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Genetically Simpler Bovine Leukemia Virus Derivatives Can Replicate Independently of Tax and Rex†

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Retrovirus genomes have a conserved modular organization that consists of *trans*-acting *gag*, *pol*, and *env* genes that function through *cis*-acting sequences to replicate the RNA genome to the DNA provirus. Genetically more complex retroviruses also encode regulatory genes and *cis*-acting sequences that are essential for their replication. We sought to convert a more complex retrovirus into a simpler retrovirus derivative that can replicate. We constructed novel, hybrid retrovirus vectors to replicate the *gag*, *pol*, and *env* genes of the more complex bovine leukemia virus (BLV) in the absence of regulatory genes and *cis*-acting response sequences. Most of the *cis*-acting sequences involved in the replication and regulation of BLV were replaced by the *cis*-acting transcriptional control sequences of a simpler retrovirus, spleen necrosis virus. We found that the resulting hybrid BLV derivatives can replicate independently of BLV Tax and Rex and the Tax and Rex *cis*-acting response sequences, as measured by successive passages of virus on target cells, detection of provirus sequences, and analyses of provirus and encapsidated RNAs.

Retroviruses can be designated as simpler or more complex on the basis of genome structure and replication cycle (4, 18). More complex retroviruses encode the *gag*, *pol*, and *env* genes common to all retroviruses as well as regulatory genes that are necessary for wild-type levels of replication (8). Bovine leukemia virus (BLV) encodes two regulatory genes, *tax* and *rex*, which function through *cis*-acting Tax and Rex response elements (TRE and RxRE, respectively) that are positioned in the BLV long terminal repeat (LTR) 5′ U3 region and 3′ R region, respectively (5, 6). Tax and TRE function together to activate BLV transcription (5), whereas Rex and RxRE promote the cytoplasmic accumulation of unspliced genomic RNA and singly spliced *env* transcripts (6, 12, 13, 16). We were interested in developing BLV derivatives that can replicate independently of Tax, Rex, TRE, and RxRE, similar to a simpler retrovirus. Genetically simpler BLV derivatives may be useful as a prototypic vaccine against retrovirus-induced disease (19). We have constructed novel, hybrid retrovirus vectors that contain the BLV *gag*, *pol*, and *env* genes as well as the BLV primer binding site, polypurine tract (17), and a putative BLV encapsidation sequence. The vectors lack *tax*, *rex*, and most of the BLV LTR sequences, including TRE, RxRE, and the BLV splice donor (normally located in the R region of the 5′ LTR) and *env* splice acceptor sites. Splice site recognition facilitates human immunodeficiency virus type 1 Rev regulation of *env* expression (3, 11) and may also play an important role in BLV Rex regulation. Therefore, we deleted BLV splice sites to achieve Rex-independent expression of hybrid vectors. The vector LTRs are hybrid molecules composed primarily of transcriptional control sequences from a simpler retrovirus, spleen necrosis virus (SNV), and transcription is predicted to be Tax independent. In addition, the vector LTRs contain deletions of the terminal SNV LTR sequences that facilitate provirus integration (att) (15) and are replaced with the predicted BLV att sequences (17).

Our primary objective was to construct a genetically simpler BLV derivative as a model toward developing retrovirus vaccines. Since the simpler BLV derivative would be a novel retrovirus with unique properties and may replicate at a high titer, we introduced a safety feature to limit virus spread (19). The BLV *gag-pol* genes and *env* genes are divided onto separate vector genomes, which can replicate by phenotypic mixing (20) (Fig. 1). When introduced into the same cell, the *gag-pol* and *env* vectors complement in *trans* to produce a complementary virus (co-virus) particle, similar to rescue of the Bryan high-titer strain of Rous sarcoma virus, which lacks *env*, by the helper viruses Rous-associated virus-1 and -2 (Fig. 2) (20). The co-virus arrangement also facilitates *env* expression in the absence of BLV splice sites.

In order to select co-virus-infected cells, the hygromycin B phosphotransferase gene, *hyg*, was introduced into the hybrid *gag-pol* vector genome. Hygromycin B-resistant (Hyg^r) infected cells can be selected and then screened for the unselected *env* provirus (Fig. 2). Doubly infected cells produce progeny co-virus that can be passaged further.

We constructed three sets of hybrid BLV vectors (Fig. 1). In vector set 1, *hyg* and *gag-pol* are placed on separate vectors. In vector set 2 and vector set 3, *hyg* is positioned directly in the *gag-pol* vector downstream of an internal ribosome entry sequence that promotes cap-independent translation of polycistronic vector RNA (1, 10); selection for Hyg^r ensures the presence of polycistronic *gag-pol-hyg* RNA in these cells. In vector sets 1 and 2, BLV *env* is provided by p*env*, while in vector set 3, *env* is expressed from the pU5*env* genome.

The BLV encapsidation sequence and other 5′ untranslated sequences that are potentially important for efficient BLV replication have not been defined, although recent in vitro studies have indicated that an RNA structure formed between U5 sequences in the 5′ BLV LTR and downstream sequences, including att, the primer binding site, and the 5′ end of *gag*, is important for RNA dimerization in vitro and for interaction with the BLV matrix (14). The selectable vectors in sets 1, 2, and 3 differ in their BLV 5′ sequences. Set 1 vector p*hyg* contains BLV 5′ untranslated sequences between BLV att and −1 of *gag*, whereas set 2 vector p*gag-pol* IH contains BLV 5′ sequences from BLV att into *gag-pol*, and set 3 vector pU5*gag-*

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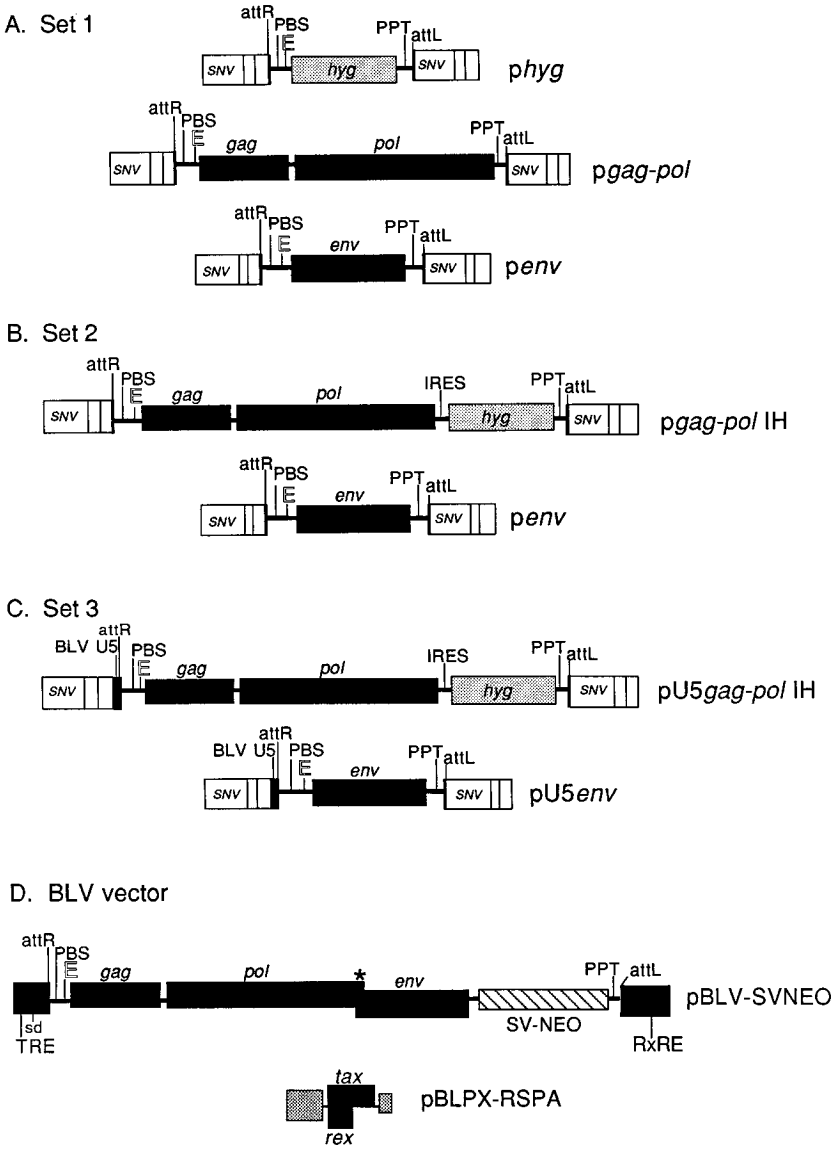

FIG. 1. Hybrid BLV vector sets 1, 2, and 3 along with pBLV-SVNEO. SNV sequences of hybrid LTRs are shown as labeled white rectangles divided into the U3, R, and U5 regions, while terminal LTR sequences derived from BLV are shown as black vertical lines and labeled attR or attL to denote *cis*-acting provirus integration sequences. Each thick black horizontal line designates BLV untranslated sequences, primer binding site (PBS), polypurine tract (PPT), and putative BLV encapsidation signal (E). Each shaded box marked *hyg* indicates a hygromycin B resistance gene, and labeled black boxes mark the positions of BLV *gag*, *pol*, and *env* genes. (A) Set 1. Vector p*hyg* encodes *hyg*, vector p*gag-pol* encodes BLV *gag* and *pol*, and p*env* encodes BLV *env*. (B) Set 2. Vector p*gag-pol* IH contains the encephalomyocarditis virus internal ribosome entry sequence (IRES) upstream of *hyg* and expresses *gag*, *pol*, and *hyg* from a polycistronic RNA. BLV *env* is expressed from p*env*. (C) Set 3. The 5′ LTRs of vectors pU5*gag-pol* IH and pU5*env* contain the complete BLV U5 region, as designated by the labeled black rectangle downstream of the 5′ SNV LTR sequences. (D) pBLV-SVNEO is a Tax- and Rex-dependent BLV vector that codes for BLV *gag*, *pol*, and *env* and a cassette that contains the simian virus 40 early promoter fused to the bacterial neomycin resistance gene (labeled SV-NEO, hatched rectangle) in place of *tax* and *rex*. BLV LTRs are depicted as black rectangles, sd designates the splice donor, and the star denotes the *env* splice acceptor. pBLPX-RSPA is a *tax* and *rex* expression plasmid that is required in *trans* for the production of BLV-SVNEO vector virus.

pol IH also contains the complete BLV U5 (Fig. 1). Comparison of the titers of these vectors may reveal boundaries of BLV 5′ untranslated sequences that are important for efficient vector replication.

Protocol to generate vector viruses. D-17 cells were used for infections since they are permissive for replication of both SNV and BLV (see Table 2). To propagate virus, each set of vectors was cotransfected onto D-17 cells, and Hyg^r cells were selected (Fig. 2). Co-virus in cell-free medium of pooled Hyg^r cells was used to infect D-17 target cells. Hyg^r cells were selected and designated passage 1 cells. To assay successive cycles of replication, cell-free medium from passage 1 cells was used for infection of fresh D-17 cells. Hyg^r cells were selected and designated passage 2 cells, and the infection protocol was repeated to passage co-viruses further.

Hybrid vector RNA and protein production. D-17 cells transiently transfected with set 1 vector p*hyg* expressed *hyg* RNA at a level similar to that expressed from the pSNV*hyg* vector, pJD214Hy (9) (Table 1). Transient transfection with set 2 vector p*gag-pol* IH or set 3 vector pU5*gag-pol* IH produced one-third of the *hyg* RNA produced by p*hyg*, a result similar to the difference in molar ratios of these transfected DNAs. Iden-

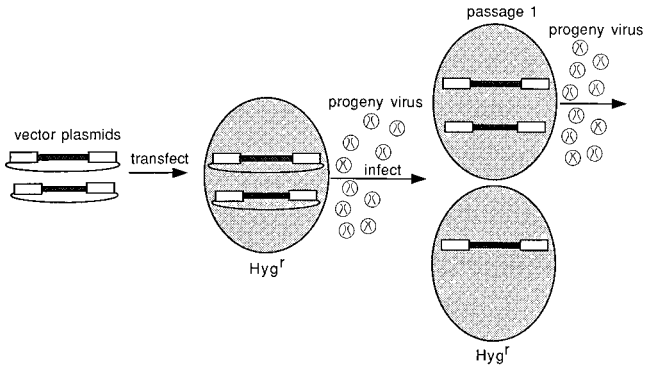

FIG. 2. Experimental protocol to test hybrid virus replication. Hybrid vectors were introduced into D-17 cells (gray ovals) by transfection, and Hyg^r colonies were selected. Virus in cell-free medium of Hyg^r transfectants was used to infect D-17 target cells, and Hyg^r colonies were selected and designated passage 1 cells. To assay successive cycles of hybrid virus replication, virus in cell-free medium from cells that contained both the *gag-pol* provirus and *env* provirus was used to infect D-17 target cells, and Hyg^r colonies were selected and designated passage 2 cells. The protocol was repeated to infect passage 3 and passage 4 cells.

tical numbers of Hyg^r colonies were selected in response to transfection with p*hyg* and the pSNV*hyg* vector, while the numbers of Hyg^r colonies observed in response to p*gag-pol* IH and pU5*gag-pol* IH were less by a factor of 10 (Table 1). Functional BLV reverse transcriptase was produced by pooled selected colonies transfected with each vector set (Table 1). The level of reverse transcriptase was similar to that produced by cells cotransfected with the BLV vector (pBLV-SVNEO) and the Tax-Rex expression plasmid (pBLPX-RSPA) (7). These results confirm that these hybrid vectors produce functional RNA and protein in transfected cells and that BLV *gag-pol* sequences do not block expression of Hyg^r.

Hybrid virus replication. Next, we tested the ability of hybrid vectors to infect target cells by using a marker transfer experiment. We used PCR to screen for transfected cell clones that contained set 1, set 2, or set 3 vectors (data not shown), and viruses in the cell-free media of these cell clones were used to infect D-17 target cells. Infected cells were selected for Hyg^r (cells infected with BLV-SVNEO were selected for resistance to the neomycin analog G418) and designated passage 1 cells.

TABLE 1. Summary of vector properties in transfected cells

Vector	*hyg* RNA level[a]	Hyg^r (CFU/μg)[b]	Reverse transcriptase activity (10^3 cpm)[c]
Set 1	1.0	500 ± 150	45 ± 5
Set 2	0.3	50 ± 15	55 ± 7
Set 3	0.3	55 ± 20	80 ± 20
Mock	<MD[d]	<MD	4 ± 2
pBLV-SVNEO[e]	NT[f]	NT	54 ± 10
pSNV*hyg*	1.0	500 ± 150	NT

[a] Posttransfection (60-h) total cellular RNA was extracted from D-17 cells, and relative *hyg* RNA levels were determined by RNA slot blot hybridization assay.
[b] D-17 cells were transfected with dilutions of vector plasmids, and Hyg^r colonies were selected and counted. Data are averages ± standard deviations of three experiments.
[c] Virus particle-associated reverse transcriptase activity expressed as 10^3 trichloroacetic acid-precipitable cpm from 2×10^6 pooled selected colonies that contained set 1, 2, or 3 vectors (10 ml of cell-free medium). Data are averages ± standard deviations of three experiments.
[d] <MD, less than the minimum detectable.
[e] pBLV-SVNEO was cotransfected with pBLPX-RSPA to supply BLV Tax and Rex in *trans*.
[f] NT, not tested.

TABLE 2. Titers of vector viruses in successive replication cycles

Vector virus	Titer (CFU/ml)[a]			
	Passage 1	Passage 2	Passage 3	Passage 4
Set 1	10 ± 2	10 ± 2	NT[b]	NT
Set 2	30 ± 10	40 ± 10	40 ± 10	40 ± 10
Set 3	100 ± 20	100 ± 20	95 ± 60	NT
Mock	<MD[c]	<MD	<MD	<MD
BLV-SVNEO[d]	30 ± 20	30 ± 20	NT	NT
SNV*hyg*[e]	3,000 ± 300	3,250 ± 300	NT	NT

[a] Hyg^r CFU/ml of cell-free medium. Data are averages ± standard deviations of at least five experiments.
[b] NT, not tested.
[c] <MD, less than the minimum detectable.
[d] Producer cells were transfected with pBLPX-RSPA, and infected cells were selected with the neomycin analog G418.
[e] SNV*hyg* vector virus was produced in the D-17-based SNV helper cell line DSDh (9). Data for this vector virus are averages of two experiments.

The titers of the hybrid viruses were similar in magnitude to BLV-SVNEO in the presence of Tax and Rex (Table 2), although these titers cannot be compared directly since the *neo* marker in BLV-SVNEO is expressed from the simian virus 40 early promoter rather than from the SNV promoter. Among the hybrid viruses, the titer of set 3 virus, which contains the BLV U5 region and *gag* sequences, was consistently the highest, 100 ± 20 CFU/ml. The titer of set 2 virus, which lacks BLV U5, was lower by a factor of 3 (Wilcoxon rank sum test; $P < 0.002$). The set 1 vector contains the smallest BLV 5′ sequence, and its titer was the lowest, 10 ± 2 CFU/ml ($P < 0.0002$). The observed statistical differences among virus titers indicate that the BLV U5 region and *gag-pol* sequences are important for efficient hybrid virus replication.

We also tested the titer of hybrid virus pseudotyped with SNV Env or amphotropic murine leukemia virus Env. D-17 cells were transfected with a selectable plasmid (pSV_2*neo*) and either an SNV *env* expression plasmid (pPR102) or an amphotropic murine leukemia virus *env* expression plasmid (pJD1) (9). Pooled selected colonies were then transfected with set 1 p*hyg* and p*gag-pol* vectors, and progeny virus was used to infect target cells. Interestingly, we found that the virus titers for virions which contained BLV Env were similar to those for virions which were pseudotyped with SNV Env or amphotropic murine leukemia virus Env (no statistical difference was detected in five experiments; data not shown). These results indicate that the titer of set 1 virus is not limited by BLV Env-mediated infection of D-17 cells.

Hyg^r infected cell clones were screened for provirus sequences by PCR amplification of three regions, (i) the region between *hyg* and the BLV polypurine tract, (ii) the BLV *pol* gene, and (iii) the BLV *env* gene. All of the set 2 virus- and set 3 virus-infected Hyg^r clones analyzed contained sequences of the *gag-pol-hyg* provirus, and 15% were also infected with the unselected *env* provirus. Similarly, all set 1 virus-infected Hyg^r clones analyzed contained the *hyg* provirus, and 15% of these clones were also infected with the unselected *gag-pol* provirus or *env* provirus.

To determine whether hybrid BLV derivatives can be passaged through successive cycles of replication, vector virus was harvested from pools of doubly infected cell clones and used to infect D-17 target cells. We observed that hybrid BLV derivatives can replicate through at least four successive replication cycles and that the virus titer is stable with passage (Table 2). Sequences of the *hyg* provirus were consistently observed in all

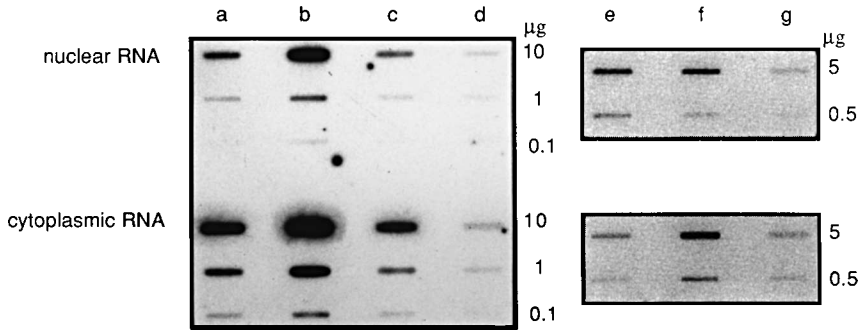

FIG. 3. Analysis of subcellular distribution of provirus RNA. RNA slot blot hybridization assay of nuclear and cytoplasmic RNAs harvested from the following D-17 cells: set 2 passage 1 clone (a), set 2 passage 2 clone (b), SNV*hyg* virus passage 1 pooled selected cells (c), mock-infected cells (d), cells transiently transfected with pBLV-SVNEO (e), cells transiently transfected with pBLV-SVNEO and pBLPX-RSPA (f), and mock-transfected cells (g). (a through d) Hybridized with a ^{32}P-labeled *hyg* DNA probe; (e through g) hybridized with a BLV ^{32}P-labeled *pol* DNA probe in Stratagene QuikHyb buffer at 68°C for 1 h. (a through d) Exposed to X-ray film; (e through g) visualized with a phosphoimager.

Hyg^r clones, and 15% of these cell clones were also infected with the unselected provirus. Virion particles were harvested from doubly infected cells and examined for genomic RNA by RNA slot blot hybridization assays. Encapsidated vector RNA was detected in set 2 and set 3 virion particles from 100 ml of cell-free producer cell medium but not in set 1 particles or particles from uninfected cells (the results of three experiments; data not shown). These results indicate that hybrid BLV derivatives can infect cells, form a provirus, and produce progeny virions that contain genomic RNA.

Tax- and Rex-independent expression. To address whether the expression of hybrid BLV RNA is independent of BLV Tax and Rex, we analyzed provirus transcription. Nuclear and cytoplasmic RNAs were fractionated from infected cells and subjected to RNA slot blot hybridization assays. The level of set 2 provirus RNA is similar in magnitude to SNV*hyg* provirus RNA (Fig. 3), indicating that transcription of hybrid BLV is independent of Tax and TRE. The fivefold-higher RNA level from the passage 2 clone compared with that of the passage 1 clone may have been influenced by the integration site of the provirus. The titers of these viruses were similar (Table 2), which indicates that the virus titer is not limited at the level of provirus transcription.

Comparison of vector RNA levels in the nucleus and cytoplasm revealed that the majority of set 2 provirus RNA was localized to the cytoplasmic compartment, similar to the Rex-independent SNV*hyg* control and the Tax-dependent control BLV-SVNEO in the presence of Tax and Rex (Fig. 3). In contrast, the majority of BLV-SVNEO RNA expressed in the absence of Tax and Rex remained in the nucleus. These results indicate that the cytoplasmic accumulation of hybrid BLV RNA is independent of Rex and RxRE.

These experiments demonstrate that hybrid BLV derivatives can infect cells, form a provirus, and produce progeny virions that contain genomic RNA. RNA expression from hybrid BLV is independent of Tax, Rex, TRE, and RxRE. This phenotype may result from deletion of *rex*, RxRE, and BLV splice sites or may indicate that SNV LTR sequences modulate transport of BLV RNAs in the absence of Rex, similar to the constitutive transport element defined in Mason-Pfizer monkey virus (2). Hybrid BLV can replicate through at least four consecutive passages, and the hybrid virus titer is similar in magnitude to the titer of a BLV vector virus. The titer of a hybrid BLV derivative may be increased by expressing BLV *gag*, *pol*, and *env* on a single hybrid BLV genome rather than on co-virus genomes. A higher-titer BLV derivative will be useful to test as a prototypic vaccine against retrovirus-induced disease (19).

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