JOURNAL OF VIROLOGY, Apr. 1991, p. 1938–1945
0022-538X/91/041938-08$02.00/0

Vol. 65, No. 4

# Activator-Dependent and Activator-Independent Defective Recombinant Retroviruses from Bovine Leukemia Virus

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Received 20 September 1990/Accepted 19 December 1990

**The replication-competent bovine leukemia virus (BLV) has been modified for use as a vector for foreign genes. The *gag*, *pol*, *env*, and pX regions of the virus were replaced by an exogenous nuclear location signal LacZ (*nls*LacZ) or SV*nls*LacZ gene. Transfection of the ovine cell line FLK-BLV, which expresses all BLV proteins from a wild-type provirus, with this viral DNA resulted in a viral titer of $10^4$ CFU/ml. The inclusion of a large portion of the *gag* region did not significantly increase the titer. Both activator-dependent and activator-independent retroviruses were constructed. In activator-dependent vectors, the expression of the insert was dependent on the presence of the Tax protein, which activated the BLV long terminal repeat. In activator-independent vectors, the expression of the insert was constitutive because of the presence of an internal promoter. Infections with the recombinant retrovirus were inhibited by specific neutralizing antibodies. The structure of the transduced genetic material was not rearranged. BLV vectors encoding a reporter *nls*LacZ gene, the product of which can be detected in single cells, greatly simplified studies of their biological properties. Determination of the host range of BLV vectors established that BLV-based recombinant retroviruses are effective in the transduction of genes in a variety of species and cell types.**

Retrovirus vectors based on murine leukemia virus (MLV), Rous sarcoma virus (RSV), and spleen necrosis virus (SNV) are widely used. The minimal regions required by vector constructs in *cis* are limited to those sequences necessary for the synthesis of the long terminal repeat (LTR) and for packaging of the virus. In addition to sequences in the LTR, they include the primer-binding site (nucleotides 323 to 340 for MLV) and the polypurine tract (starting at nucleotide 7966 for MLV). The virions are produced in helper cell lines which provide in *trans* the viral structural proteins (*gag*, *pol*, and *env*; 23; reviewed in reference 27). The host range of murine vectors is broad because of the use of amphotropic (6, 24) and xenotropic (9) helper cell lines. For facilitation of the expression of a foreign gene, these vectors may include exogenous eukaryotic promoters (34, 38, 42). In addition, the LTR may be manipulated to alter its promotor function (15, 44) and/or to obtain duplication of the insert in the provirus (39). These modifications in general have no effect on the efficiency of replication. Therefore, the expression of an insert can be controlled in a number of ways (7, 16). The uses of retrovirus vectors are multiple and include the generation of transgenic animals (17, 18, 28), the generation of mosaic animals for cell lineage analysis (31, 36), and the genetic modification of somatic cells for gene therapy (2, 14, 16, 22, 43). Retrovirus vectors have also been used as a model to gain insight into the biological properties of retroviruses (30, 37).

Bovine leukemia virus (BLV) belongs to the same group of retroviruses as does human T-cell leukemia virus (35). Both BLV and human T-cell leukemia virus cause leukemia and lymphoma. Their genomes are complex and, in contrast to MLV, RSV, and SNV, their expression requires virus-encoded *trans* regulators. The pX region of BLV encodes two regulatory proteins, Tax and Rex, used for this purpose. Tax is essential for virus expression (10, 11, 33), and Rex allows the regulation of the synthesis of structural or regulatory proteins (11). Tumor cells transformed by BLV contain the viral genome, but the expression of viral proteins is generally not detected (5, 40). This complex regulation complicates the study of BLV and makes it difficult to establish simple methods of detection and titration of the virus, to characterize its life cycle, and thus to establish conditions for using it as a vector.

We report here that BLV-based recombinant retroviruses with viral regions limited to sequences in and immediately adjacent to the LTR can be produced in helper cell lines. By taking advantage of the specific requirement of Tax for *trans* activation, we obtained conditional expression of transduced genes in cells which express the Tax gene. These vectors, designated activator dependent, have not been previously obtained with either murine or avian vectors. With the addition of an internal promoter, constitutive expression of the transduced genes was obtained. These vectors have been designated activator independent. We have used both types of vector as a model with which to study some of the biological properties of BLV, in particular, the half-life and host range of the virus. The availability of BLV-based recombinant retroviruses would also facilitate in vivo studies of the virus, in particular, the evaluation of the usefulness of these retroviruses as vectors for gene transfer in cells and animals.

## MATERIALS AND METHODS

**Plasmid construction.** Plasmid pBLV13 was constructed from two overlapping clones of a BLV provirus cloned by Deschamps et al. (13) and Couez et al. (8). The T15 clone (13) contains an 8,092-bp *Sac*I-*Sac*I fragment of the BLV provirus, and the T15/4 clone (8) contains an intact 3′ LTR inserted at the *Eco*RI site of pBR322. The reconstruction of pBLV13 was achieved in two steps. First, an intact 5′ BLV

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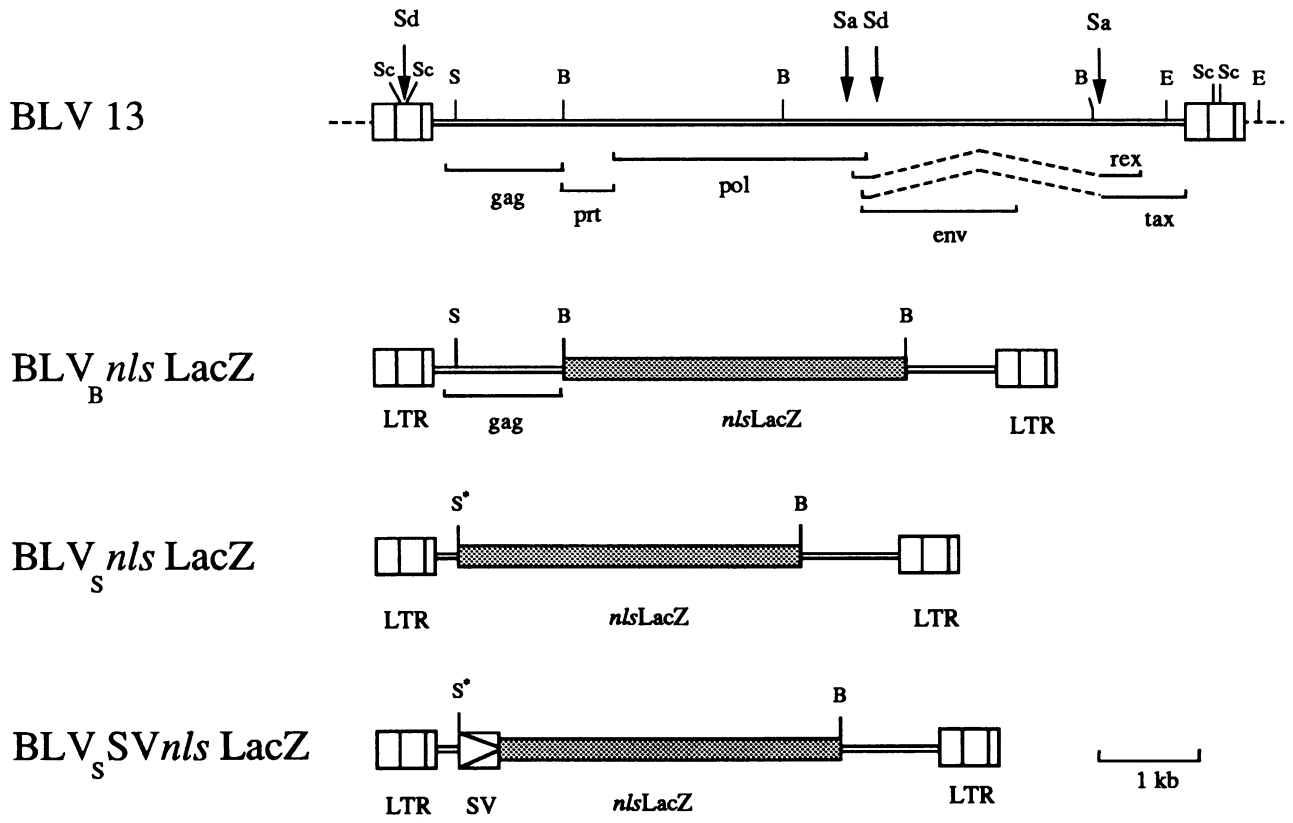


FIG. 1. Structures of the LacZ BLV-based recombinant retroviruses. BLV13 is the reconstituted BLV provirus. BLV$_B$*nls*LacZ is a recombinant provirus in which the *nls*LacZ gene is placed between two *Bam*HI sites (nucleotides 1831 and 6997 in the viral RNA). BLV$_S$*nls*LacZ is a recombinant provirus in which the *nls*LacZ gene is placed between an *Sma*I site and a *Bam*HI site (nucleotides 616 and 6997 in the viral RNA). BLV$_S$SV*nls*LacZ has the same viral structure as BLV$_S$*nls*LacZ, but *nls*LacZ expression is driven by the early promoter of SV40 (SV). Abbreviations: Sd, splice donor; Sa, splice acceptor; Sc, *Sac*I site; E, *Eco*RI site; S, *Sma*I site; S*, *Sma*I site not reconstituted during construction; B, *Bam*HI site functional in all constructs.

LTR containing the 98-bp *Sac*I-*Sac*I fragment was constructed by ligating three fragments: a *Bam*HI-*Sac*I fragment of T15/4 obtained by *Bam*HI total restriction and *Sac*I partial restriction, a *Sac*I-*Eco*RI fragment of T15 obtained by *Sac*I and *Eco*RI total restriction, and the *Bam*HI-*Eco*RI fragment of pGEM1. The presence of the 98-bp *Sac*I-*Sac*I fragment in the final plasmid, pBLVΔLTR3′, was verified by DNA sequencing (data not shown). The second step restored an intact BLV provirus by the addition of the 3′ LTR to pBLVΔLTR3′. This was done by ligation of the *Eco*RI fragment of T15/4 containing the intact 3′ LTR to a fragment obtained by linearization of pBLVΔLTR3′ by *Eco*RI.

pBLV$_B$*nls*LacZ (Fig. 1) was generated by replacement of the *Bam*HI-*Bam*HI (nucleotides 1831 to 6997) fragment of pBLV13 with a *Bam*HI-*Bam*HI fragment of pMMuLVHPRT *nls*LacZ containing the *nls*LacZ gene (3) (Fig. 1). pBLV$_S$*nls* LacZ was prepared by exchanging the *Sma*I-*Bam*HI (nucleotides 616 to 6997) fragment of pBLV$_B$*nls*LacZ with a *Sal*I-*Bam*HI fragment of pMMuLV-SV*nls*LacZ after filling in of the protruding *Sal*I end with the Klenow fragment of DNA polymerase (Fig. 1). pBLV$_S$SV*nls*LacZ was constructed by exchanging the *Sma*I-*Bam*HI (nucleotides 616 to 6997) fragment of pBLV$_B$*nls*LacZ with a *Pst*I-*Bam*HI fragment of pGemSV*nls*LacZ after filling in of the protruding *Pst*I end with the Klenow fragment of DNA polymerase (Fig. 1).

pMX-1 (21) is a plasmid containing the complete coding sequence of the Tax (pX1) activator under the control of the mouse metallothionein I promoter, the activation of which can be induced by cadmium chloride.

**Cell culture.** 3T3 (mouse fibroblast), HeLa (human), MRC5 (human embryonic lung), HEp-2 (human larynx carcinoma), COS (simian), G355-5 (feline), PK15 (pig kidney), and BRL (buffalo rat) are established cell lines. RFM2 cells are fetal ovine spleen cells (9) immortalized by transfection with pSV2gpt (25), which expresses the large T and small t antigens of simian virus 40 (SV40). PFM (fetal ovine lung), PFC (fetal caprine lung), PV (calf lung), PL (rabbit lung), RLG (rabbit lung), and FP (chicken fibroblast) are primary cells (9). ST (swine testis) cells were a gift from P. Nandy. YR2 is a BLV-induced ovine tumoral B-cell line (40).

Cells were cultivated as described by Jakob and Nicolas (19). At 24 h before infection or transfection, the cells were seeded at $3 \times 10^4$ in a 35-mm petri dish containing 2 ml of medium. For transfection, 250 μl of calcium phosphate precipitate was prepared as described previously (19), except that the precipitate was added to the cells directly without washing 25 min later. For infection, 5 μg of Polybrene per ml (or 5 μg of DEAE-dextran) was added to the culture medium at the time of infection.

*lacZ* expression was tested 2 days after infection or transfection with 4-chloro-5-bromo-3-indolyl-β-D-galactoside (X-Gal) staining of fixed cells (36) or with fluorescein di-β-D-galactopyranoside (FDG) staining of living cells and fluorescence-activated cell sorter (FACS) analysis (29). Viral

TABLE 1. Transactivation by Tax of pBLV$_B$*nls*LacZ[a]

| Plasmids | No. of β-gal$^+$ cells | | | | |
|---|---|---|---|---|---|
| | FLK-BLV | RFM2 | 3T3 | G355-5 | HeLa |
| pBLV$_B$*nls*LacZ + pGem1 | 8,172 | 2 | 0 | 36 | 0 |
| pBLV$_B$*nls*LacZ + pMX-1 | ND | 8,576 | 10,864 | 9,840 | 1,728 |
| pMLV-SV*nls*LacZ + pGem1 | 2,464 | 3,424 | 3,954 | 11,552 | 3,552 |

[a] Cells were transfected with 10 μg of DNA (5 μg of LacZ plasmid plus 5 μg of pMX-1 or pGem plasmid) as described in Materials and Methods. pMX-1 expresses the Tax activator from the mouse metallothionein I promoter; pGem is a bacterial plasmid used as a carrier to normalize the quantity of DNA in each precipitate; and pMLV-SV*nls*LacZ is a plasmid containing a LacZ MLV recombinant provirus with an internal SV40 promoter. At 48 h after transfection, cells were fixed and stained with X-Gal. Cells with blue staining of nuclei were counted. ND, Not determined.

titers were calculated from the linear portions of titration curves.

**Viral production.** FLK-BLV is an ovine cell line in which BLV structural and regulatory proteins are overexpressed. It produces wild-type BLV (41). FLK-BLV/BLV$_B$*nls*LacZ cells were isolated after transfection of FLK-BLV cells with pBLV$_B$*nls*LacZ DNA. One week after transfection, the β-galactosidase-positive (β-gal$^+$) cells were sorted by FDG staining as described previously (29). A subsequent selection by cell sorting was carried out on this population to obtain cells stably expressing LacZ at high levels. FLK-BLV/BLV$_S$*nls*LacZ and FLK-BLV/BLV$_S$SV*nls*LacZ cells were similarly obtained after transfection with pBLV$_S$*nls*LacZ and pBLV$_S$SV*nls*LacZ DNAs, respectively.

Stocks of virus were obtained from the medium of the producer cells before or just after they reached confluence. The supernatants were filtrated through 0.22-μm-pore filters, and aliquots were stored at −80°C.

Antisera from BLV-infected cattle (B85163) and sheep (S129) as well as monoclonal antibodies against F, G, and H epitopes of the gp51 BLV *env* protein (4) were gifts from A. Burny. Sera and monoclonal antibodies were incubated for 30 min on ice at various dilutions in the presence of 100 μl of BLV$_S$SV*nls*LacZ virus. The mixtures were added to the culture medium of BRL (buffalo rat) cells as in the infection protocol described in the preceding paragraph.

## RESULTS

**Construction of LacZ recombinant proviruses.** The BLV provirus used in all of our vector constructions was isolated from an ovine tumor (13). This provirus was chosen because its LTR is transactivable by the Tax protein, as demonstrated in experiments with a BLV LTR-CAT chimeric construct (33). The provirus was cloned in two fragments; the first one (13), a *Sac*I fragment, includes the entire viral genome, except for 98 bp in the R region, and the second one (8), an *Eco*RI fragment, contains the complete 3′ LTR.

As the initial step in the construction of our BLV vectors, we reconstructed the intact provirus by using these two clones as described in Materials and Methods. To verify the functionality of the reconstituted BLV LTR, we replaced a sequence of BLV with the *nls*LacZ reporter gene (*lacZ* gene from *Escherichia coli* fused to a sequence coding for the SV40 nuclear location signal) (3, 20). The deleted fragment corresponded to the *pol* and *env* sequences located between two *Bam*HI sites (from nucleotides 1831 to 6997 in the RNA). In this construct (pBLV$_B$*nls*LacZ; Fig. 1), there was no expression of the Tax and Rex proteins, as the shared first exon for the Tax and Rex genes is contained in the deleted fragment. pBLV$_B$*nls*LacZ was transfected into cells expressing the Tax activator. The first cell line tested, FLK-BLV, is a cell line which expresses a wild-type BLV provirus (41). Two days after transfection, approximately 5% of the transfected cells were β-gal$^+$, as shown by counting of cells with blue staining of nuclei after X-Gal histochemistry (36) (Table 1). Transfection of other cells which do not express the Tax activator, RFM2 (ovine), 3T3 (murine), G355-5 (feline), or HeLa (human), resulted in no or very few β-gal$^+$ cells (data not shown). The reporter gene was expressed in these cell lines only when Tax expression was obtained by the addition of a second plasmid in the transfection (pMX-1) (21) (Table 1).

In an effort to further examine the activity of the viral LTR promoter, we compared the number of β-gal$^+$ cells after cotransfection of pBLV$_B$*nls*LacZ and pMX-1 with the number of β-gal$^+$ cells obtained after transfection of pMLV-SV*nls*LacZ (in which LacZ is expressed under the control of MLV and the SV40 early promoter). For the various cells tested, the BLV LTR promoter was found to be a strong promoter when transactivated by Tax, with β-gal$^+$ cell numbers comparable to those obtained with the MLV-SV40 double promoter (Table 1). From these experiments, it was concluded that the BLV LTR has very low baseline activity in most cell lines in the absence of Tax and that it is transactivated by Tax to high levels in cells from a variety of species.

**Production of activator-dependent LacZ recombinant retroviruses.** To produce BLV$_B$*nls*LacZ recombinant retrovirus, we transfected FLK-BLV cells expressing all BLV proteins with pBLV$_B$*nls*LacZ DNA. An enriched population of β-gal$^+$ cells was obtained by FACS selection following viral staining for β-galactosidase expression with FDG (29) (Fig. 2A). Another FACS selection of the resulting population was performed 2 weeks later to isolate cells expressing β-galactosidase at high levels (Fig. 2B). A 100% β-gal$^+$ cell population was thus isolated (Fig. 2C), and the supernatant was tested for the production of BLV$_B$*nls*LacZ recombinant retrovirus in the following manner.

To detect the recombinant retroviruses, we created a cell line constitutively expressing Tax. The RFM2 cell line was chosen for the generation of such a line because (i) it is susceptible to BLV infection, as shown by the formation of syncytia after the addition of BLV virions to cells in culture (data not shown), and (ii) it permits the proper activation of the BLV promoter in the presence of the Tax protein (Table 1). An RFM2 cell line constitutively expressing the Tax activator was obtained by cotransfection of pMX-1 and pSV-Tk-neoβ (added to select stable transformed clones) (26). To test for Tax expression, we transfected the G418-resistant clones with pBLV$_B$*nls*LacZ and screened them for the expression of β-galactosidase (Table 2). The RFM2-Tax14 clone produced the greatest numbers of β-gal$^+$ cells and was used for all subsequent assays of viral production.

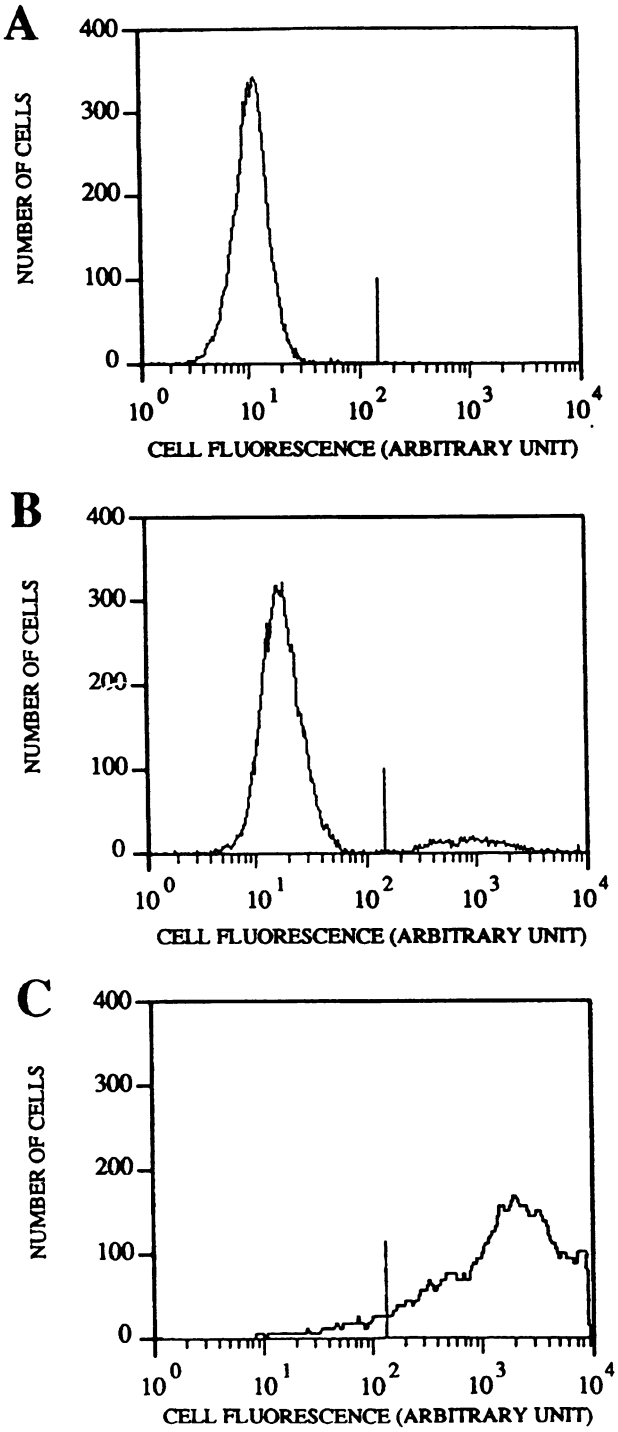


FIG. 2. FACS selection of $BLV_B$*nls*LacZ producer cells. (A) One week after transfection of FLK-BLV cells with $pBLV_B$*nls*LacZ DNA, β-gal$^+$ cells were revealed with FDG as a substrate. Cells with a fluorescence higher than 150 (0.1% of cells) were sorted. (B) After 3 weeks of culturing, cells were FDG labeled, and those with a fluorescence higher than 150 (8.9% of cells) were selected. (C) After an additional 10 days of culturing, cells were analyzed for β-galactosidase activity by FDG labeling; 93% of cells had a fluorescence higher than 150. The β-gal$^+$ FLK-BLV/$BLV_B$*nls*LacZ cells were used as a producer of recombinant viruses. The mean autofluorescence of FLK-BLV cells was 10 to 20.

TABLE 2. Isolation of an ovine cell line expressing Tax and identification of recombinant retrovirus production[a]

| Cells | No. of β-gal$^+$ cells after: | |
|---|---|---|
| | Transfection of $pBLV_B$*nls*LacZ | Infection with $BLV_B$*nls*LacZ |
| RFM2 | 0 | 2 |
| RFM2-Tax11 | 30 | 92 |
| RFM2-Tax13 | 0 | 3 |
| RFM2-Tax14 | 210 | 1,776 |
| FLK-BLV | 472 | 0 |

[a] Cells were transfected with 10 μg of $pBLV_B$*nls*LacZ DNA or infected with 1 ml of the supernatant of $BLV_B$*nls*LacZ cells as described in Materials and Methods. At 48 h after infection or transfection, cells were fixed and stained with X-Gal. Cells with blue staining of nuclei were counted.

One milliliter of FLK-BLV/$BLV_B$*nls*LacZ cell supernatant was added to the culture medium of RFM2-Tax14 cells. Two days later, X-Gal staining revealed LacZ expression in $2 \times 10^3$ cells (Table 2). After infection of an identical number of RFM2 or BRL cells (which do not express Tax), only one clone of two β-gal$^+$ cells or no clone, respectively, was obtained (Tables 2 and 3). The unique RFM2-Tax14 β-gal$^+$ clone was presumably infected with both $BLV_B$*nls*LacZ and wild-type BLV.

FLK-BLV cells could not be infected with $BLV_B$*nls*LacZ (Table 2), most likely because of interference between the endogenous BLV provirus and the recombinant retrovirus. Alternatively, a lack of expression of the BLV receptor in this cell line may be responsible.

We next determined whether the *gag* coding sequences can be further deleted with no effect on the efficiency of genomic RNA encapsidation. To do this, we deleted all *gag* sequences except for the first 200 bp from $pBLV_B$*nls*LacZ. The *nls*LacZ gene was inserted between the *Sma*I and *Bam*HI sites (nucleotides 616 to 6997 in the RNA) of the BLV provirus (Fig. 1). To obtain a producer cell line for this new recombinant retrovirus, called $BLV_S$*nls*LacZ, we performed two subsequent FACS selections of β-gal$^+$ cells on FLK-BLV cells transfected with $pBLV_S$*nls*LacZ. One milliliter of the culture medium of the β-gal$^+$ cells was used to infect RFM2-Tax14 cells. After X-Gal staining, $1.3 \times 10^3$ β-gal$^+$ cells were obtained. Thus, the titers of both FLK-BLV/$BLV_B$*nls*LacZ and FLK-BLV/$BLV_S$*nls*LacZ are equivalent (Table 3), indicating that no sequences important for encapsidation are located between the *Sma*I and *Bam*HI sites (nucleotides 616 to 1831).

These experiments demonstrated that the $BLV_B$*nls*LacZ RNA and the $BLV_S$*nls*LacZ RNA can be encapsidated into core particules produced from a wild-type BLV provirus and

TABLE 3. Production of activator-dependent and activator-independent recombinant retroviruses[a]

| Viral producer cells | No. of β-gal$^+$ cells ($10^3$) after infection of: | |
|---|---|---|
| | RFM2-Tax14 cells | BRL cells |
| FLK-BLV/$BLV_B$*nls*LacZ | 1.1 | 0 |
| FLK-BLV/$BLV_S$*nls*LacZ | 1.3 | 0 |
| FLK-BLV/$BLV_S$SV*nls*LacZ | 2.4 | 1.3 |

[a] RFM2-Tax14 and BRL cells were infected with 1 ml of the supernatant of FLK-BLV/$BLV_B$*nls*LacZ, FLK-BLV/$BLV_S$*nls*LacZ, or FLK-BLV/$BLV_S$SV *nls*LacZ cells. At 48 h after infection, cells were fixed and stained with X-Gal. Cells with blue staining of nuclei were counted.

that the recombinant retrovirus contains all the information for reverse transcription and integration. In these constructs, foreign gene expression has been placed directly under the control of the BLV LTR. It is therefore entirely dependent on the activation of the BLV LTR by Tax.

**Activator-independent BLV-based recombinant retroviruses with an internal promoter.** To produce a recombinant retrovirus which would express the transferred gene independently of the activation of the LTR by Tax, we generated a provirus with an internal promoter. A DNA fragment containing the SV40 early-region promoter and the *nls*LacZ gene was inserted between the *Sma*I and *Bam*HI sites of BLV (nucleotides 616 and 6997 respectively, in the RNA) (Fig. 1). As expected, when pBLV$_S$SV*nls*LacZ was transfected into different cell lines, LacZ expression was detected independently of Tax expression (including the ovine RFM2, bovine PV, caprine PFC, human HeLa, feline G355-5, and simian COS cell lines) (data not shown), indicating that the expression of an exogenous gene transferred by a BLV-based recombinant retrovirus can be obtained even when the LTR is not transactivated.

To obtain BLV$_S$SV*nls*LacZ recombinant retrovirus, we performed FACS selections of β-gal$^+$ cells after transfection of pBLV$_S$SV*nls*LacZ DNA into FLK-BLV cells. The production of recombinant retrovirus was tested by infection of RFM2-Tax14 or BRL cells with 1 ml of cell supernatant. After X-Gal staining, $2.4 \times 10^3$ or $1.3 \times 10^3$ β-gal$^+$ cells, respectively, were obtained (Table 3).

The culture media of FLK-BLV/BLV$_S$*nls*LacZ and FLK-BLV/BLV$_S$SV*nls*LacZ were found to contain the same numbers of blue CFU, as shown by infection of RFM2-Tax14 cells (Table 3). This result suggests that in FLK-BLV cells, the presence of an internal promoter has no detectable effect on the transcription of genomic recombinant RNA from the BLV LTR.

Using G355-5 infected with BLV$_S$SV*nls*LacZ cells, we established by Southern blot analysis that the transferred provirus contains no detectable rearrangements (Fig. 3). The structure of the recombinant retrovirus provirus was examined after digestion with *Sac*I, which cuts in each LTR and in the *lacZ* gene, or *Bam*HI, which cuts only at the 3′ end of the *lacZ* gene. The fragments were identified by a LacZ probe to be of the correct sizes.

**Determination of the half-life of the virus.** We determined the stability of the BLV$_S$SV*nls*LacZ virus at 37°C by measuring its half-life. After incubation at 37°C for 0.5, 1, 2, and 3 h, 200 μl of the virus was used to infect BRL cells under standard conditions. After every hour of incubation, the number of infectious particules had decreased twofold (data not shown). In contrast, when the virus was incubated for 3 h on ice, no significant degradation was apparent.

**Determination of the BLV host range with BLV$_S$SV*nls* LacZ.** Transfection of pBLV$_S$SV*nls*LacZ resulted in β-gal$^+$ cells in all cell lines listed in Table 4, indicating that the early promoter of SV40 is active in these cells (data not shown). Thus, the BLV$_S$SV*nls*LacZ virus can be used to determine the host range of BLV. As BRL cells were found to be sixfold more able to be infected when 5 μg of Polybrene per ml was added to the medium at the time of infection (and YR2 cells were threefold more able to be infected), we used these conditions to test the ability of cells to be infected. In different experiments, G355-5, a feline cell line which is very sensitive to BLV infection and stably expresses the *lacZ* gene, was used to standardize the results obtained on cell lines with different stocks of recombinant retrovirus. On the G355-5 cell line, the titers of infection regularly reached $10^4$ CFU/ml.

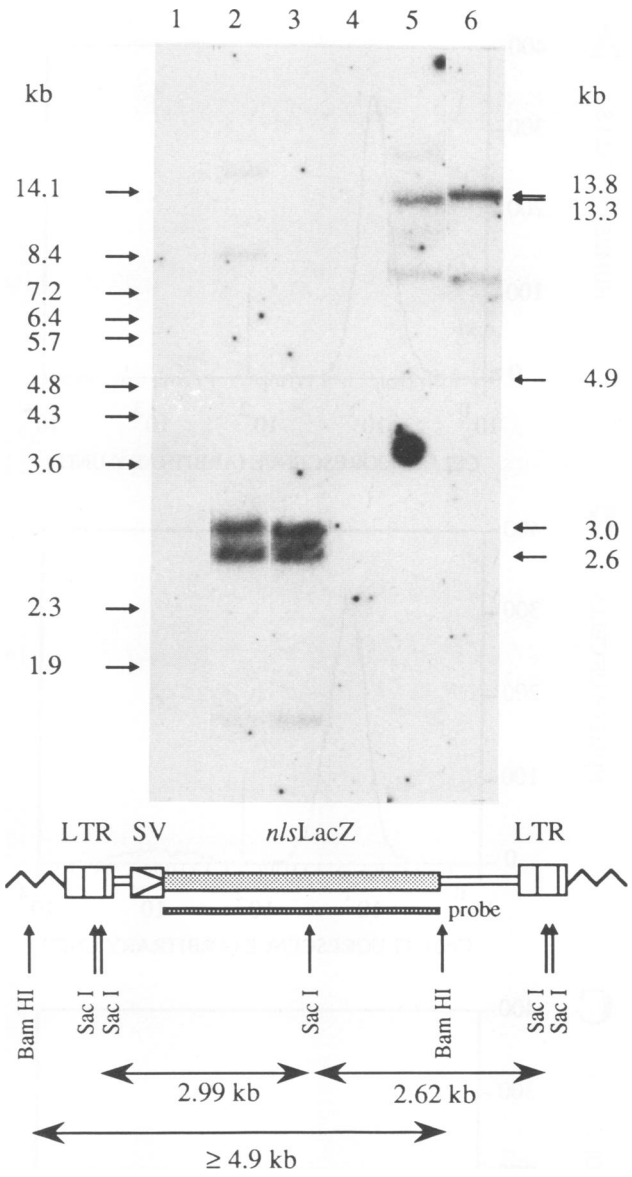


FIG. 3. Southern blot analysis of the transduced BLV$_S$SV*nls*LacZ recombinant retrovirus. The structure of the transduced BLV$_S$SV*nls*LacZ recombinant provirus in β-gal$^+$ infected G355-5 cells was analyzed after digestion of purified cellular DNA with *Sac*I or *Bam*HI and Southern transfer. Shown are *Sac*I (lanes 1, 2, and 3) and *Bam*HI (lanes 4, 5, and 6) digests of, respectively, FLK-BLV (control), FLK-BLV/BLV$_S$SV*nls*LacZ (producer cell line), and G355-5/BLV$_S$SV*nls*LacZ (infected cells). *Sac*I cuts the provirus in 2.99- and 2.62-kb fragments, as indicated in the map. Fragments of the predicted molecular sizes were detected with the DNAs of both the producer cell line (lane 2) and the β-gal$^+$ infected G355-5 cells (lane 3). *Bam*HI is unique in the provirus. Bands larger than 4.9 kb were found in the producer cell line (lane 5) and in the β-gal$^+$ infected G355-5 cells (lane 6). The labeled probe is *nls*LacZ.

The cells of cat (G355-5), buffalo rat (BRL), sheep (RFM2, PFM, and YR2), cattle (PV), and goat (PFC) origins were the most easily infected, with titers ranging from $10^3$ and $10^4$ blue CFU/ml. On rabbit (PL and RLG), pig (ST), and

TABLE 4. Host range of BLV virus as determined with $BLV_SSVnlsLacZ$ virus[a]

| Cell line | Species | Relative ability to be infected |
|---|---|---|
| G355-5 | Cat | 100 (≈$10^4$blueCFU/ml) |
| RFM2 | Sheep | 50 |
| PFM | Sheep | 22 |
| YR2 | Sheep | 10 |
| BRL | Buffalo rat | 34 |
| PFC | Goat | 14 |
| PV | Cow | 10 |
| PL | Rabbit | 6 |
| RLG | Rabbit | 3 |
| ST | Pig | 3 |
| PK15 | Pig | 0 |
| COS | Monkey | 2 |
| MRC5 | Human | 0.5 |
| HeLa | Human | 0.3 |
| HEp-2 | Human | 0 |
| 3T3 | Mouse | 0.1 |
| FP | Chicken | 0.1 |

[a] Cells were infected with 1 ml, 100 μl, or 10 μl of the supernatant of FLK-BLV/$BLV_SSVnlsLacZ$ cells as described in Materials and Methods. At 48 h after infection, cells were fixed and stained with X-Gal. Cells with blue staining of nuclei were counted. The titers were calculated from the linear portions of titration curves. For comparison of the titers obtained in different experiments, the titers were standardized relative to the titers obtained on the feline G355-5 cell line.

monkey (COS) cells, the infectivity of the $BLV_SSVnlsLacZ$ virus was lower, the titers being between $1 \times 10^2$ and $6 \times 10^2$ blue CFU/ml. A third group included cells of human (HeLa, MRC5, and HEp-2), mouse (3T3), and chicken (FP) origins, which were slightly or not infected, with titers of less than $5 \times 10^1$ blue CFU/ml. To attest to the low ability of human cell lines to be infected, we tested three different cell lines, HeLa, HEp-2, and MRC5. To verify that LacZ can be expressed in these human cell lines, we transfected $pBLV_SSVnlsLacZ$ in HeLa and MRC5 cells. Two days later, 5% of the cells were $LacZ^+$. That the low ability to be infected was not due to the addition of Polybrene at the time of infection was verified by performing the experiment in the absence of Polybrene as well as with the addition of DEAE-dextran at 5 μg/ml. The same low titers were obtained on HeLa cells when LacZ expression was tested 5 days after infection. In contrast, in a control experiment, full LacZ expression was detected 2 days after infection with an MLV-based xenotropic LacZ recombinant retrovirus (9). Thus, the low ability of human cells to be infected under our conditions was presumably due to a barrier at penetration, maturation, or integration of the BLV-based recombinant retrovirus rather than a blockage of LacZ expression.

**Confirmation of BLV specificity by antibody-mediated neutralization.** To test the reactivity of $BLV_SSVnlsLacZ$ recombinant retrovirus to specific antibodies, we used the neutralizing activities of several monoclonal antibodies which specifically recognize the F, G, or H epitopes of the gp51 BLV *env* protein (4). The anti-F and anti-H antibodies completely inhibited $BLV_SSVnlsLacZ$ infectivity at a 1/10 dilution and decreased infectivity by twofold at a 1/300 dilution. The anti-G antibody reduced the LacZ recombinant retrovirus infectivity at dilutions of up to 1/100, but high concentrations of this antibody were needed to neutralize BLV (Fig. 4A).

Therefore, the availability of a BLV-based recombinant retrovirus, detectable after only one cell cycle by expression of a reporter gene, may facilitate quantitative analysis of antiviral compounds. As an example, we titrated sera from BLV-infected sheep (S129) and cattle (B85163). These antisera could inhibit recombinant retrovirus infectivity when incubated with 100 μl of viral supernatant. At a 1/33 dilution, the infectivity of the recombinant retrovirus was completely inhibited by both antisera (Fig. 4B). At ≈1/300 to 1/500 dilutions, infectivity was decreased by 50%. No neutralization was observed at a 1/1,000 dilution. To eliminate the unlikely possibility that these antisera may have acted through a general effect on cells, we verified that serum S129 from a BLV-infected sheep did not inhibit Moloney ecotropic MMuLV-$SVnlsLacZ$ infection (data not shown).

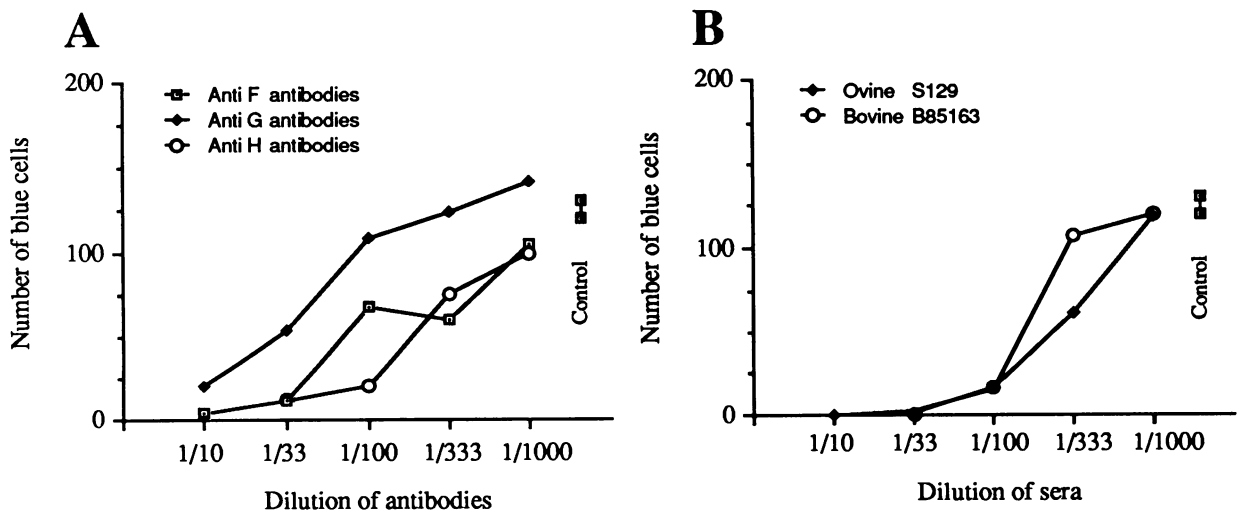


FIG. 4. Sensitivity of the LacZ BLV-based recombinant retrovirus to neutralizing antibodies. $BLV_SSVnlsLacZ$ (100 μl) was incubated for 30 min on ice with monoclonal antibodies or with sera from BLV-infected animals before being used for infections of BRL cells. (A) Monoclonal antibodies against F, G, or H epitopes of the gp51 *env* protein. (B) Sera from BLV-infected ovine (S129) and bovine (B85163) cells. The control points correspond to incubations of recombinant retrovirus with 10% fetal calf serum.

## DISCUSSION

BLV-based recombinant retroviruses were constructed by replacing the *gag*, *pol*, *env*, and pX regions by a foreign gene. Titers of $10^4$ CFU/ml of medium were produced in a transcomplementing replication-competent helper cell line (FLK-BLV). Therefore, the virion of BLV contains all the products necessary for reverse transcription and integration of a defective genomic BLV RNA. In addition, sequences responsible in *cis* for efficient packaging in the particles and for reverse transcription and integration of the genomic RNA are probably all located in the first 616 bp of the genomic RNA. This information may help in the development of a transcomplementing helper-free cell line which would be used instead of FLK-BLV to produce recombinant viruses.

Two types of BLV vectors were constructed. One type ($BLV_S$*nls*LacZ and $BLV_B$*nls*LacZ) conferred activator dependence for expression of the transduced gene when it was positioned directly under the control of the BLV LTR. The restricted expression of these vectors in the infected cells was overcome by supplying Tax in *trans*. The second type of vector ($BLV_S$SV*nls*LacZ) was activator independent. The transduced gene was under the control of a second promoter in a position internal to the virus. There is no evidence for interference of the internal promoter in the production of the BLV vector, as the titers of $BLV_S$SV*nls*LacZ and $BLV_S$*nls*LacZ were similar. These vectors transduced genes into cells with no apparent rearrangement, as Southern blotting of the DNA of infected cells showed that sequences of the recombinant retrovirus had the expected structure. Therefore, infection with these recombinant retroviruses did not lead to genetic instability during reverse transcription or integration, even when a second promoter was included in the recombinant retroviruses.

There is a certain flexibility with which the *gag-pol* proteins of a retrovirus can associate with *env* proteins to produce chimeric particles. For instance, Ban et al. (1) have shown that the *gag-pol* genes from SNV or MLV can be complemented with the *env* gene from BLV to produce virions. In the absence of information on the efficiency of pseudotyping BLV vectors in murine particles, it was necessary to verify that the viruses produced by the FLK-BLV helper cell line were specifically inhibited by BLV-neutralizing antibodies. Neutralizing monoclonal antibodies abolished the infections on buffalo rat (BRL) and ovine (YR2) cell lines.

Features of our LacZ BLV vectors offered the opportunity to study their biological properties. First, we determined that the half-life of the recombinant retrovirus is 1 h at 37°C in culture medium. This value is significantly lower than the ones obtained for MMuLV-SV*nls*LacZ recombinant retrovirus (37) or for human immunodeficiency virus (32), which are about 7 h. Whether this result reflects an intrinsic characteristic of BLV can be elucidated by measuring the half-lives of vectors with a different structure. Second, we measured the effect of polycation addition on BLV infectivity. Polybrene and DEAE-dextran increased three- to sixfold the titers of $BLV_S$SV*nls*LacZ on BRL and YR2 cells. The host range of BLV was determined with Polybrene, as it allows the identification of low infectivity. Third, the host range of BLV was studied on cell lines from various species. The activator-independent $BLV_S$SV*nls*LacZ vector efficiently infected cells of cat, buffalo rat, sheep, goat, and cattle origins. A lower efficiency was observed with cells of rabbit, pig, and monkey origins. An even lower efficiency was observed for mouse and human cell lines. Our experiments confirm that the family of receptors for BLV is widely expressed on various species and cell types.

Recently, Derse and Martarano (12) reported the construction of a BLV vector termed BLVSVneo. The structural genes *gag*, *pol*, and *env* were maintained in this recombinant virus. Therefore, BLVSVneo is a self-packaging vector when complemented by Tax and Rex. However, high titers of the recombinant virus were only obtained in FLK-BLV, which provides both regulatory and structural proteins and produces wild-type virus. This strategy limits the capacity of integration of foreign DNA to 2 kb. Our data demonstrate that no sequence in the *gag*, *pol*, and *env* genes is required in *cis* for vector production in a helper cell line. This simpler system allows the introduction of up to 6 to 8 kb in the vector and decreases the probability of complementation or recombination when used in animals. The conclusions of Derse and Martarano (12) were slightly different from ours concerning the host range of the vector; in particular, they found that a human cell line (HeLa) and a cat cell line (FEA) were infected at higher levels than were bovine cell lines. The difference between the results of these two reports is not due to a low expression of $BLV_S$SV*nls* LacZ in human cells, as we showed that strong β-galactosidase signals were obtained following transfection. Further work on different cell lines and primary cells in culture will determine more completely the status of BLV infectivity for human tissues.

Together, these results demonstrate the utility of BLV vectors for introducing genes into cells in culture or into animals.

## ACKNOWLEDGMENTS

We thank A. Burny for the gifts of T15 and T15/4 proviruses, YR2 cells, and anti-BLV antibodies; Y. Ikawa for plasmid pMX-1; C. Delouis for the PFM, PFC, PL, PV, and FP cell lines; and P. Nandy for the ST cell line. We are grateful to H. Jouin for cell sorter selections and B. Wojcik and C. Delouis for review of the manuscript.

This work was supported by grants from the Agence Nationale pour la Valorisation de la Recherche (A8702099Q), the Centre National de la Recherche Scientifique (UA 1148), the Institut National de la Santé et de la Recherche Médicale (68131), and the Association pour la Recherche sur le Cancer (6622 and 6744). D.M. and J.-F.N. are supported, respectively, by the Institut National de la Recherche Agronomique and the Institut National de la Santé et de la Recherche Médicale.

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