/ml (13).

Moreover, vector producer cells were grown for 5 months under triple selection for both retrovirus vectors (using G418) and packaging activities (using hygromycin and phleomycin). Aliquots of supernatants were regularly collected and titrated both for NL vector particles and for replication-competent viruses. Results Reported in table 1 also demonstrate the stability of helper-free production of ALV-based vectors from the Isolde packaging cell line during a continuous long-term culture. A slight decrease of vector production was observed, however, after 5 months. The simultaneous use of all three antibiotics was shown to be a very important condition for retaining optimal production of vectors. Therefore, we concluded that the Isolde cell line was stable under selective conditions for expression of the viral genes *gag*, *pol*, and *env* without release of any helper virus. Regarding the absence of formation of replication-competent viruses, we attributed the safety of the Isolde packaging cell line to the three levels of defectiveness generated in the helper vector (13): (i) deletion of the packaging site, (ii) deletion of the 3′ LTR and of 3′ noncoding sequences, and (iii) fragmentation of the genes coding for the virion components *gag-pol* on one plasmid and *env* on another. Moreover, to avoid recombinations during transfection process, these two constructions were introduced separately when Isolde was generated.

It must be noted that no more than 10 to 20% of the NL vector producer cells were β-Gal$^+$ from X-Gal staining assays (data not shown). Different hypotheses could explain these low percentages. Possible rearrangements between pNL plasmid DNAs during transfection (5) could result in inactivation of the *lacZ* gene, with the Neo^r gene remaining functional because of the selective pressure. These results prompted us to suppose that only the β-Gal$^+$ cells were able to produce *lacZ*-transmissible vector particles. Then, to test such a hypothesis, we tried to obtain an enrichment of β-Gal$^+$ cells among producer cells. For this purpose, individual clones were isolated after transfection of the NLA vector into Isolde cells, and about 100 clones were examined for β-Gal production by in situ staining. Among these clones, seven were represented by 100% β-Gal$^+$ cells and tested for vector virus production (Table 2). Three clones were found to produce NLA vector with titers higher than 10^6 RFFU/ml, whereas titers of the others ranged from 10^5 to 10^6 RFFU/ml.

Expression of *lacZ* gene in cells infected with NL vectors. Depending on the presence or absence of G418 selection, two types of experiment were conducted for quantitative comparisons of the production of β-Gal within cells infected with the different NL vectors. In the first kind of experiment, after G418 selection, which assessed that all cells were

TABLE 3. Expression of β-Gal and viral transcripts from G418-selected QT6 cells after infection with NL vectors

Vector	β-Gal activity[a]				Detection of viral transcripts		
	for Neo$^+$ cells[b]		for β-Gal$^+$ cells[c]		*lacZ* cpm[d]	GAPDH cpm[d]	Ratio[e]
	Activity	Ratio	Activity	Ratio			
NL53	90.4	1	150	1	776	150	1
NLA	305	3.4	395	2.6	889	182	1
NLB	543	6	654	4.4	1,631	162	2
NLD	126	1.4	311	2.1	653	174	0.7

[a] Measured with ONPG standard assays (37) and expressed as nanograms of β-Gal per milligram of protein.
[b] Average values from Neo$^+$cells. Ratios were expressed by using NL53 as standard.
[c] Average values from Neo$^+$ and *lacZ*-positive cells.
[d] Average value from 1 μg of total RNA hybridized either to a *lacZ* or a chicken GAPDH probe, the latter used as an internal standard (see text).
[e] NL53 vector values were used as the standard after correction with the GAPDH internal standard.

expressing at least the Neo^r gene, β-Gal activity was comparatively evaluated in four categories of QT6 cells infected by the four types of NL vectors previously mentioned. β-gal activity was tested by incubation of lysates of the cells with the *o*-nitrophenyl-β-D-galactopyranoside (ONPG) substrate at 37°C (37). The degradation of the substrates was measured by spectrophotometry at 404 nm. Simultaneously, proportions of β-Gal$^+$ Neo$^+$ cells were determined by X-Gal staining of Neo$^+$ cells (data not shown), since Neo$^+$ clones from NL-infected QT6 cells have been shown to exhibit variable proportions of β-Gal$^+$ cells depending on the NL vector tested and the number of cell generations following infection (30a, 34). From the results reported in Table 3, the highest β-Gal activity was found in cells infected with NLB viruses, whether or not the relative β-Gal$^+$ cell proportions were taken into account.

In the second type of experiment, infected cells were grown in the absence of G418 selection to mimic in vivo conditions of infection. QT6 cells were infected at a low multiplicity of infection (1 infectious virion for 100 cells) with the same amount of *lacZ* virions (10^3 CFU) of every vector. The production of β-Gal in 10^3 β-Gal$^+$ cells infected by the different NL vectors was then estimated. These test conditions allowed us to measure an average value for β-Gal production per cell expressing one copy of *lacZ* functional provirus of every NL vector. At 24 h after infection, infected cells were fixed and permeabilized. Plates were then incubated at 37°C with chlorophenol red-β-D-galactopyranoside (CPRG), a more sensitive substrate than ONPG for the β-Gal enzyme (40), and the kinetics of degradation of CPRG were determined from aliquots of supernatants by spectrophotometry at 577 nm. Kinetic curves (not shown) were found to be linear during the test period, allowing us to determine the average value of the enzymatic activity by measuring the initial speed of reaction for every NL vector. These activities were found to vary depending on the vector tested (Table 4). As in those experiments mentioned above, in which cells were selected with G418, maximal activity was obtained with the NLB vector and was approximately 10 times higher than that of NL53 vector.

Analyses of RNAs. In order to test whether different levels of transcription were relevant to the differences observed between NL vectors both in viral production (which could arise from more-abundant packageable RNAs) and in expression of β-Gal activity (which could also arise from an increased transcription of the vector), we performed analyses of vector virus RNAs. Total RNAs were extracted (4) from QT6 cells infected with helper-free stocks of every NL vector, selected with G418, and then analyzed on Northern (RNA) blots by hybridization to a *lacZ*-specific probe (Fig. 2). Viral genomic RNAs and subgenomic RNAs of the expected sizes were detected. Although sequences responsible for negative regulation of splicing within the *gag* gene in wild-type viruses (2) were not conserved in our vectors, the formation of spliced RNAs (subgenomic RNAs) was shown not to be very efficient, since subgenomic RNAs were found in smaller amounts than genomic RNAs. Autoradiographs (Fig. 2) showed that different amounts of virus-specific RNAs were obtained from NL-infected cells. More-abundant transcripts were detected with QT6 cells infected with the NLB vector containing the RAV-2 *cis*-acting elements than with the same cells infected with vector NL53 carrying *cis*-acting sequences from AEV. The other vectors did not display a marked difference in detection of viral transcripts.

To quantify precisely the amounts of viral RNAs expressed from the different NL vectors, dot-blot analyses were performed (Fig. 2) with labeled probes. Then, radioactivity from hybridization signals was evaluated by scintillation counting. To obtain a quantitative comparison of RNAs spotted on filters, duplicates of the dots were done and a replica was hybridized to a specific probe corresponding to a cellular gene expressed in avian fibroblasts, the chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, which is used as an internal standard for RNA amounts. The results are reported in Table 3 and demonstrate that only vector NLB displayed a twofold enhancement of the level of viral transcripts over that of NL53. The other vectors displayed either equal quantities (NLA) or smaller amounts

TABLE 4. Expression of β-Gal from unselected cells after infection with β-Gal$^+$ virions

Vector[a]	β-Gal activity[b]		Relative efficiency[c]
	1.2 mM	0.5 mM	
NL53	4.6	3.2	1
NLA	22.6	15.5	5
NLB	40.3	29.4	9
NLD	18.8	14.4	4.3

[a] NL vector proviruses (10^3) were introduced by infection into 10^5 QT6 cells.
[b] Infected cells were fixed with formaldehyde, permeabilized with Triton X-100 (0.25% in phosphate-buffered saline), and incubated with CPRG substrate (two concentrations of substrate were tested) whose degradation was monitored by spectrophotometry at 577 nm. β-Gal activity is given in picomoles per minute per cell ($\varepsilon_{577} = 75 \times 10^3$ liters/mol/cm).
[c] β-Gal activities relative to that of NL53.

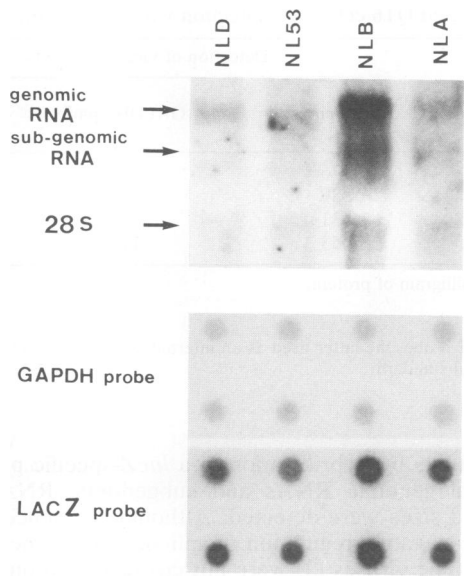

FIG. 2. Analyses of vector RNAs. (Top) Northern blots of total RNAs extracted from G418-selected QT6 cells after infection with each of the four NL vectors and hybridized to a *lacZ* probe. Expected locations of genomic, subgenomic, and 28S rRNAs are indicated. (Bottom) Four repeats of 1-μg dot-blots of total RNAs extracted from the same categories of cells used in the blots at the top were revealed either by hybridization to a *lacZ* probe (two repeats) or to the GAPDH probe (two repeats).

(NLD) of RNA compared with NL53. The same experiments were also carried out with chicken embryo fibroblast cells and confirmed that only weak differences between levels of NL virus transcripts were detected (data not shown).

Conclusion. The terminal regions of the retrovirus genome are richly endowed with signals which are essential for virus replication and gene expression (Fig. 1). They include (i) transcription signals (enhancer, promoter, and terminator) located in the U3 region of the LTRs (15–17, 26); (ii) packaging sites, with the most important one located in the median part of the leader region (30) and others located in the 3′ noncoding region (42); (iii) sequences responsible for the regulation of reverse transcription located in the 5′ leader region as well as in the 3′ noncoding region (48); and (iv) translation signals located in both the LTR and the leader region (9, 18, 19, 21, 39, 46). It is likely that some of these signals are recognized not only as linear nucleotide sequences by viral or cellular factors but also in the context of secondary structures involving hairpin structures for different parts of the viral RNA (18, 19, 29). Particularly, the formation of a stable hydrogen-bound complex between R-U5 and the leader region might be implicated in the control of translation versus packaging (18, 19).

By exchanging these *cis*-acting regions of different retrovirus origins in retrovirus vectors, we found significant differences in both the production of vector particles and the efficiency of expression of the inserted *lacZ* gene (see results for NLA and NLB compared with those for NL53 in Tables 1, 3, and 4), whereas only slight differences between the ratios of virus transcripts were detected (Table 3). For the NLA vector that did not give rise to more-abundant transcripts than NL53, we found significantly higher virus titers. Similarly, in the case of vector NLB, 10- and 6-fold enhancements of both virion production and *lacZ* gene expression, respectively, were observed, while we could detect at most only two times more viral transcripts in NLB-infected cells than in NL53-infected cells. Finally, cells infected with the NLD vector gave rise to a slight increase of production of infectious particles compared with NL53, even though fewer transcripts were detected with the former.

The weak correlation between transcription of NL proviruses and either expression of the *lacZ* gene or production of virions lead us to infer that factors other than transcription could account for the discrepancies observed between the different NL vectors. Minor modifications in the leader region and particularly in the ribosome-binding site (19) have previously been shown to strongly affect virus replication (29). We can thus hypothesize that NL RNAs might bear sequences playing a role at the level of secondary structures which could be more or less stable depending on the vector construction or could be differently accessible to protein factors involved in the replication of retrovirus vectors.

In conclusion, we have generated a set of new defective ALV-based vectors that give rise to higher titers of helper-free vector viruses and higher expression of the inserted genes after infection of target cells than did previous AEV-based vector constructions. Moreover, the most efficient vectors (NLA and NLB) were characterized by stable expression of the nonselectable *lacZ* gene, since a comparative study performed in QT6 cells with the four NL vectors mentioned in this report revealed that a high proportion of NLA- or NLB-infected clones stably expressed the reporter gene after more than 40 cell generations, whereas NL53- or NLD-infected clones displayed a dramatic decrease of β-Gal expression after only 10 cell generations (30a, 34).

Finally, the NLA and NLB vectors provide efficient vectors for transferring foreign sequences into eukaryotic cells in vivo, as is illustrated by analyses reported elsewhere (45a) of infections of early chicken embryos with 10^5 *lacZ* CFU of concentrated vector virus stocks and X-Gal staining at 72 h of incubation that showed foci of β-Gal$^+$ cells dispersed into the embryos.

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