Arch Virol (1998) 143: 681–695

Archives of Virology

Defective RNA packaging is responsible for low transduction efficiency of CAEV-based vectors

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Accepted November 26, 1997

Summary. Replication defective retroviral vectors are regularly used for transfer and expression of exogenous genes into dividing cells and in animals. Since lentiviruses are able to infect terminally differentiated and non-dividing cells, their use to produce replication defective vectors may overcome this limitation. We developed two replication-defective lentiviral vectors based on the genome of Caprine Arthritis Encephalitis Virus (CAEV). The first vector (pBNL2) carries the *neo* and *lacZ* marker genes. *Neo* gene is expressed from a genomic RNA and *lacZ* gene from a subgenomic RNA. The second vector (pCSHL) carries a single fusion gene encoding both phleomycin resistance and β-galactosidase activity. Replication-competent CAEV was used as helper virus to provide the viral proteins for transcomplementation of these vectors. Our data demonstrated that the genomes of both vectors were packaged into CAEV virions and transduced into goat synovial membrane cells following infection. However, the vector titers remained 3 to 4 logs lower than those of CAEV. Further analysis showed a lack of accumulation of unspliced pBNL2 RNA into the cytoplasm of producer cells resulting in the packaging of pBNL2 sub-genomic RNA only. In contrast, RNA produced from pCSHL vector was correctly transported to the cytoplasm and more efficiently packaged than the pBNL2 sub-genomic RNA as revealed by slot-blot and quantitative RT/PCR analyses. However this higher packaging efficiency of pCSHL genome did not result in a higher transduction efficiency of *lacZ* gene.

Introduction

Caprine arthritis encephalitis virus (CAEV) is a natural lentivirus of goat that causes chronic and progressive multisystemic disease characterized by leukoen-

cephalomyelitis in young kids, progressive arthritis and mastitis in older animals [22]. The CAEV genome encodes the Gag, Pol and Env virion proteins, and three regulatory proteins Vif, Tat and Rev [33]. Vif proteins is associated with the virus infectivity [13] and Tat protein with low transactivation of the 5′ LTR long terminal repeat [10, 34]. Rev protein and its target sequence RRE (rev responsive element) are associated with the stability of viral RNA, regulation of viral RNA splicing and transport of large RNA (unspliced and singly-spliced) from the nucleus to the cytoplasm [17, 18, 35].

CAEV, like all lentiviruses, infects and replicates in terminally differentiated and non-dividing cells [16, 24, 38]. Furthermore, expression of its genome can be modulated by Rev protein activity. These properties make CAEV genome an attractive model for development of new retroviral vectors able to infect non-dividing cells and regulate expression of the carried genes into target cells. The most frequently used retroviral vectors are based on Moloney Murine Leukemia Virus (Mo-MuLV) and Avian Leukosis Viruses (ALV). They are widely used for transfer and expression of viral or exogenous genes into cell cultures or animals (reviewed in [19]). However their use is limited by their inability to infect non-dividing cells.

During the last 5 years several efforts were made to develop retroviral vectors based on HIV-1 genome. The earlier studies reported high packaging efficiencies of HIV-1 based vectors from which all the viral genes were deleted and replaced by marker genes [3, 27]. However, in recent studies it was reported that *gag* and *env* sequences are required for efficient packaging of HIV-1 based vectors [26, 31, 32].

In order to investigate the potential use of non-human lentiviruses as vectors, we constructed two replication-defective CAEV-based vectors. CAEV viral genes were deleted and replaced with either *neo* and *lacZ* genes (pBNL2) or the fusion *SHlacZ* gene (pCSHL). Following transfection into a goat fibroblastic cell line geneticin or phleomycin resistance and β-galactosidase activity were expressed. CAEV infection of transfected cells resulted in production of virus stocks that transduced both CAEV and CAEV-based vector genomes. However transduction efficiency of vector genomes was 3 to 4 logs lower than that of CAEV. We demonstrate that this low transduction efficiency correlates with a lack of accumulation of genomic RNA into the cytoplasm and a low packaging efficiency of vector genomes.

Materials and methods

Cells and viruses

Goat synovial membrane cells (GSM) were derived from a carpal synovial membrane explant from a goat embryo as previously described [23]. GSM cells were grown in Eagle's Minimum Essential Medium (MEM, Life Technologies), supplemented with 8% fetal calf serum (FCS, Seromed). The large T-immortalized goat embryo fibroblast cell line (TIGEF) [9] was obtained following transfection of GSM cells with the pMK16-SV40-ori$^-$ plasmid carrying the SV40 genome deleted from its replication origin [11]. CAEV-CO strain [23] grown on GSM cells was used as helper virus.

Plasmids

The CAEV genome was originally cloned in two fragments [28]. To construct CAEV-based vectors we used the pCAEV containing a large 8.3 Kb *Hind*III fragment extending from the end of U3 sequence of the 5′ LTR to the 3′ part of the *env* gene, and a 400 bp fragment isolated from pCAEVLTR-CAT plasmid [14]. This plasmid carries the 3′ end of the *env* gene, the U3, R and U5 sequences but not the leader. pC2LTR plasmid is an intermediate construct that was used to derive CAEV-based vectors. The construction of pC2LTR from the two fragments of CAEV is illustrated in Fig. 1A. Briefly, pCGP plasmid was derived from pCAEV by elimination of a *Sma*I fragment containing part of *tat* and complete *env* gene sequences. A 400 bp fragment containing the 5′ part of U3 sequence was isolated from pCAEVLTR-CAT and inserted into pCGP plasmid to generate pCLTRGP. This last construct contains a complete 5′ LTR, leader, *gag, pol*, *vif* and *tat* sequences. The complete LTR was then isolated in a 0.9 Kb NcoI fragment and inserted into the *Sma*I site of pUC18 plasmid to generate pCLTR. pC2LTR was derived from pCLTR by insertion of a second copy of LTR into the *Hinc*II site of pCLTR. pC2LTR was used as a basic intermediate construct to insert heterologous genes into the *BamH*I and *Xba*I unique sites. A cassette containing *neo* and *lacZ* genes was isolated in a *BamH*I fragment from pNLA which is a RAV-1 based vector [8], and inserted into *BamH*I site of pC2LTR to obtain pBNL2. pCSHL vector was obtained from pC2LTR following insertion of a fusion gene encoding phleomycin resistance and β-galactosidase activity into the *BamH*I site.

Transfection and selection for stable expression

Plasmid DNA (5 μg) of each vector was transfected into TIGEF cells (5×10^5/60 mm dishes) using the calcium-phosphate precipitation method [12]. Transfected cells were maintained in selective medium containing 400 μg/ml of G418 (geneticin, Boehringer Mannheim). After 2 to 3 weeks, G418 resistant clones were either dissociated all together by trypsin to produce a polyclonal cell line or individually trypsinized using cloning cylinders and expanded. β-galactosidase activity was assayed with 5-bromo-4-chloro-indonyl-β-D-galactopyranoside (X-Gal) as previously described [37].

Transduction of vector genome using replication-competent helper virus

TIGEF cells transfected with vector DNA were subsequently infected with the CAEV-CO strain in MEM medium containing 2% FCS, and incubated overnight at 37 °C. Culture medium was removed and replaced with fresh MEM medium containing 8% FCS. Culture medium containing the progeny virions was harvested at day 4 post-infection, clarified by filtration through 0.45 μm filters, and stored as virus stock at −80 °C for virus titration.

Virus stock titration

Sub-confluent GSM cell cultures were inoculated with various dilutions of virus stocks in duplicate. Three days post inoculation, one set of infected cells was fixed and tested for β-galactosidase activity as previously described [37]. Blue cells were scored and vector titers were defined as blue cells forming units (BCFU) per ml of supernatant. The second set of infected cells was maintained in culture during 10 days. At day 10 cells were formalin fixed and May-Grünwald-Giemsa stained. Multinucleated giant cells were scored and viral titers of CAEV calculated according to the Reed and Muench method [29]. Titers were expressed as tissue culture infectious dose endpoints ($TCID_{50}$) per ml of virus stock.

Slot-blot RNA analysis

Culture medium was harvested from virus producer cells and clarified by filtration through 0.45 μm membrane to remove cell debris. 1 ml of clarified supernatant was used to isolate

virus particles in a pellet following centrifugation at 10 000 **g** for 45 min. RNA was isolated from virions using the acid guanidium thiocyanate method [5] and spotted in duplicate onto two nylon membranes (Hybond N, Amersham) using a slot-blot apparatus (Hoefer Scientific Instruments). Three hybridization probes were used: a *pol* probe corresponding to a 2 Kb *Bgl*II/*Xba*I fragment isolated from pCAEV [28], a *lacZ* probe corresponding to a 1.5 Kb *Xba*I/*EcoRV* fragment and a *neo* probe corresponding to a 0.9 Kb *Pst*I/*Pst*I fragment, both isolated from the pBNL2. Probes were labeled with [α-^{32}P]dCTP ($>$ 3 000 Ci/mmol) using a multiprime DNA labeling system (Amersham). Hybridization was performed at 40 °C as described previously [15].

RT/PCR analysis

Virion RNA was extracted from 1 ml of culture supernatant as described for slot-blot analysis. One half of extracted RNA (10 μl) was used as template for reverse transcription in a final volume of 20 μl (100 U MoMuLV reverse transcriptase (Promega), 0.1 μg random hexamer primers (Promega), 20 U RNasin (Promega), 0.5 mM each deoxynucleoside triphosphate, 5 mM of $MgCl_2$ in 1 $\times$ RT buffer (Promega)). The reaction was performed in a thermocycler (Appligene) as follows: denaturation at 70 °C for 5 min, reverse transcription at 42 °C for 30 min followed by 5 min at 95 °C to inactivate the enzyme. Reverse transcription product (5μl) was used as template for PCR amplification in a final volume of 50 μl (1.5 U of Taq polymerase (Eurobio), 20 pmoles of each primer, 40 mM each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$ in 1 $\times$ PCR buffer (Eurobio). The first step of PCR reaction is a 5 min incubation at 95 °C for template denaturation; the second step is a set of 35 cycles of 1 min denaturation at 95 °C, 1 min primer/template annealing at a temperature determined for each primer pair and 1 min of extention at 72 °C; the third and last step is the extension of all molecules by incubation at 72 °C for 10 min. After amplification, 1/5 (10 μl) of each PCR product was run on a 1.5% agarose gel. Quantitative RT/PCR analysis was performed by using two fold serial dilutions of the reverse transcription product as template for the PCR amplification.

Primers were chosen from the CAEV genome sequence [33] : leader$_{257}$ 5′CAAGAGAG-AAGAAGTAGAGC3′ (nt 257–276) and gag$_{794}$ 5′TCTCCTCGAGAGTTA-GATTC3′ (nt 772–794), U3$_{7\,819}$ 5′TCACCCTTTGAATTCTTATTTTTGTG3′ (nt 7793–7819); from the *neo* gene [7]: neo$_{302}$ 5′ATCTCCTGTCATCT-CACCTTGC3′ (nt 302–323) and neo$_{779}$ 5′AG-AAGGCGATAGAAGGCGATGC3′ (nt 758–779); and from the *lacZ* gene [4] : lac$_{126}$ 5′CTC-TTCGCTATTACGCAGC3′ (nt 107–126), lac$_{395}$ 5′AAAGCTGGCTACAGGAAGGC3′ (nt 395–415), lac$_{870}$ 5′CGACGTTCAGACGTAGTGTG3′ (nt 870–889), lac$_{2\,952}$ 5′ATATG-GGGATTGGTGGC-GAC3′ (nt 2 952–2 972).

Northern blot analysis

Total cellular RNA was isolated from cell monolayers using the acid guanidium thiocyanate method [5]. For cytoplasmic RNA extraction, cells were resuspended in 1$\times$ lysis buffer (100 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris pH 7.5, 1 mM Vanadyl-ribonucleoside complexes, 0.5% NP-40) and incubated for 5 min in ice. Nuclei were sedimented by centrifugation at 10 000 **g** for 5 min at 4 °C. The supernatant containing the cytoplasmic fraction was harvested and clarified again by repeating the centrifugation. RNA were extracted by phenol-chloroform and chloroform, then ethanol precipitated. RNA pellets were resuspended in 30 μl diethylpyrocarbonate-treated water. One third was separated by electrophoresis in 1% agarose gel under denaturating conditions [36] and transferred to nylon membrane under vacuum. Hybridization was performed as described above with the *lacZ* probe and with a human glyceraldehyde 3-phosphate dehyrogenase (G3PDH) probe corresponding to a 1.1 Kb cDNA fragment (Clontech).

Results

Expression of pBNL2 and pCSHL vectors into TIGEF cells

Since CAEV genome was originally cloned in two fragments, a straightforward approach could not be used to the construction of CAEV-based vectors. We first constructed intermediate recombinant plasmids with one and two CAEV LTR (pCLTR and pC2LTR respectively, Fig. 1A). *Neo* and *lacZ* genes were isolated in a *BamH*I fragment from the pNLA which is RAV-1-based vector [8] and inserted between the two LTR of pC2LTR. This resulted in pBNL2 vector expressing *Neo* and *lacZ* genes under control of two CAEV LTR (Fig. 1B). According to its design, transcription of pBNL2 vector should produce a 5.6 Kb genomic RNA encoding G418 resistance and a 4.0 Kb subgenomic RNA, produced by splicing between donor (DS) and acceptor splice sites (AS), encoding β-galactosidase activity (Fig. 1B). The pCSHL plasmid was constructed by insertion of a fusion gene that encodes both phleomycin resistance and β-galactosidase activity between the two LTR in pC2LTR. This vector carries the CAEV splice donor site located in the leader sequence but no splice acceptor site (Fig. 1C).

Plasmid DNA of pBNL2 and pCSHL vectors was introduced into TIGEF cells by transfection. After G418 selection of pBNL2 transfected cells, a polyclonal cell line expressing β-galactosidase activity in 70% of the cells and three clonal cell lines expressing β-galactosidase activity in 100% of the cells were produced. Following phleomycin selection of pCSHL transfected cells a polyclonal cell line expression β-galactosidase activity in 100% of cells was produced.

These results indicated that *neo, lacZ* and *SHlacZ* genes were expressed under the control of CAEV LTR in the absence of *tat, rev* and *vif* regulatory genes.

Packaging of vector genomes into CAEV virions

To investigate whether the vector genomes were packageable into CAEV helper particles, TIGEF-pBNL2 and TIGEF-pCSHL polyclonal cell lines were infected with the CAEV-CO strain. At day 4 post-infection virion RNA was extracted from culture medium of producer cells and hybridized to a *pol* probe to detect the CAEV-CO RNA genome and to a *lacZ* probe (Fig. 2A) or a *neo* probe (Fig. 2B) to detect the vector RNA genome. Strong signals were obtained following hybridisation with *pol* probe to the CAEV RNA, weaker signals were obtained following hybridization with *lacZ* probe to the vector RNA. Interestingly however, stronger signals were obtained following hybridization of *lacZ* probe to pCSHL than pBNL2 RNA, suggesting that pCSHL genome is more efficiently packaged into CAEV particles than pBNL2 genome. No specific signal was detected following hybridization with *neo* probe (Fig. 2B) suggesting that pBNL2 RNA detected with the *lacZ* probe contained no neo sequences.

To demonstrate that the RNA detected by slot-blot analysis corresponded to infectious viral particles, we assessed the ability of medium containing virions to infect GSM cells. The vector particles were identified by their ability to transduce the *lacZ* gene and express β-galactosidase activity. CAEV helper virus was detected by the development of giant multinucleated GSM cells. Results

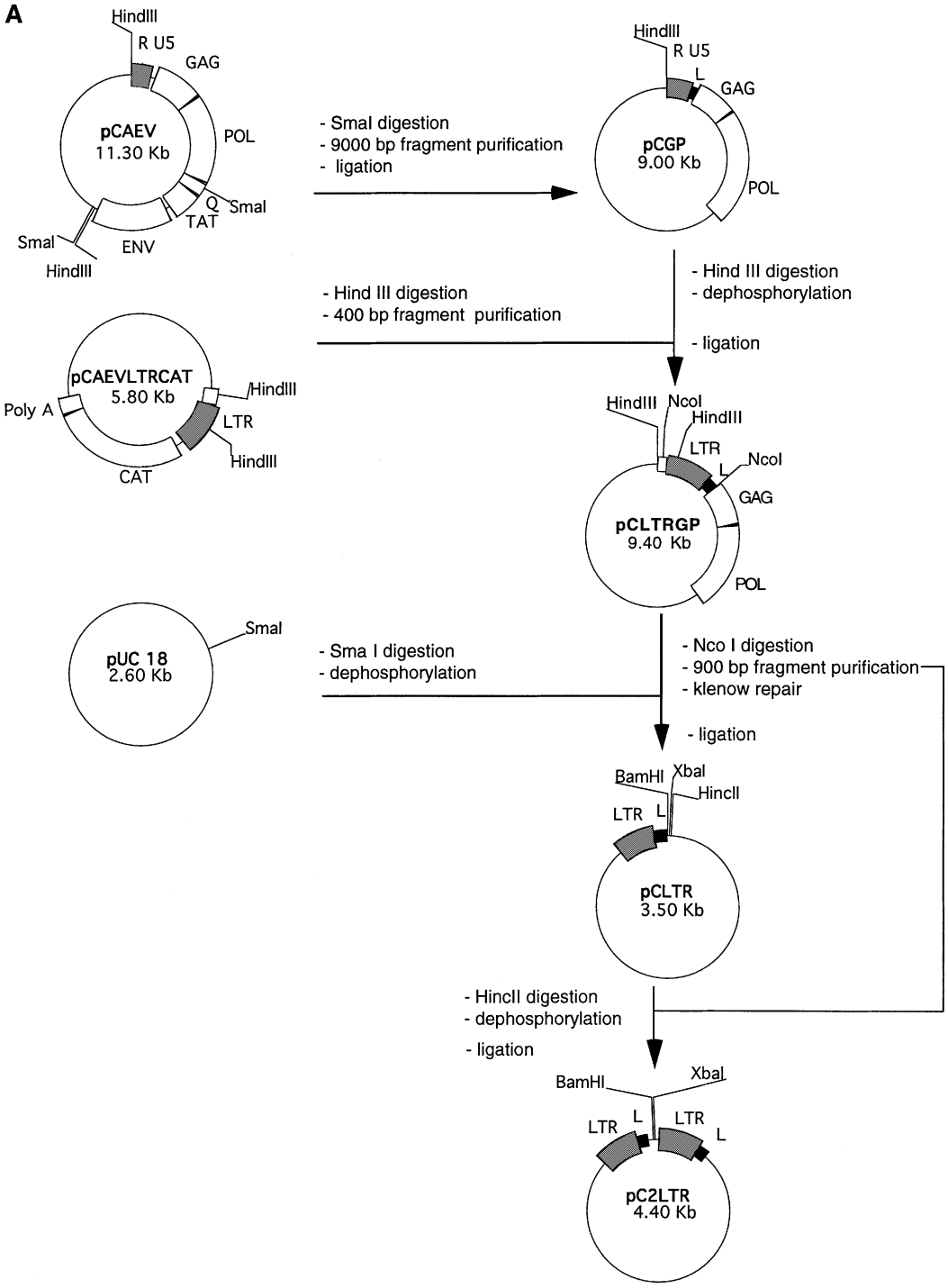

Fig. 1 (*continued*)

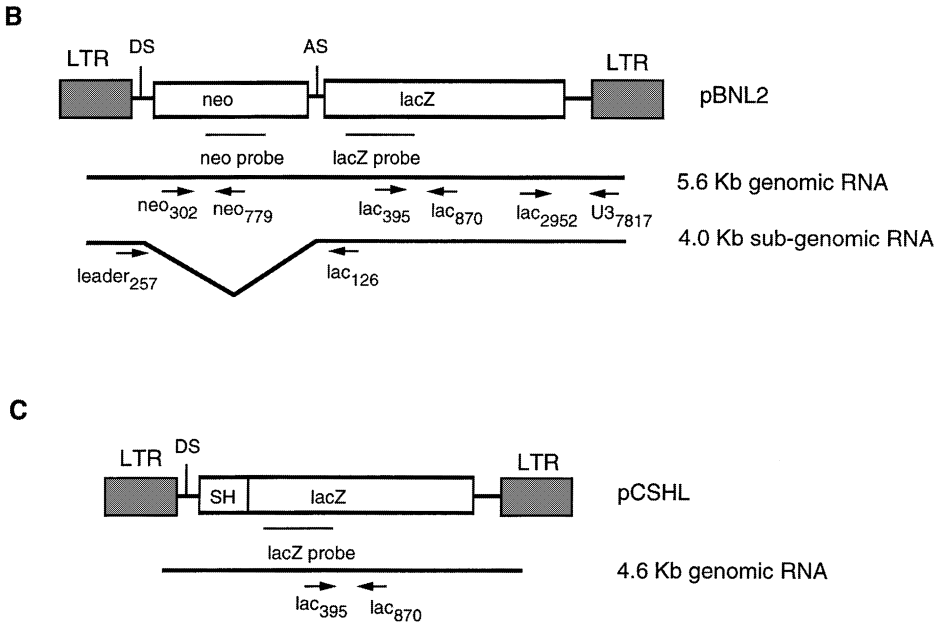

Fig. 1. Schematic representation of the strategy used for vector construction. **A** Construction of plasmid pC2LTR containing two CAEV LTR. **B** Proviral structure of pBNL2 vector showing the expected genomic and subgenomic RNA. Positions of probes and primers used for Northern-blot, slot-blot and RT/PCR analyses are indicated. *DS* Donor splice; *AS* acceptor splice. **C** Structure of pCSHL vector. *SHlacZ* Fusion gene coding for phleomycin resistance and β-galactosidase activity

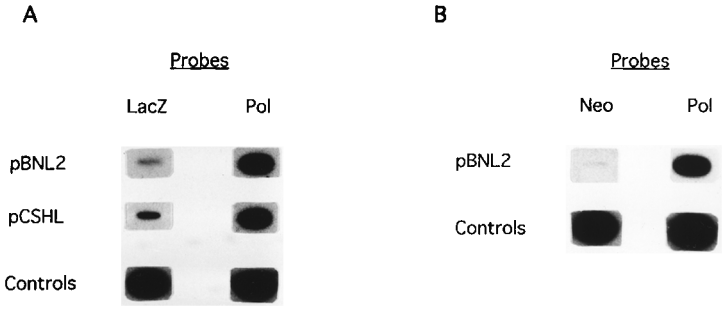

Fig. 2. Detection by slot-blot analysis of pBNL2, pCSHL and CAEV virion RNA. **A** RNA were isolated from 1 ml of culture medium of CAEV-infected TIGEFpBNL2 and TIGEF-pCSHL polyclonal cell lines. RNA samples were spotted in duplicate onto two membranes. One membrane was hybridized with a *lacZ* probe and the second with a *pol* probe. 500 pg of DNA probes were spotted onto the membranes and used as positive controls. **B** RNA isolated from 1 ml of culture medium of CAEV-infected TIGEFpBNL2 polyclonal cell line was spotted in duplicate onto two membranes. One membrane was hybridized with a *neo* probe and the second with a *pol* probe. 500 pg of DNA probe were spotted onto the membranes and used as positive controls. All the probes used had equivalent high specific activity. Following hybridization the membranes were autoradiographied for 24 h

Table 1. pBNL2 and pCSHL vector titers

Vector cell lines	Vector titers		
	low passages	medium passages	high passages
C9[a]	20	12	10
C14[a]	30	14	12
C18[a]	20	10	10
polyclonal TIGEFpBNL2	187	60	28
polyclonal TIGEFpCSHL	40	17	ND

Titration of replication defective CAEV-based vectors following infection of GSM cells. Titers are expressed in *lacZ* BCFU (Blue cell forming units)/ml. Titration of progeny virions was performed 4 days post infection. CAEV helper titers were generally in the range of 10^5 to 7×10^5 $TCID_{50}$/ml of supernatant

[a]Individual clones of TIGEFpBNL2 cells

are summarized in Table 1. The pBNL2 exhibited titers ranging 10 to 187 *lacZ* BCFU/ml, the pCSHL titers ranging 17 to 40 BCFU/ml and the CAEV titers ranging 10^5 to 7×10^5 $TCID_{50}$/ml. Surprisingly, higher vector titers were generated from the polyclonal cell line than from the TIGEFpBNL2 clones. We also observed that the titers decreased with increasing passage number of the cells (Table 1).

pBNL2 vector transduced only the lacZ gene

To determine whether the vector RNA packaged into CAEV virions corresponded to genomic or sub-genomic RNA, we performed RT/PCR using specific oligonucleotide primers to *neo* (neo_{302}/neo_{779}) and *lacZ* (lac_{395}/lac_{889}). PCR amplification of CAEV helper genome was performed using a primer in the leader sequence ($leader_{257}$) and an other one in the *gag* gene (gag_{794}). The results of PCR amplification are represented in Fig. 3A. A 494 bp specific band was detected with lac_{395}/lac_{889} primers (lane 4) and a 537 bp with $leader_{257}$/gag_{794} (lane 6). In contrast, no specific band was observed with neo_{302}/neo_{779} primers. These results suggest that pBNL2 sub-genomic but not genomic RNA was packaged into CAEV particles. Otherwise, infection of GSM cells with pBNL2 virions confirmed these results since blue cells but no G418 resistant clones were obtained.

To check that the packaged genomes were not products of recombination between the vector and the helper genomes, further RT/PCR analysis were performed on virion RNA isolated from culture media of pBNL2/CAEV and pCSHL/CAEV producer cells. Amplification with primers located at the 5′ part ($leader_{257}$/lac_{126}) or the 3′ part ($lac_{2\,952}$/$U3_{7\,819}$) of the vector genome produced only fragments of the expected size (Fig. 3B) suggessing that no recombination occurred between the vector and the helper genomes.

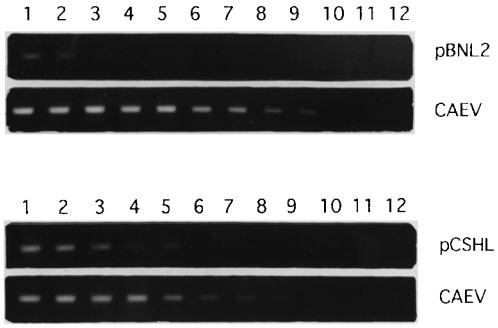

Fig. 3. Quantitative RT/PCR analysis of pBNL2, pCSHL and CAEV RNA packaged into CAEV particles. RNA were isolated from 500 μl of culture medium from CAEV-infected TIGEFpBNL2 and TIGEFpCSHL polyclonal cell lines and used as template for reverse transcription. The products of reverse transcription were two-fold serially diluted (*1–12*) and used to perform PCR amplification. pBNL2 subgenomic RNA was amplified using $leader_{257}/lac_{126}$ primers that produced a fragment of 508 bp; pCSHL RNA was amplified using lac_{395}/lac_{889} primers that produced a fragment of 494 bp; CAEV RNA was amplified using $leader_{257}/gag_{794}$ proimers which produced a fragment of 537 bp. Position of the primers used for PCR amplifications is represented in Figs. 1B and 1C

The non-spliceable pCSHL genome was more efficiently packaged than the pBNL2 subgenomic RNA

Slot-blot analysis of vector and helper RNA reported in Fig. 2 showed a stronger signal with pCSHL than pBNL2 RNA isolated from CAEV particles. This result indicates that pCSHL genome is more efficiently packaged than pBNL2 into CAEV particles. To measure this difference we performed quantitative RT/PCR analysis on RNA extracted from medium containing pCSHL/CAEV and pBNL2/CAEV virions. Extracted RNA was first used as template for reverse transcription, then products of reverse transcription were two fold serially diluted and used to perform PCR amplification using pBNL2 ($leader_{257}/lac_{126}$), pCSHL (lac_{395}/lac_{889}) or CAEV ($leader_{257}/gag_{794}$) specific primers that generate fragments of similar sizes (508 bp, 494 bp and 637 bp respectively). Positive PCR signals were obtained with the 2 first dilutions of RT product from pBNL2, whereas analysis of RT product from pCSHL RNA showed positive signals in three more dilutions (Fig. 4). This results confirmed the higher packaging efficiency of pCSHL RNA compared to pBNL2.

Lack of cytoplasmic accumulation of pBNL2 unspliced RNA

To determined whether the low packaging efficiency of vector genomes resulted from a lack of accumulation of vector RNA in the cytoplasm of producer cell lines, total and cytoplasmic RNA from TIGEF-pBNL2 and TIGEFpCSHL cell lines were extracted and analyzed by Northern blot. Two bands corresponding to the 5.6 Kb genomic and the 4.0 Kb subgenomic pBNL2 RNA were detected in both RNA fractions (Fig. 5A). While the two RNA species were equally represented in the total RNA fraction suggesting that the vector was correctly spliced,

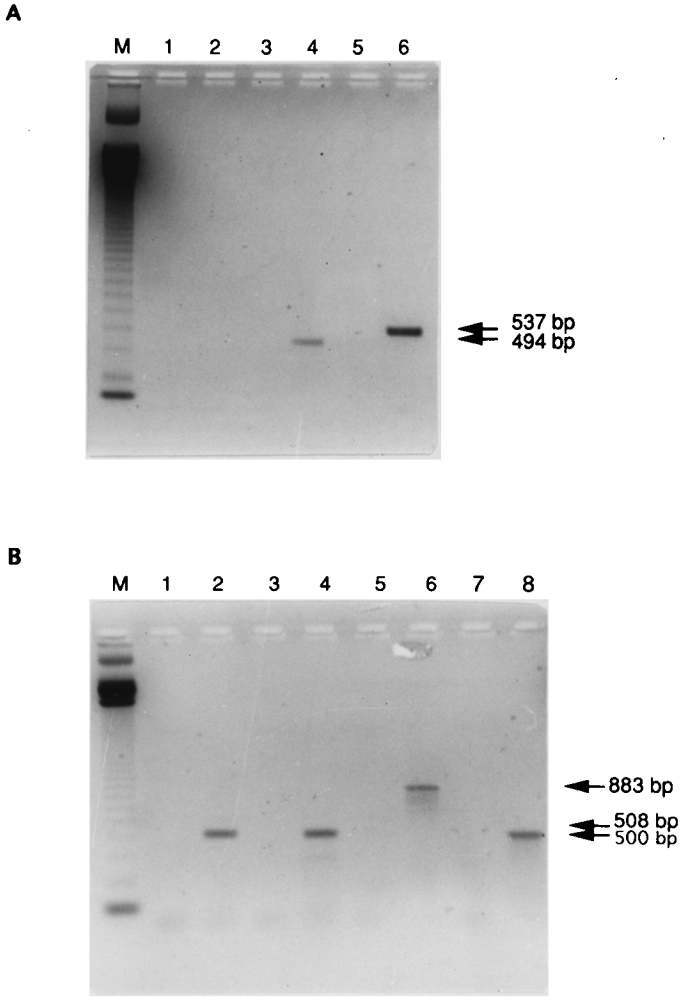

Fig. 4. Results of RT/PCR analysis on RNA extracted from culture medium of CAEV-infected TIGEFpBNL2 and TIGEFpCSHL polyclonal cell lines. **A** Analysis of the pBNL2 RNA packaged into the CAEV particles. *M* Ladder 123 bp (Gibco BRL) used as molecular size marker; *1, 3, 5* represent negative controls corresponding to PCR amplification in absence of reverse transcription step from RNA samples using neo_{302}/neo_{779}, lac_{395}/lac_{889} and $leader_{257}/gag_{794}$ primers respectively; *2, 4, 6* PCR amplification using the cDNA obtained from reverse transcription of the above RNA and the same primers. Fragments of 477 bp, 494 bp, and 537 bp were expected for the amplification with neo_{302}/neo_{779}, lac_{395}/lac_{889} and $leader_{257}/gag_{794}$ primers respectively. **B** RT/PCR analysis of pBNL2 and pCSHL packaged RNA to check for integrity of their genomes. *M* Ladder 123 bp (Gibco BRL) used as molecular size marker; *1–4* Amplification of pBNL2 RNA samples; in absence of RT step *1, 3* and on cDNA from reverse transcription *2, 4* using $leader_{257}/lac_{126}$ and $lac_{2\,952}/U3_{7\,819}$ respectively. Fragments of 508 bp and 500 bp were respectively expected using $leader_{257}/lac_{126}$ and $lac_{2\,952}/U3_{7819}$ on pBNL2 RNA. *5–8* Amplification on pCSHL RNA samples; in absence of RT step *5, 7*, and on cDNA obtained from reverse transcription *6, 8* using $leader_{257}/lac_{126}$ and $lac_{2952}/U3_{7\,819}$ respectively. Fragments of 883 bp and 500 bp were respectively expected using $leader_{257}/lac_{126}$ and $lac_{2\,952}/U3_{7\,819}$ on pCSHL RNA. The primers used for PCR amplifications are represented in Figs. 1B and C

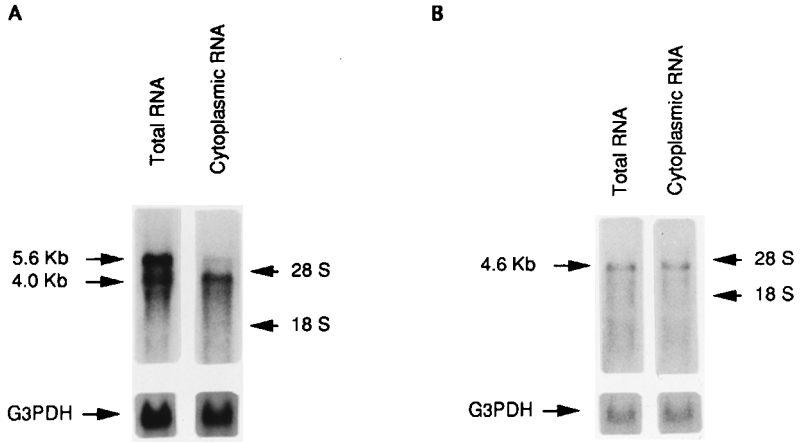

Fig. 5. Northern blot analysis of total and cytoplasmic pBNL2 and pCSHL RNA. Total and cytoplasmic RNA were extracted from CAEV-infected TIGEF-pBNL2 (**A**) and TIGEFpC-SHL (**B**) cell lines. A *lacZ* probe (represented in Fig. 1B) was used that hybridized to 4.6 Kb pCSHL RNA and to both the 5. 6 Kb genomic and the 4.0 Kb subgenomic pBNL2 RNA. Hybridization of total and cytoplasmic cellular RNA to a *G3PDH* cDNA probe was used as positive control for the RNA quality. The positions of 18 S and 28S rRNA are indicated

a faint band corresponding to the 5.6 Kb genomic RNA was detected in the cytoplasmic fraction. This results indicates a defect of accumulation of this RNA in the cytoplasm (Fig. 5A). The same result was observed both in presence and absence of CAEV. The unique band corresponding to the 4.6 Kb RNA produced from pCSHL was equally represented both in the total and the cytoplasmic fractions (Fig. 5B).

Discussion

In this study we constructed two replication defective CAEV-based vector: pBNL2 expressing two marker genes from a genomic and a sub-genomic RNA, and pCSHL expressing a fusion gene *SHlacZ* in absence of splicing. Both vectors expressed efficiently the marker genes into the goat fibroblastic cell line. The genomes of these two vectors were packaged into CAEV particles in a helper/vector system and the marker genes were transduced to GSM cells. Since the transduction efficiency was low, further analyses were carried out to define at which step the blocking occurred.

Northern blot analysis revealed a lack of accumulation of unspliced pBNL2 RNA in the cytoplasm. The absence of unspliced cytoplasmic RNA did not correlate with an apparent active over-splicing, but rather with a retention of unspliced form in the nucleus, or to its rapid degradation in the cytoplasm. It is interesting to note that RNA retention can occur in the absence of cis-acting repressive sequences (CRS) that have been mapped within the lentiviral *gag/pol* and *env* intron [2, 6]. Our results suggest that presence of the splice donor and acceptor sites can lead to retention of unspliced from of RNA in the nucleus. This observation was not common with vectors based on murine and avian simple retroviruses.

Presence of only the splice donor site in pCSHL genome did not result in RNA sequestration suggesting that both donor and acceptor splice sites are required. The low level of pBNL2 genomic RNA present in the cytoplasm was sufficient to encode G418 resistance since TIGEFpBNL2 clonal and polyclonal cell lines were produced. However it was not sufficient for packaging into CAEV virion since no genomic RNA was detected in the supernatant of TIGEFpBNL2 infected cells, and no G418 resistance clone was observed following infection of recipient cells.

Packaging of sub-genomic form of RNA was observed with retroviruses carrying a packaging signal upstream of the splice donor site [1]. Encapsidation of pBNL2 subgenomic RNA can be explained by a similar position of CAEV packaging signal. However, the higher packaging efficiency of pCSHL genome compared to pBNL2 sub-genomic RNA suggests that this signal may extend into the region between the donor splice site and *gag* ATG.

Despite an apparent increase of packaging efficiency of pCSHL vector genome into CAEV particles, this efficiency remained lower than packaging of the helper genome. These observations lead to conclude that other sequences from CAEV genome that have been removed during the construction of these vectors may be also required to achieve higher packaging efficiencies.

In spite of a better packaging efficiency of pCSHL genome, the titers remained low. This may result from a lower specific activity of the fusion gene *SHlacZ* compared to *lacZ* gene, or from an instability of the vector particles. HIV-1 based vectors particles have been shown to be more stable when pseudotyped with MLV or Vesicular stomatitis envelope proteins [21, 30]. The low pCSHL titers may also result from inefficient infection of target cells. Recent work on HIV-1 based vectors reported the involvement of sequences from *pol* gene for efficient reverse transcription of vector genomes into the target cells [25].

In summary, this study provides demonstration that CAEV-based vectors express and transduce heterologous genes into target cells. Improvement of vector titers requires a better transport of unspliced form of RNA from the nucleus to the cytoplasm which may be achieve using the CAEV Rev/RRE system. It also requires the identification of other CAEV sequences necessary to increase the packaging of vector genome, and stability or specific infectivity of particles.

Acknowledgements

We thank G. Querat and R. Vigne for supplying pCAEV and pCAEVLTR-CAT, and for helpful discussions. We are grateful to O. Narayan and T. Greenland for critically reading the manuscript and to C. Fornazero and F. Gounel for expert technical assistance. This work was supported in part by grants from the Agence Nationale de la Recherche sur le SIDA (ANRS). L. M.-L. is the recipient of a Fondation Mérieux and an ANRS fellowships.

References

1. Aronoff R, Hajjar AM, Linial M (1993) Avian retroviral RNA encapsidation: reexamination of functional 5′ RNA sequences and the role of nucleocapsid cys-his motifs. J Gen Virol 67: 178–188

2. Brighty DW, Rosenberg M (1994) A cis-active repressive sequence that overlaps the rev-responsive element of human immunodeficiency virus type 1 regulates nuclear retention of env mRNA independently of known splice signals. Proc Natl Acad Sci USA 91: 8 314–8 318
3. Buchschacher GL, Panganiban AT (1992). Human immunodeficiency virus vectors for inducible expression of foreign genes. J Virol 66: 2 731–2 739
4. Casadaban MJ, Martinez-Arias A, Shapiras SK, Chou J (1983) Beta-galactosidase gene fusions for analyzing gene expression in Escherichia coli and yeast. Methods Enzymol 100: 293–308
5. Chomczynsky P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159
6. Cochrane AW, Johnes KS, Beidas S, Dillon PJ, Skalka AM, Rozen CA (1991) Identification and characterization of intragenic sequences which repress human immunodeficiency virus structural gene expression. J Virol 65: 5 305–5 313
7. Colbere-Garapin F, Horodniceau F, Kourilsky P, Colbert-Garapin A (1981) A new dominant hybrid selectable marker for higher eukaryotic cells. J Mol Biol 150: 1–14
8. Cosset FL, Legras C, Thomas JL, Molina RM, Chebloune Y, Faure C, Nigon VM, Verdier G (1991) Improvement of avian leukosis virus (ALV)-based retrovirus vectors by using different cis-acting sequences from ALVs. J Virol 65: 3 388–3 394
9. Da Silva Teixeira MF, Lambert V, Mselli-Lakhal L, Chettab AK, Chebloune Y, Mornex JF (1996) Immortalization of caprine fibroblasts permissive for small ruminant lentiviruses replication. Am J Vet Res 58: 579–584
10. Davis JL, Clements JE (1989) Characterization of a cDNA clone encoding the visna virus transactivation protein. Proc Natl Acad Sci USA 86: 414–418
11. Gluzman Y, Frisque RJ, Sambrook J (1980) Origin-defective mutants of SV40. Cold Spring Harb Symp Quant Biol 44: 293–300
12. Graham FL, Eb AJ (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52: 456–467
13. Harmache A, Bouyac M, Audoly G, Hieblot C, Peveri P, Vigne R, Suzan M (1995) The vif gene is essential for efficient replication of caprine arthritis encephalitis virus in goat synovial membrane cells and affects the late steps of the virus replication cycle. J Virol 69: 3 247–3 257
14. Hess JL, Pyper JM, Clements JE (1986) Nucleotide sequence and transcriptional activity of the caprine arthritis-encephalitis virus long terminal repeat. J Virol 60: 385–393
15. Leroux C, Cordier G, Mercier I, Chastang J, Lyon M, Quérat G, Greenland T, Vigne R, Mornex JF (1995). Ovine aortic smooth muscle cells allow the replication of visna-maedi virus in vitro. Arch Virol 140: 1–11
16. Lewis P, Hensel M, Emerman M (1992) Human immunodeficiency virus infection of cells arrested in the cell cycle. Embo J 11: 3 053–3 058
17. Malim MH, Cullen BR (1993) Rev and the fate of pre-mRNA in the nucleus: implications of the regulation of RNA processing in eukaryotes. Mol Cell Biol 13: 6 180–6 189
18. Mazarin V, Gourdou I, Querat G, Sauze N, Audoly G, Vitu C, Russo P, Rousselot C, Filippi P, Vigne R (1990) Subcellular localization of rev-gene product in visna virus-infected cells. Virology 178: 305–310
19. Miller DA, Miller DG, Garcia VJ, Lynch CM (1993) Use of retroviral vectors for gene transfer and expression. Methods Enzymol 217: 581–599

20. Naldini L, Blömer U, Gage FH, Verma IM (1996 a) Efficient transfer integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. Proc Natl Acad Sci USA 93: 11 382–11 388
21. Naldini L, Blömer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D (1996b) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272: 263–267
22. Narayan O, Clements JE (1989) Biology and pathogenesis of lentiviruses. J Gen Virol 70: 1 617–1 639
23. Narayan O, Clements JE, Strandberg JD, Cork LC, Griffin DE (1980) Biological characterization of the virus causing leukoencephalitis and arthritis in goats. J Gen Virol 50: 69–79
24. Narayan O, Wolinsky JS, Clements JE, Strandberg JD, Griffin DE, Cork LC (1982) Slow virus replication: the role of macrophages in the persistence and expression of visna viruses of sheep and goats. J Gen Virol 59: 345–356
25. Parolin C, Brunella T, Palu G, Sodroski J (1996) Use of cis and trans-acting viral regulatory sequences to improve expression of human immunodeficiency virus vectors in human lymphocytes. Virology 222: 415–422
26. Parolin C, Dorfman T, Palu G, Gottlinger H, Sodroski J (1994) Analysis in human immunodeficiency virus type 1 vectors of cis-acting sequences that affect gene transfer into human lymphocytes. J Virol 68: 3 888–3 895
27. Poznansky M, Lever AML, Bergeron L, Haseltine W, Sodrosky J (1991) Gene transfer into human lymphocytes by a defective human immunodeficiency virus type 1 vector. J Virol 65: 532–536
28. Pyper JM, Clements JE, Gonda MA, Narayan O (1986) Sequence homology between cloned caprin arthritis encephalitis virus and visna virus, two neurotropic lentiviruses. J Virol 58: 665–670
29. Reed L, Muench H (1938) A sample method for estimating fifty per cent points. Am J Hyg 27: 413–497
30. Reiser J, Harmison G, Kluepfel-Stahl S, Brady RO, Karlson S, Schubert M (1996) Transduction of non-dividing cells using pseudotyped defective high-titer HIV type 1 particles. Proc Natl Acad Sci USA 93: 15 266–15 271
31. Richardson H, Child LA, Lever AM (1993) Packaging of human immunodeficiency virus type 1 RNA requires cis-acting sequences outside the 5′ leader region. J Virol 67: 3 997–4 005
32. Richardson JH, Kaye JF, Child LA, Lever AML (1995) Helper virus-free transfer of human immunodeficiency virus type 1 vectors. J Gen Virol 76: 691–696
33. Saltarelli MJ, Querat G, Konings DA, Vigne R, Clements JE (1990) Nucleotide sequence and transcriptional analysis of molecular clones of CAEV which generate infectious virus. Virology 179: 347–364
34. Saltarelli MJ, Schoborg R, Gdovin SL, Clement JE (1993) The CAEV tat gene transactivates the viral LTR and is necessary for efficient viral replication. Virology 197: 35–44
35. Saltarelli MJ, Schoborg R, Pavlakis GN, Clements JE (1994) Identification of the caprine arthritis encephalitis virus Rev protein and its cis-acting Rev-responsive element. Virology 199: 47–55
36. Sambrook J, Fritsch EF, Maniatis TE (1989) Molecular cloning: a laboratory manual, 2nd ed. Press Cold Spring Harbor Laboratory Press Cold Spring Harbor
37. Thomas JL, Afanassieff MF, Cosset FL, Molina RM, Ronfort C, Drynda A, Legras C, Cheblounе Y, Nigon VM, Verdier G (1992) In situ expression of helper-free avian

leukosis virus (ALV)-based retrovirus vectors in early chick embryos. Int J Dev Biol 36: 215–227

38. Trowbridge RS, Lehmann J, Torchio C, Brophy P (1980) Visna virus synthesized in absence of host-cell division and DNA synthesis. Microbios 29: 71–80

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Received August 8, 1997