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NOTES

Lower Mutation Rate of Bovine Leukemia Virus Relative to That of Spleen Necrosis Virus

LOUIS M. MANSKY AND HOWARD M. TEMIN*

McArdle Laboratory for Cancer Research, University of Wisconsin—Madison, Madison, Wisconsin 53706

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Genetic variation of the more complex retroviruses in the human T-cell leukemia virus/bovine leukemia virus (HTLV/BLV) group is less than in some other retroviral genera. To test whether reverse transcription of HTLV/BLV group members is less error prone than that of members of other groups, we developed an assay for detecting forward mutations in BLV, similar to that developed for the simpler spleen necrosis virus (SNV). We used this system to study the rates and types of mutations that occur during a single replication cycle. We found that BLV reverse transcription is approximately two and one-half times less error prone than SNV reverse transcription (4.8×10^{-6} versus 1.2×10^{-5} mutation per bp per cycle, respectively). The relative numbers of all types of observed mutations (that is, base pair substitutions, frameshifts, deletions, and deletions with insertions) were similar for BLV and SNV.

Retroviruses are a family of RNA animal viruses that use reverse transcription in replication (1, 40, 41, 44). Retroviruses have been classified as simpler or more complex on the basis of genetic organization and replication (9, 10). Retrovirus populations show great genetic variability (8, 17, 22, 37). This retroviral genetic variation is the composite of three variables (7): the mutation rate per replication cycle, the number of replication cycles, and the selective advantage or disadvantage possessed by the variant viruses. Also, the rate of recombination in retrovirus replication is high (20, 46; for a review, see 18).

Genetic variation in the human T-cell leukemia virus/bovine leukemia virus (HTLV/BLV) genus appears to be less than that of some other groups in the *Retroviridae* family (5). For example, low levels of sequence variation among isolates of BLV (26, 45), HTLV-I (16, 21, 24, 28, 35), and HTLV-II (15, 21) have been reported, in contrast to the relatively high levels of genetic variation of the lentiviruses (4, 6, 25) and of murine leukemia virus (8, 22). One possible explanation for these observations is that reverse transcription of lentiviruses is more error prone than reverse transcription of HTLV/BLV group viruses. Alternatively, the members of the HTLV/BLV genus are thought to replicate more often as proviruses during cell division, rather than as viruses by reverse transcription and integration (5).

The mutation rate per base pair per replication cycle has been reported for spleen necrosis virus (SNV), an avian type C retrovirus similar to the murine type C retroviruses (13, 14, 31, 32, 43). With the *lacZ*α peptide gene as a reporter gene for mutations and the blue/white colony color selection method for identifying mutant proviruses, the in vivo forward mutation rates for various types of mutations were determined (31, 32). The major types of mutations found were base pair substitutions, frameshifts, simple deletions, deletions with insertions, and hypermutations (31, 32). These studies indicated that errors in reverse transcription during retroviral replication may be an important factor leading to the genetic variation that has been observed with SNV (29, 30).

To determine whether reverse transcription of the HTLV/BLV group is less error prone than that of some other genera of retroviruses, we adapted the previously described forward mutation assay (31, 32) to study the rate of mutation of a more complex retrovirus, BLV. A BLV shuttle vector (Fig. 1A), BLV-SVNEO/ACYCLacZ, was constructed from the retrovirus vector BLV-SVNEO (11), which was kindly provided by David Derse (National Cancer Institute, Frederick, Md.). The pACYC origin of replication and the *lacZ*α gene sequences from the plasmid pSU 20 (2) (kindly supplied by Borja Bartolomé, Universidad de Cantabria, Santander, Spain) were amplified by polymerase chain reaction with primers containing *Eco*RI sites; the amplified DNAs were digested with *Eco*RI and were cloned into the *Eco*RI site immediately after the *neo* gene in BLV-SVNEO to create the BLV shuttle vector. The BLV *tax/rex* expression plasmid, pBLPX-RSPA (Fig. 1C), was kindly supplied by D. Derse.

The BLV shuttle vector, BLV-SVNEO/ACYCLacZ, is deficient in the expression of the two regulatory genes, *tax* and *rex*. The BLV shuttle vector contains the BLV *gag*, *pol*, and *env* genes (Fig. 1A). The BLV shuttle vector also contains the *neo* gene, which allows selection in mammalian cells with the neomycin analog G418 and in bacterial cells with kanamycin. In addition to the selectable marker, the BLV shuttle vector contains a bacterial origin of replication and the *lacZ*α peptide gene. The vector also contains a packaging signal. Therefore, the vector can replicate in mammalian cells as a retrovirus and in bacterial cells as a plasmid.

The systems developed for this study utilize a helper virus or helper plasmid for production of vector virus. The BLV helper virus and helper plasmid used for vector virus production are

* Corresponding author. Mailing address: Department of Oncology, University of Wisconsin—Madison, McArdle Laboratory, 1400 University Avenue, Madison, WI 53706. Phone: (608) 262-1209. Fax: (608) 262-2824. Electronic mail address: temin@oncology.wisc.edu.

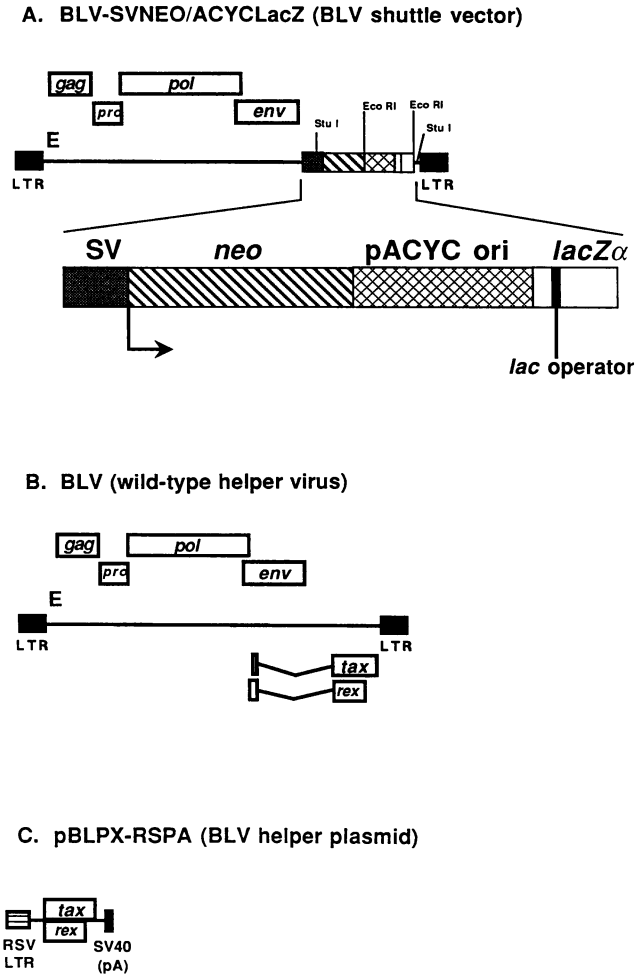

FIG. 1. (A) BLV shuttle vector; (B) BLV; (C) the BLV helper plasmid used for forward mutation rate studies. The BLV shuttle vector and BLV are shown in proviral DNA forms. Solid black boxes represent BLV long terminal repeats (LTRs). Solid black lines indicate viral sequences. Rectangular boxes above or below the solid black lines indicate viral coding sequences, with the relative locations of the boxes corresponding to the translational reading frame. Retroviral genes are indicated as *gag*, *pro*, *pol*, *env*, *tax*, and *rex*. Bent lines in between the coding regions for *tax* and *rex* in panel B show reading frames joined by splicing events. E in panels A and B indicates the location of the encapsidation sequence. In panel A, the simian virus 40 promoter (SV) is represented as a dark gray box; the *neo* gene is represented as a hatched rectangular box; the pACYC origin of replication (pACYC ori) is represented as a cross-hatched box; and the *lacZ*α peptide gene is represented as an open rectangular box with a black band representing the *lac* operator sequence. The box with horizontal lines in panel C represents the Rous sarcoma virus (RSV) LTR, and the thin black box indicates the simian virus 40 late gene polyadenylation signal [SV40 (pA)].

shown in Fig. 1B and C. Two approaches were used for shuttle vector virus production. One consisted of a wild-type, replication-competent BLV helper virus, and the second used a BLV *tax/rex* expression helper plasmid, pBLPX-RSPA. The BLV helper virus and helper plasmid were used to complement the BLV shuttle vector in *trans* and allow for packaging of the vector. Since the BLV helper virus has a packaging signal, it is also packaged. The BLV helper plasmid constitutively expresses the *tax* and *rex* genes. The *tax* gene product is a *trans*-acting transcriptional activator; the *rex* gene product increases the ratio of the nonspliced viral mRNA to spliced viral mRNA. The Tax and Rex proteins aid in efficient virus production of the shuttle vector.

The experimental protocols developed to obtain a single cycle of BLV shuttle vector virus replication are shown in Fig. 2. Fetal lamb kidney (FLK) cells (graciously supplied by Judy Mikovits, National Cancer Institute) and FLK cells producing wild-type BLV (FLK-BLV) cells (kindly provided by Martin Van der Maaten, National Animal Disease Center, Ames, Iowa) were used in these studies (42). Cells were grown in Temin-modified Eagle's medium (38) supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, Mo.) and were maintained at 37°C with 6% CO_2. The first strategy uses a replication-competent BLV helper virus to produce BLV shuttle vector virus (Fig. 2A). The BLV shuttle vector was first transfected (23) into FLK-BLV cells, which were then placed under G418 selection (Step 1 cells) in the presence of α-BLV antiserum to prevent reinfection of cells producing the vector virus. To prevent reinfection and spread of BLV shuttle vector virus, cells were maintained in medium containing α-BLV polyclonal antisera from BLV-infected cows (kindly supplied by Ronald Schultz, University of Wisconsin—Madison, Madison). All α-BLV antiserum used was tested for the ability to neutralize vector virus produced from FLK-BLV cells and FLK cells containing the BLV *tax/rex* expression plasmid, pBLPX-RSPA. To eliminate mutations occurring during the transfection process, virus harvested from these cells was used to infect (19) virus-free FLK cells that were subsequently placed under G418 selection in the presence of α-BLV antiserum (Step 2 cells). Virus harvested from Step 2 clones was used to infect fresh FLK target cells, with infected cells being selected for G418 resistance in medium containing α-BLV antiserum (Step 3 cells). Step 3 cells were also obtained by cocultivation of mitomycin-treated Step 2 cells with fresh FLK target cells and selection for G418 resistance in the presence of α-BLV antiserum. (To increase the number of infected target cell clones obtained, we cocultivated mitomycin-treated, virus-producing cells with FLK target cells. First, virus-producing cells [typically, 2.5×10^5 cells] were treated with mitomycin [10 μg/ml] for 2 h at 37°C. The cells were then washed three times with fresh medium, and 2.5×10^5 FLK target cells were added. Following cocultivation, medium containing α-BLV antisera was added, and selective medium containing G418 was added 2 days later.)

In the second method (Fig. 2B), the BLV shuttle vector was introduced into FLK cells and was placed under G418 selection. Cell clones were then transiently transfected with the BLV helper plasmid, pBLPX-RSPA, and the cells were placed in medium containing α-BLV antiserum (Step 1 cells). Virus was harvested and used to infect fresh FLK cells. G418-resistant cell clones were transiently transfected with the BLV helper plasmid (Step 2 cells), and the cells were placed in medium containing α-BLV antiserum. Virus was harvested from Step 2 cells and was used to infect fresh FLK target cells, or Step 2 cells were cocultivated with FLK target cells; cells were then selected with G418 (Step 3 cells). Proviral DNA from all Step 3 cells was purified and used to determine mutations in the *lacZ*α peptide gene mutational target sequence.

The titer of vector virus produced from Step 1 and Step 2 cells was low for both experimental protocols. Typically, virus titers ranged from 5 to 40 CFU/ml. To increase the number of infected Step 3 target cells, cocultivation of virus-producing cells with target cells was performed. We found that coculti-

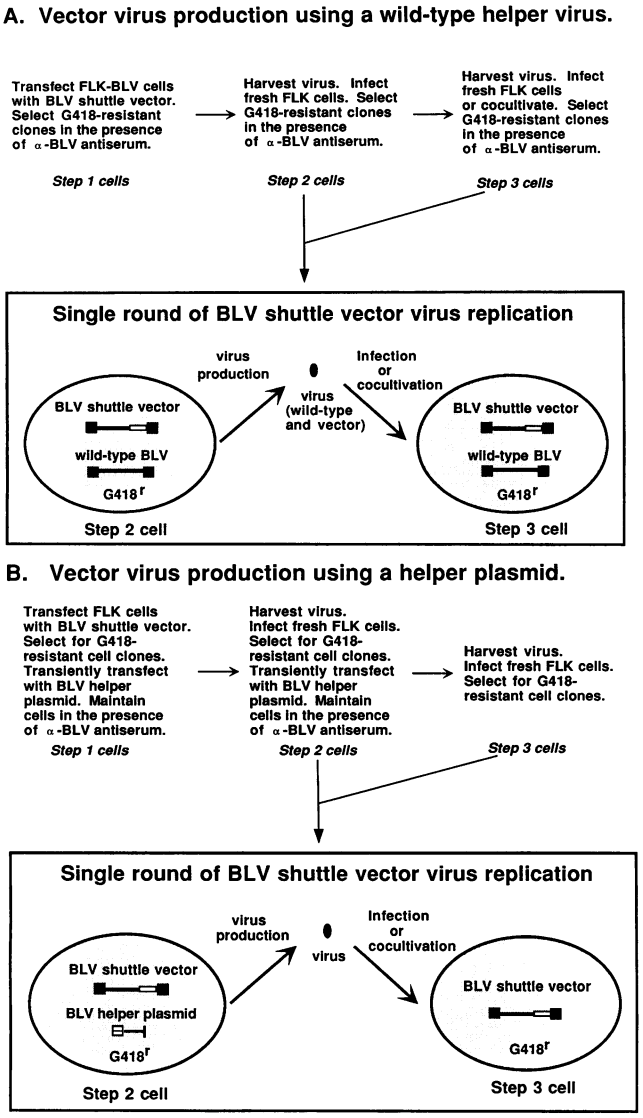

FIG. 2. Experimental protocols for studying one round of BLV shuttle vector virus replication. (A) Virus production using wild-type, replication-competent BLV helper virus. In Step 1, FLK-BLV cells were transfected with pBLV-SVNEO/ACYCLacZ and were placed under G418 selection in the presence of α-BLV antiserum (to prevent reinfection of vector virus). Virus was harvested from these cells and was used to infect fresh (virus-free) FLK cells; these cells were placed under G418 selection in the presence of α-BLV antiserum (Step 2 cells). Virus was harvested from Step 2 clones and was used to infect fresh FLK cells, which were then placed under G418 selection in the presence of α-BLV antiserum (Step 3 cells). Alternatively, Step 3 cells were produced by cocultivation of mitomycin-treated Step 2 cells with fresh FLK cells and G418 selection in medium containing α-BLV antiserum. (B) Virus production using a BLV helper plasmid. pBLV-SVNEO/ACYCLacZ was transfected into FLK cells, and G418-resistant cells were selected. These cells were transiently transfected with the BLV *tax/rex* expression plasmid, pBLPX-RSPA, and were placed in medium containing α-BLV antiserum (Step 1 cells). Virus was harvested and was used to infect fresh FLK cells. G418-resistant cell clones were transiently transfected with pBLPX-RSPA, and cells were placed in medium containing α-BLV antiserum (Step 2 cells). Virus was harvested and used to infect fresh FLK cells (Step 3 cells). Alternatively, Step 3 cells were made by cocultivation of mitomycin-treated Step 2 cell clones with fresh FLK cells.

TABLE 1. Mutation frequency in recovered proviruses

Step 2 clone no.[a]	Mutation type[b] (mutant isolate no.)	No. of mutants/ total no. of bacterial colonies	Mutation frequency
1	D (1)	1/3,415	2.9×10^{-4}
2	S (4)	1/480	2.1×10^{-3}
3	S (5) F (9)	2/2,236	8.9×10^{-4}
4	DI (2) DI (3)	2/1,050	1.9×10^{-3}
2 c[c]	D (12, 13)[d] F (14)	2/3,181	6.3×10^{-4}
4 c	S (10) F (11)	2/3,616	5.5×10^{-4}
5 c	F (15)	1/4,031	2.5×10^{-4}
Total		11/18,009	6.1×10^{-4}

[a] Step 2 clones 1 through 3 were made with wild-type BLV helper virus; clones 4 and 5 were made with the BLV helper plasmid.

[b] D, deletion; DI, deletions with insertions; F, +1- or −1-bp frameshift; S, base pair substitution.

[c] c, step 3 cells were made by cocultivation of FLK cells with mitomycin-treated Step 2 cells.

[d] Proviral mutant isolates that contained identical sequences were recovered from the same pool of Step 3 cells. These isolates were probably the result of clonal expansion. Only one was used for calculations of mutation frequency and rate. These clones are presented on the same line.

vation increased the number of G418-resistant colonies from 20- to 50-fold to produce 800 to 2,000 CFU/ml. We also found, in control experiments done with each cocultivation experiment, that the mitomycin-treated, virus-producing cells did not proliferate and no longer adhered to the surfaces of culture dishes. Therefore, G418-resistant cell colonies represented infected FLK target cells (Step 3 cells).

The steps going from a parental shuttle vector provirus in the Step 2 cells to a vector provirus in the Step 3 cells constitute a single replication cycle of the shuttle vector virus. These steps include transcription of the proviral RNA by the cellular transcription machinery, packaging of the viral RNA and release of viral particles, infection of target cells, reverse transcription of viral RNA, and integration of newly synthesized viral DNA to generate a vector provirus.

Five different Step 2 clones were used to generate Step 3 clones, by either infection or cocultivation (Table 1). In order to determine whether the Step 2 clones used to generate the Step 3 cell clones contained only a single shuttle vector proviral DNA, we analyzed the Step 2 clone genomic DNAs by Southern blot analysis. The Step 2 clones used contained only a single BLV shuttle vector provirus (data not shown). Step 3 clones generated using the wild-type helper virus were found to contain both the BLV helper and the shuttle vector (data not shown); we found that the titer of vector virus was much lower than that of the wild-type virus produced from either Step 1 or Step 2 cells (data not shown).

Purified genomic DNA (36) from pools of Step 3 clones was digested with *Stu*I to release the *neo*, pACYC origin of replication, and *lacZ*α peptide gene sequences from the shuttle vector proviral DNA (Fig. 1A). Proviral DNA was purified with the *lac* repressor protein (Promega Corp., Madison, Wis.) according to a protocol developed by Pathak and Temin (31). Briefly, *Stu*I-digested genomic DNA from FLK target cells was incubated for 20 min at room temperature with the *lac* repressor protein (8 μg of repressor protein per 400 μg of genomic DNA). The *lac* repressor protein-DNA mixture was filtered through nitrocellulose (Gelman) and rinsed with wash

buffer, and the filter was placed (with the DNA side forming the inside curvature) with forceps in a 1.5-ml Eppendorf tube. The protein-bound DNA was then eluted from the filter, the samples were extracted with phenol and chloroform, and the proviral DNA was precipitated with ethanol. The proviral DNA was ligated and was used to electroporate competent *Escherichia coli* DH5α cells. Kanamycin-resistant bacterial colonies were selected in the presence of the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) color indicator and the isopropyl-β-D-thiogalactopyranoside (IPTG) inducer. The ratio of white plus light-blue bacterial colonies to total bacterial colonies observed provided a forward mutation rate for a single retroviral replication cycle.

A total of 18,009 bacterial colonies were recovered from approximately 17,000 Step 3 cell clones obtained. Eleven of these bacterial colonies had a light-blue or white colony color phenotype. The overall mutation frequency was found to be 6.1×10^{-4} (Table 1), and the overall rate per base pair was calculated to be 4.8×10^{-6} mutation per bp per cycle (see Table 3).

The nucleotide sequence of the *lacZ*α gene from each of the 11 proviral mutants was determined by DNA sequencing. Plasmid DNA was purified (Qiagen, Chatsworth, Calif.) from white and light-blue transformant colonies. The *lacZ*α peptide gene sequence of the plasmid was determined by using the Sequenase kit and protocols (United States Biochemical, Cleveland, Ohio) with the dGTP reactions. The M13 universal and reverse primers (United States Biochemical) were used for most of the sequencing reactions. The DNA Strider computer program was used for sequence analysis (27), and the GCG Sequence Analysis package (12) was used for performing nucleic acid homology searches of the GenBank and EMBL sequence data bases.

The types of mutations found included base pair substitutions, frameshifts, deletions, and deletions with insertions (Fig. 3; Table 2). Three mutants were found to contain base pair substitutions (Fig. 3; Table 2). Each mutant was generated from a different Step 2 clone, one of which contained the BLV helper plasmid (Table 1). Two of these mutants were G-to-A transition base pair substitution errors (one creating an in-frame stop codon), and the other was a C-to-T transition error (creating a stop codon) (Table 2).

The four frameshift mutants recovered included two −1 frameshift mutants of A, one +1 frameshift mutant of C, and one −1 frameshift mutant of T (Fig. 3; Table 2). These mutants were generated from four different Step 2 clones, two of which contained the BLV helper plasmid (Table 1).

Three mutants were found to have simple deletions (Table 2; Fig. 3). These mutants were generated from two different Step 2 cells, with two of them generated from the same Step 2 clone containing the BLV helper plasmid (Table 1). These two mutants were found to contain identical deletion mutations and were recovered from different bacterial colonies containing proviral DNA from the same pool of Step 3 cells. Therefore, clonal amplification probably occurred during expansion of target cell pools; amplification also may have occurred during propagation of cloned proviruses in *E. coli* prior to plating on selective media. These two mutants were probably created from a single mutational event rather than representing a mutational hotspot and are presented on the same line in Tables 1 and 2 (mutant D2 in Table 2 and Fig. 3). The deletion mutants were characterized by deletion of nucleotide sequence including one small direct repeat. The size of the direct repeats was 3 bp for both deletion mutants. The small direct repeats for D1 and D2 were TCG and GCG, respectively.

Two mutants contained deletions with insertions (Table 2; Fig. 3). These mutants were generated from the same Step 2 clone, which contained the BLV helper plasmid (Table 1). The deletions were characterized by deletion of a portion of the *lacZ*α gene and the insertion of foreign nucleotide sequences at the deletion junction (Fig. 3). Both mutants also contained relatively large insertions (419 and 342 bp) (sequences not shown). A search of the GenBank and EMBL data bases revealed no sequences similar to the inserted sequences. Both inserted sequences were found to contain open reading frames (data not shown).

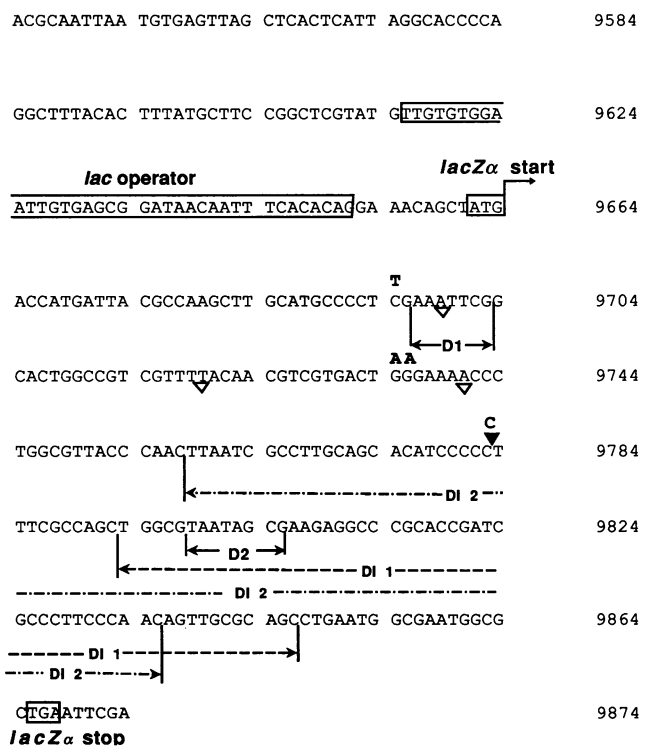

FIG. 3. Plus strand nucleotide sequence of the *lacZ*α gene region in BLV-SVNEO/ACYCLacZ. The start for nucleotide numbering is the beginning of the 5′ long terminal repeat. The start and stop codons of the *lacZ*α open reading frame (small boxed sequences) and the *lac* operator sequence (large boxed sequence) are shown. Nucleotide positions of base pair substitutions (letters above the sequence), +1 frameshifts (letters with ▼ above the sequence), −1 frameshifts (▽ below the sequence), deletions (solid black lines below the sequence and adjacent to the deletion names D1 and D2), and deletions with insertions (dashed lines [DI 1] or dashed lines with dots [DI 2] below the sequence and adjacent to the deletion with insertion names) are indicated.

In this report, we have described an in vivo forward mutation rate system that allows for the study of mutations after a single cycle of replication for BLV, a member of the HTLV/BLV genus of retroviruses. The overall rate of mutation per base pair was 4.8×10^{-6} mutation per bp per cycle.

The system described here for BLV is similar to the forward mutation rate system developed for SNV a simpler retrovirus (31, 32). One major difference is that helper or packaging cells (engineered cell lines that express the viral proteins necessary for virus production) were not available for use in production of the BLV shuttle vector. Instead, a wild-type helper virus or a helper plasmid (expressing the BLV *tax* and *rex* genes) was used for vector virus production (Fig. 1B and C and 2).

In our study, we recovered 11 mutants from 18,009 bacterial colonies (Table 1). In contrast, Pathak and Temin recovered 37

TABLE 2. Base pair substitution, frameshift, and deletion mutations

Mutation type and designation (mutant no.)	Nucleotide change(s)	No. of recovered mutants
Substitution		3
S1 (4)	G to A (Glu to Lys)	
S2 (5)	C to T (Arg to stop)	
S3 (10)	G to A (Glu to stop)	
Frameshift		4
F1 (9)	AAAA to AAA	
F2 (14)	CCCCC to CCCCCC	
F3 (11)	AAA to AA	
F4 (15)	TTTT to TTT	
Deletion		2
D1 (1)	−7	
D2 (12, 13)	−8	
Deletion with insertion		2
DI 1 (2)	−54, +419	
DI 2 (3)	−78, +342	

mutants from 16,867 bacterial colonies in the SNV system (31). The frequencies of mutations per replication cycle for BLV and SNV were analyzed by a chi-square distribution, and the difference was found to be significant ($P < 0.05$). This result indicates that replication of BLV is less error prone than that of SNV.

The types of mutations observed for BLV in this study—base pair substitutions, frameshifts, deletions, and deletions with insertions—were also found with the SNV system. Of the 11 mutants that were recovered in this study, 3 had base pair substitutions, 4 had frameshifts, 2 had deletions, and 2 had deletions with insertions (Table 2). In the SNV study, there were 11 mutants with base pair substitutions, 5 with frameshift mutations, and 12 with deletion mutations; 7 mutants had deletions with insertions (Table 3). The distribution of mutations was similar for both viruses.

The overall rates per base pair of mutation for BLV and SNV (i.e., 4.8×10^{-6} and 1.2×10^{-5} mutation per base pair per cycle, respectively) show a reduction by a factor of about two and one-half in mutations for BLV relative to SNV. The similarity of type of mutations observed for each virus indicates that one property of reverse transcription is involved in the generation of most mutations. Abnormal strand transfers have been suggested to be the property responsible for these types of mutations (39).

TABLE 3. Relative rate of mutation for bovine leukemia virus and spleen necrosis virus

Mutation type	Number of recovered mutant shuttle vector proviruses	
	BLV	SNV
Base-pair substitution	3	11
Frameshift	4	5
Deletion	2	12
Deletion with insertion	2	7
Overall mutation rate[a]	4.8×10^{-6}	1.2×10^{-5}

[a] The rates of mutation were calculated as the sums of the rates of base-pair substitution, frameshift, and deletion mutations per 18,009 total shuttle vector proviruses for BLV or per 16,867 total shuttle vector proviruses for SNV (31) per 113 target nucleotides for substitutions, per 150 target nucleotides for frameshifts, or per 280 target nucleotides for deletion mutations. Target nucleotides for substitution, frameshift, and deletion mutations have been previously described (3, 32). Rate of mutation values are calculated as mutation per base pair per cycle.

The locations in the *lacZα* gene region in which the base pair substitution and frameshift mutations occurred in the BLV forward mutation rate system and the SNV system (31–33) were different. The deletion and deletion with insertion mutants also involved different nucleotide sequences. The nucleotide sequence locations of the base pair substitution and frameshift mutations were similar to previously described mutational hotspots in the *lacZα* gene region (3).

The deletion and deletion with insertion mutants were similar in structure to previously described mutants of SNV (32, 34). The model proposed for deletion mutants suggests that during DNA synthesis by reverse transcriptase, template misalignments occur at the growing point on the nascent strand from one copy of a small direct repeat (about 3 to 8 nucleotides) to a second copy of the direct repeat in the template strand. Synthesis of the nascent strand is then completed to generate a final product that has a deletion of the sequence in between the two repeats and a deletion of one of the two direct repeats. The model for generation of deletion with insertion mutants suggests that unpaired bases at the end of the growing nascent strand pair with homologous nonviral RNA sequences and are extended for some length before another template switch transfers the growing point back to the original viral molecule, again by pairing of the unpaired bases at the end of the nascent strand with homologous sequences in the viral template. The resulting deletion with insertion mutant contains an inserted sequence at the deletion junction. Since our deletion with insertion mutants, DI 1 and DI 2, contained inserted sequences with open reading frames, we presume that the templates for these sequences were mRNAs. The inserted sequences in DI 1 and DI 2 were not homologous to known sequences in the GenBank and EMBL data bases (data not shown).

That reverse transcription of BLV is less error prone than that of SNV may help explain the low genetic diversity of BLV in vivo. Fewer rounds of replication, replication as a provirus, and the host immune response may also exert selective pressures that restrict genetic diversity. Information regarding the molecular basis for genetic variation will be useful for understanding not only the replication, genetic diversity, and evolution of retroviruses but also genetic traits such as virulence, drug resistance, and immune escape.

Nucleotide sequence accession number. The inserted sequences in mutants DI 1 and DI 2 were submitted to GenBank and assigned accession numbers L19256 and L19257, respectively.

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