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High Mutation Rate of a Spleen Necrosis Virus-Based Retrovirus Vector

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Spleen necrosis virus (SNV) is an avian retrovirus that efficiently infects some mammalian cells (e.g., dog and rat cells). We constructed an SNV-based vector, which contains less than 1 kilobase (kb) of the retrovirus sequence, and a number of derivatives containing selectable markers. We obtained high-titer virus stocks, over 10^6 transforming units per ml, with a vector whose genomic RNA consists of 1,850 bases (full-length SNV RNA is 7.7 kb). We also studied two vectors that both carry two genes which should be expressed from a single promoter, one gene from unspliced mRNA and the other gene from spliced mRNA. In one vector, both genes were efficiently expressed as expected. However, in the other vector, expression of the gene 3′ to the splice acceptor was inhibited. When we selected for expression of the 3′ gene is this latter case, we found that the resistant cells contained mutant proviruses in which the 3′ gene could be expressed. Furthermore, we found that mutations were generated during a single round of virus replication (provirus to provirus) at a rate of approximately 0.5% mutations per cycle.

Retroviruses are RNA viruses that replicate through a DNA intermediate. The DNA intermediate, the provirus, stably integrates into the chromosomal DNA of the host cell. Moreover, retroviruses can effectively infect most or all cells in a culture (37). Because of these properties, retroviral vectors represent an attractive system for stable introduction of exogenous genes into cells.

Retroviral vectors that contain two expressed genes have been of particular interest because they can carry both a gene of interest and a selectable gene, enabling easy selection of cells that have been infected by vector-derived virus. There are two major ways such a vector can be constructed. One is to make a vector with an internal promoter so that one gene is expressed from the retrovirus long terminal repeat (LTR) promoter while the other gene is expressed from an internal promoter. The other method is to contruct a vector so that both genes are expressed from a single LTR promoter, one from unspliced retroviral RNA and the other from spliced retroviral RNA. Both types of vectors have been made (2, 4–6, 9, 10, 12, 15, 16, 20, 22, 26, 28–30, 32, 36, 40).

We are interested in using spleen necrosis virus (SNV)-based retroviral splicing vectors to introduce different genes into avian cells, but we previously found that inserting different genes into reticuloendotheliosis virus (Rev) strain T (Rev-T) suppresses transformation by reducing the amount of spliced Rev-T mRNA (21). Consequently, we thought that it would be prudent to study in detail the biological properties of retroviral vectors with spliced mRNA. We term these constructions splicing vectors. Moreover, because of the interest in using splicing vectors as vehicles in somatic therapy of human genetic diseases, a deeper understanding of their properties should be helpful in developing successful protocols for their use in this therapy. We constructed two SNV-based splicing vectors that each contained about 1 kilobase (kb) of retroviral sequences and the dominant selectable genes for hygromycin phosphotransferase B (*hygro*) (8) and neomycin phosphotransferase (*neo*) (14). When *neo* was the 5′ gene being expressed from unspliced mRNA and *hygro* was the 3′ gene being expressed from spliced mRNA, we found that both genes were expressed at about the same levels. Similar results have been reported for murine leukemia virus-based and Rous sarcoma virus-based splicing vectors (2, 22). However, when *hygro* was the 5′ gene and *neo* was the 3′ gene, *neo* expression was greatly reduced. Moreover, the majority of the rare Neo^r cells contained mutant proviruses. We show that these mutants arose during transfection and during viral replication at very high rates, 10% per cycle of transfection and 0.5% per cycle of viral replication. This was the first time that a mutation rate per cycle of viral replication was determined for a retrovirus.

MATERIALS AND METHODS

Nomenclature. By mutations, we mean any stable, heritable changes, such as point mutations, deletions, inversions, and duplications. Mutation rate means the mutation frequency per cycle of retroviral replication. *hygro* and *neo* refer to genes, while Hygro^r and Neo^r refer to resistance phenotypes. Plasmids have a small p before their name (e.g., pJD214Hy), while viruses made from those plasmids do not (e.g., JD214Hy).

Plasmid constructions. The retrovirus sequences used in our constructions were derived from SNV, except for the splice acceptor sequences, which were derived from REV strain A (Rev-A), a virus which has 98% nucleotide sequence homology to SNV.

All plasmid constructions (Fig. 1) were made by standard techniques (17). pJD214 was the result of an eight-step sequential cloning, the specifics of which will be supplied upon request. pJD214 lacks the 5′ end of the 5′ LTR and the 3′ end of the 3′ LTR. It has only 1.45 kilobase pairs of retroviral DNA and a pUC12 polylinker. pJD214Hy was constructed by blunt-end ligation of a Bam*HI* fragment containing the *hygro* gene into the *Hin*dIII site of pJD214. pJD214Neo is pJD214 with a *Hin*dIII fragment containing the *neo* gene in the *Hin*dIII site. pJD216NeoHy was constructed by first inserting the *Hin*dIII fragment containing the *neo* gene into the *Xba*I site of pJD214Hy via blunt-end ligation followed by insertion of a 221-base-pair (bp) *Sma*I-

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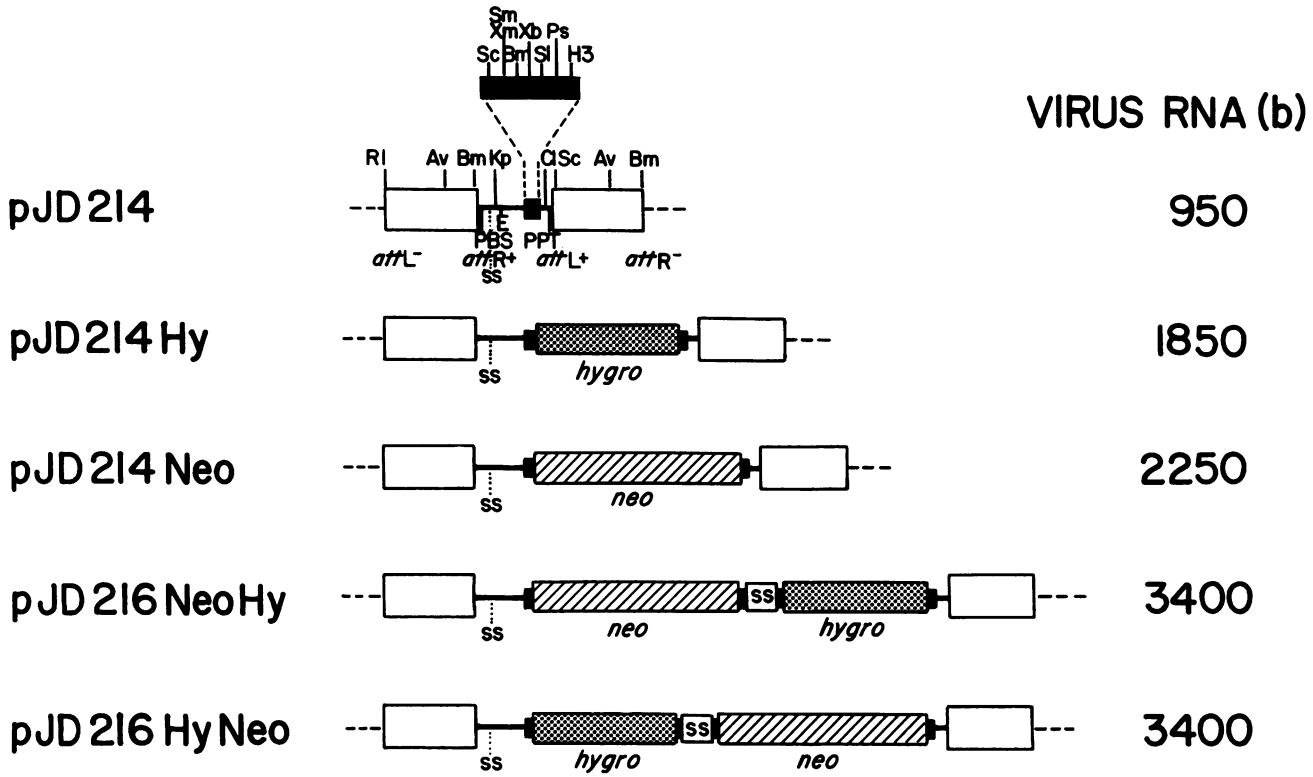

FIG. 1. Vector constructs. ----, pBR322 sequence; ▭, SNV viral LTR; The pBR322 sequences in all the clones extend from map positions 2066 to 4361 with the right-side LTR ligated to position 2066 and the left-side LTR ligated to position 4361. ▬, SNV sequence; ▬, polylinker sequence from pUC12; ▬, *hygro* coding sequence; ▨, *neo* coding sequence. The sequences required in *cis* for viral replication are indicated only in pJD214 although they are present in all the constructs. They are the primer binding site (PBS), polypurine tract (PPT), encapsidation sequence (E), sequences that will form the right side of the attachment site (*att*R$^+$), and the left side of the attachment site (*att*L$^+$). Other abbreviations: SS, splice site; *att*L$^-$, original provirus left-side attachment site which has been deleted; *att*R$^-$ original right-side attachment site which has been deleted. Restriction enzyme cleavage sites indicated are as follows: RI, *Eco*RI; Av, *Ava*I; Bm, *Bam*HI; Kp, *Kpn*I; Sc, *Sac*I; Sm, *Sma*I; S1, *Sal*I; Xm, *Xma*I; Xb, *Xba*I; Ps, *Pst*I; H3, *Hin*dIII; Cl, *Cla*I. Restriction enzyme cleavage sites are only indicated on pJD214. The sizes of the virus RNA derived from these vectors are indicated in bases (b) on the right.

*Bst*EII fragment containing the Rev-A splice acceptor (nucleotides 5578 to 5799 of the Rev-A sequence) (39) into the *Sal*I site of the resulting clone. pJD216HyNeo was made by first inserting the *Sma*I-*Bst*EII fragment containing the Rev-A splice acceptor into the *Sal*I site of pJD214Neo via blunt-end ligation followed by blunt-end ligation of a *Bam*HI fragment containing the *hygro* gene into the *Sma*I site of the resulting clone.

pJD216NeoHypr is a construction in which the 1,500-bp *Sac*I-*Hin*dIII fragment containing the *hygro* gene and splice acceptor from pJD216NeoHy was cloned into *Sac*I-*Hin*dIII-digested pUC12. pJD216HyNeopr is a construction in which the 2,100-bp *Bam*HI fragment containing the *neo* gene and splice acceptor from pJD216HyNeo was cloned into the *Bam*HI site of pUC12 in an orientation in which the splice site was adjacent to the *Xba*I site in the pUC12 polylinker. pJD216NeoHypr and pJD216HyNeopr were used to prepare probes for S1 nuclease mapping (see Fig. 5).

Cells. Chicken embryo fibroblasts (CEF), D17 dog cells, and D17-C3 dog helper cells were grown as previously described (5, 33). The D17-C3 dog cell line is a D17 Rev-A helper cell line which supplies *trans*-functions for the packaging of defective Rev without the production of replication-competent helper virus (34). Selection for hygromycin-resistant (Hygro^r) cells was done in the presence of 50 μg of hygromycin (Eli Lily & Co.) per ml. Selection for G418-resistant (Neo^r) cells was done in the presence of 400 μg of G418 (GIBCO Laboratories) per ml.

DNA analysis. Isolation of unintegrated viral DNA (11) and Southern analysis (18, 24, 27) were done as previously described (33).

Transfections and virus. Transfection were done by the calcium phosphate precipitation method (7, 38). Virus was harvested at 5 days posttransfection from CEF transfected with vector DNA. Virus harvested from CEF was frozen and thawed at least once before use. Virus collected from D17 or D17-C3 cells was either frozen and thawed at least three times or clarified by centrifugation followed by freezing and thawing at least once before use. All virus stocks were stored at −70°C. Virus titers were determined by infecting 2 × 10^5 D17 cells in tissue culture dishes (diameter, 60 mm) with 10-fold serial dilutions of the virus stock in the presence of 100 μg of polybrene per ml in 0.4 ml of medium. The titers, in Hygro^r or Neo^r transforming units (TU) per milliliter, were calculated as the number of phenotypically transformed cell colonies formed after infection and selection multiplied by the dilution.

Antibody preparation. Hyperimmune chicken serum was prepared against SNV by injecting chickens with culture medium from SNV-infected CEF followed by a single challenge and subsequent bleeding. Antibody was used in the culture medium at a final concentration of 1/100. This

concentration was sufficient to neutralize about 10^7 IU of SNV per ml, after incubation for 1/2 h at 37°C (31).

S1 nuclease mapping. RNA was isolated as described by Ross (25). Cells were washed with Tris buffer (25 mM Tris hydrochloride [pH 7.5], 0.14 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4), scraped with a rubber policeman, and pelleted. Cells were then lysed at room temperature by adding urea lysis buffer (7 M urea, 2% sodium dodecyl sulfate, 0.35 M NaCl, 0.001 M EDTA, 0.01 M Tris hydrochloride [pH 8.0]). One milliliter of lysis buffer was used per 10^7 cells. The lysate was shaken for 1 to 2 s followed by the addition of an equal volume of phenol-chloroform. Samples were extracted with phenol-chloroform two times. They were then extracted with chloroform one time. After the extractions, 1 g of cesium chloride was added per 2.5 ml of lysate. This solution was then layered over 3 ml of a 5.8 M cesium chloride solution and centrifuged overnight in a Beckman SW41 rotor at 30,000 rpm at 15°C. The supernatant was removed, and the pellet was suspended in 0.3 M sodium acetate followed by ethanol precipitation.

Probes were made by 5′ end-labeling with [γ-^{32}P]ATP and T4 polynucleotide kinase by published protocols (17). To obtain the JD216NeoHy probe, pJD216NeoHypr DNA was digested with *Eco*RI, treated with calf intestinal phosphatase, and end labeled followed by digestion with *Nde*I and *Cla*I, electrophoresis on a 5% polyacrylamide gel, autoradiography, and purification of the 640-bp end-labeled fragment. To obtain the JD216HyNeo probe, pJD216HyNeopr DNA was digested with *Bgl*II, treated with calf intestinal phosphatase, and end labeled followed by digestion with *Nde*I, electrophoresis on a 5% polyacrylamide gel, autoradiography, and purification of the 790-bp end-labeled fragment.

S1 nuclease mapping was done as previously described (1, 19, 35). For each sample, total cellular RNA (5, 10, 15, or 30 μg), 35 μg of tRNA, and the end-labeled probe were suspended in 50 μl of formamide hybridization buffer. Samples were first incubated at 65°C for 15 min, followed by incubation overnight at 50°C. Standard S1 nuclease buffer (0.5 ml) was added to each sample, and the samples were incubated at 37°C for 2 h. The samples were extracted once with phenol-chloroform followed by the addition of 5 μg of tRNA and ethanol precipitation. The samples were electrophoresed on a 5% polyacrylamide–7 M urea sequencing gel. Autoradiography was then performed.

RESULTS

Characteristics of the vectors. To determine whether our vectors (Fig. 1) contained all of the sequences required in *cis* for virus propagation, we followed the procedure outlined in Fig. 2. We generated virus with titers of approximately 1×10^6 TU/ml by using vector pJD214Hy or pJD214Neo (Fig. 2) even though the virus RNA of JD214Hy consists of only 1,850 bases and the virus RNA of JD214Neo consists of only 2,250 bases. The RNA of the full-length, replication-competent SNV is 7.7 kb. We also obtained high-titer JD214Hy and JD214Neo virus stocks (10^5 TU/ml) from D17-C3 helper cells (34), which provided Rev-A viral proteins without producing helper virus (data not shown). Thus, these viruses could be propagated despite their small size, either with helper virus or in helper cells.

Next we tested whether two genes could be expressed by using the splicing vectors pJD216NeoHy and pJD216HyNeo. We began by generating virus stocks as illustrated in Fig. 2. We then followed the procedure outlined in Fig. 3A until step I. The Neo^r and the Hygro^r TU titers for JD216NeoHy were almost equal (Table 1). In contrast, the Hygro^r TU titer was 50 times higher than that of the Neo^r TU titer for JD216HyNeo (Table 1). This result indicates that, when the *hygro* gene was in the 5′ position, it inhibited the expression of the *neo* gene probably by inhibiting accumulation of spliced JD216HyNeo mRNA (see below). On the other hand, when the *neo* gene was in the 5′ position, it did not inhibit *hygro* expression.

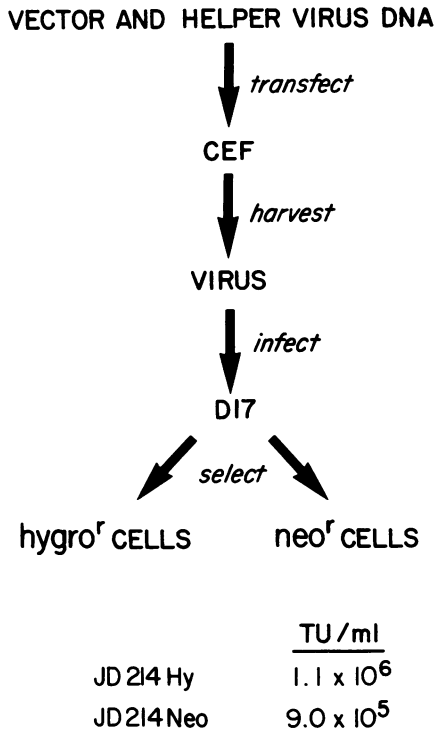

FIG. 2. Characterization of vectors pJD214Hy and pJD214Neo. A flow diagram describing the experiment is shown at the top. Titers are given in TU per milliliter (TU/ml) at the bottom.

To determine whether, after passage through D17 cells and selection, the JD216NeoHy phenotype (in which the expression of both genes was about the same) and the JD216HyNeo phenotype (in which *neo* gene expression was inhibited) were maintained, we harvested and analyzed virus from the Neo^r and the Hygro^r D17 cells (Fig. 3A, step II). When JD216NeoHy was passaged through D17 cells with selection for the *hygro* [JD216NeoHy(Hy) (Table 1)] or *neo* [JD216NeoHy(Neo) (Table 1)], genes, the progeny virus in both cases maintained the parental phenotype (expression of both genes was about the same [Table 1]), and they were the same size as parental virus (Fig. 3B, lanes 2 and 4). When JD216HyNeo was passaged through D17 cells with selection for the *hygro* gene [JD216HyNeo(Hy) (Table 1)], the progeny virus maintained the parental phenotype; that is, the Hygro^r TU titer was about 50 times higher than the Neo^r TU titer (Table 1), and the virus was the same size as parental virus (Fig. 3B, lane 1). However, when JD216HyNeo was passaged through D17 cells with selection for the *neo* gene [JD216HyNeo(Neo) (Table 1)], the progeny virus had a different phenotype from the parental virus. This virus had a Hygro^r-to-Neo^r TU ratio of 3, whereas the parental ratio was 50 (Table 1). Moreover, there was variation in the size of the progeny virus. The progeny virus included virus with dele-

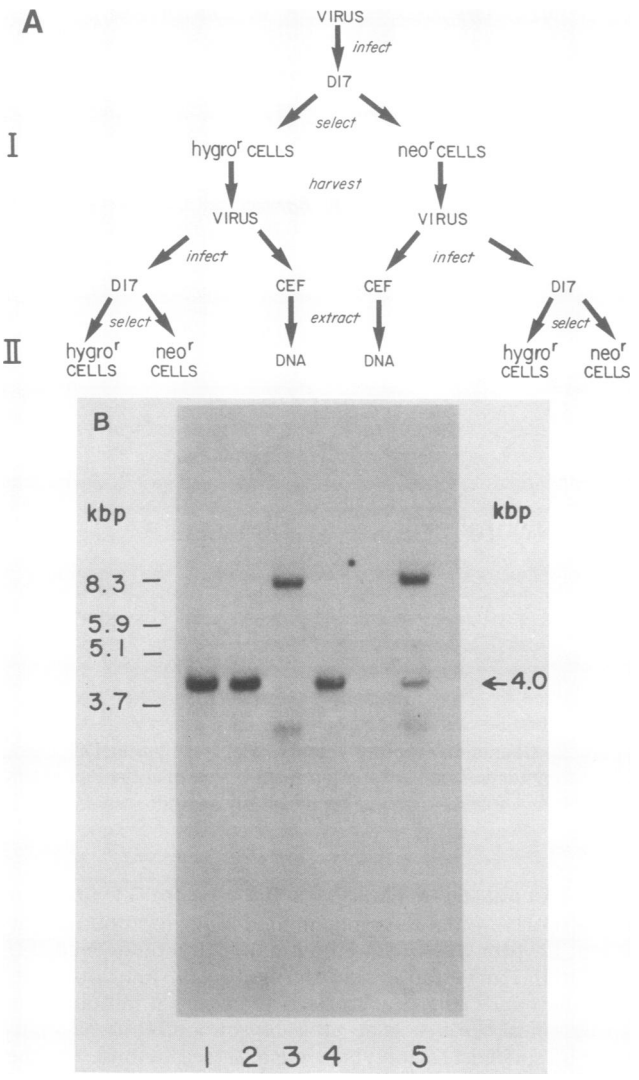

FIG. 3. Characterization of vectors pJD216NeoHy and pJD216HyNeo. (A) Flow diagram describing the experiment. I and II indicate that titers were determined at these steps. (B) Analysis of linear unintegrated viral DNA obtained with the different virus stocks from Hygro^r (Hy) or Neo^r (Neo) cells. Lanes: 1, JD216HyNeo(Hy); 2, JD216NeoHy(Hy); 3, JD216HyNeo(Neo); 4, JD216NeoHy(Neo); 5, JD216HyNeo(Neo). The same vector was used to obtain the samples used in lanes 3 and 5, but the samples were obtained at different times. CEF were infected with virus. Cytoplasmic DNA was purified on day 3 postinfection. The samples were electrophoresed on a 1% agarose gel. The DNA was transfered to nitrocellulose and then probed with a ^{32}P-labeled *neo* probe. The size of unintegrated, linear parental virus DNA is 4 kb.

tions, virus that was the same size as full-length Rev-A, and virus of the expected size (Fig. 3B, lanes 3 and 5).

Mutant proviruses reside in the Neo^r cells originally infected with JD216HyNeo virus. If mutant proviruses reside in Neo^r cells infected with JD216HyNeo, then Neo^r cell clones, each with a single provirus, should produce clonal populations of mutant virus. To determine whether mutant proviruses reside in the Neo^r cell clones, we performed the experiment outlined in Fig. 4A. Virus stocks from three Neo^r D17 cell clones (clones 5, 6, and 7) had no Hygro^r TU but had high Neo^r TU titers (10^4 to 10^6 TU/ml) (Table 2). Clones 5 and 6 produced virus with deletions, while clone 7 produced virus the size of full-length helper virus (Fig. 4B). We also analyzed the virus produced from another four Neo^r D17 cell clones for TU and size, and we found that three produced mutant virus and one produced virus that maintained the parental phenotype (data not shown).

As a control, we analyzed virus from Hygro^r cell clones containing a single JD216HyNeo provirus (Fig. 4). Cell clones 2, 3, and 4 produced parental-phenotype virus, that is, virus with a much higher Hygro^r than Neo^r TU titer (on the average, 180 times higher [Table 2]), and virus that maintained the parental size (Fig. 4B). Clone 1 produced deleted virus (Fig. 4B) which did not express *neo* (Table 2). Four other Hygro^r cell clones studied produced parental-type virus (data not shown).

These results indicate that the majority of the proviruses in the Neo^r cells, originally infected with JD216HyNeo, were mutants (6 of 7 clones), while the majority of the proviruses in the Hygro^r cells were of the parental phenotype (7 of 8 clones).

Accumulation of spliced JD216NeoHy mRNA is more efficientthan accumulation of spliced JD216HyNeo mRNA. To determine steady-state levels of JD216NeoHy and JD216HyNeo mRNAs, D17 cells were infected with JD216NeoHy or JD216HyNeo, total cellular RNA was purified from the mass-infected cells on day 4 postinfection (Fig. 5, lanes 2, 3, 7, and 8), and S1 nuclease mapping was performed with the probes diagramed in Fig. 5. JD216NeoHy mRNA was correctly spliced, since a 375-base protected fragment, corresponding to protection by spliced JD216NeoHy mRNA, was visible (Fig. 5, lanes 2 and 3). The 420-base fragment corresponds to protection by unspliced JD216NeoHy mRNA. The ratio of unspliced to spliced JD216NeoHy mRNA was 9:1 as determined by gel scanning densitometry (Fig. 5, lanes 2 and 3). Spliced JD216HyNeo mRNA was also detected as evidenced by a 430-base protected fragment (Fig. 5, lanes 7 and 8). The 560-base fragment corresponds to protection by unspliced JD216HyNeo mRNA (Fig. 5, lanes 7 and 8). However, the ratio of unspliced to spliced JD216HyNeo mRNA was 40:1.

We also determined the steady-state levels of spliced mRNA in D17 cell clones containing either a phenotypically parental JD216NeoHy provirus (which produced virus with a Neo^r TU titer about the same as the Hygro^r TU titer) (Fig. 5, lanes 4 and 5) or a phenotypically parental JD216HyNeo provirus (which produced virus with a much higher Hygro^r than Neo^r TU titer) (Fig. 5, lanes 9 and 10). In the cell clone with the JD216NeoHy provirus, there was efficient accumulation of subgenomic JD216NeoHy mRNA (Fig. 5, lanes 4 and 5). The ratio of unspliced to spliced JD216NeoHy mRNA was 2:1. In the cell clone with the JD216HyNeo

TABLE 1. Titers of virus stocks

Step[a]	Virus[b]	Titers[c] (TU/ml) of virus		5′ gene/3′ gene (titer ratio)
		Hygro^r	Neo^r	
I	JD216NeoHy	3.7×10^4	8.2×10^4	2.2
	JD216HyNeo	1.4×10^5	2.8×10^3	50
II	JD216NeoHy(Hy)	6.8×10^5	1.7×10^6	2.5
	JD216NeoHy(Neo)	1.8×10^5	4.0×10^5	2.2
	JD216HyNeo(Hy)	3.8×10^5	9.0×10^3	42
	JD216HyNeo(Neo)	2.4×10^5	8.0×10^4	3

[a] Steps correspond to those in Fig. 3A.
[b] (Hy), Virus was harvested from Hygro^r D17 cells; (Neo), virus was harvested from Neo^r D17 cells.
[c] Values are the averages of at least two separate experiments.

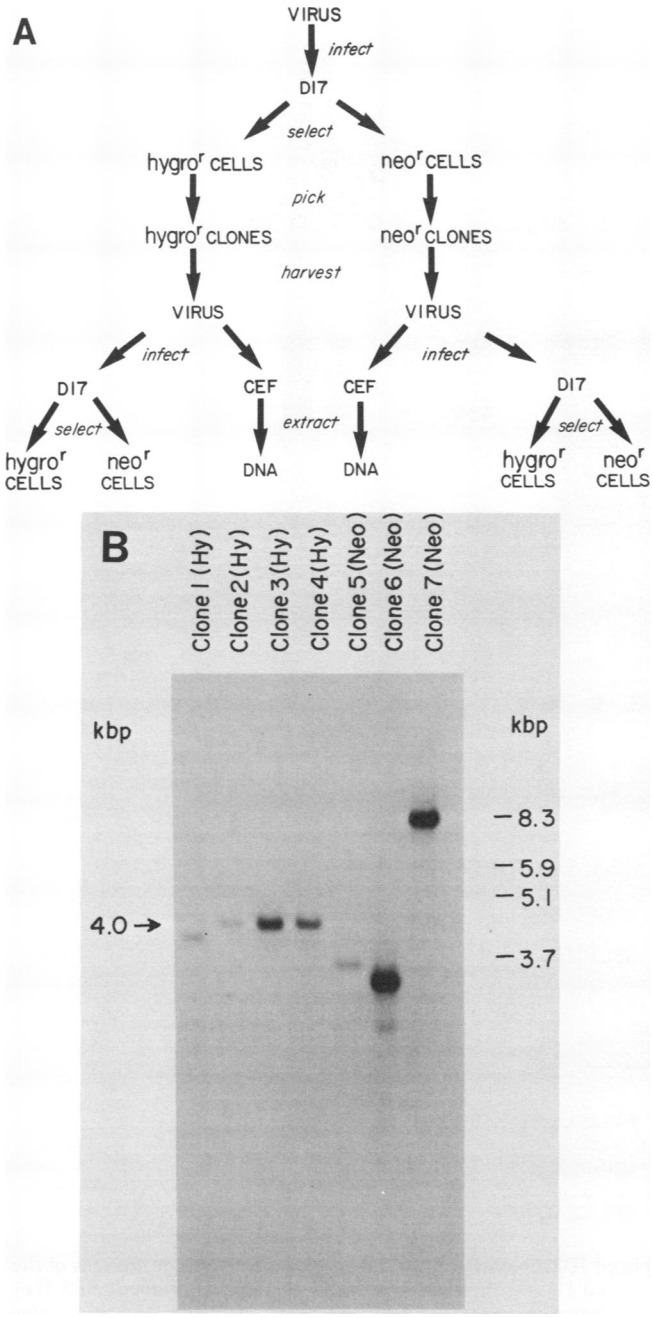

FIG. 4. Analysis of virus from Hygro^r and Neo^r D17 cell clones originally infected with JD216HyNeo virus. (A) Flow diagram illustrating the experiment. The virus stock used to begin the experiment was obtained by cotransfecting CEF with pJD216HyNeo and helper virus DNA, followed by virus harvest on day 5 posttransfection (see Fig. 2). D17 cells were infected with a low concentration of this virus stock, so that an infected D17 cell would contain a single provirus. We examined the genomic DNA of four D17 cell clones infected at low concentration. We found that three contained a single provirus each and one contained two proviruses (data not shown). (B) Analysis of the unintegrated viral DNA obtained with the seven virus stocks from the D17 cell clones. A ^{32}P-labeled *neo* probe was used. (Hy), Hygro^r D17 clone; (Neo), Neo^r D17 clone.

TABLE 2. Titers of virus from JD216HyNeo-infected D17 cell clones

JD216HyNeo-infected D17 cell clone[a]	Titers (TU/ml) of virus		Hygro^r/Neo^r ratio	DNA size (kbp)
	Hygro^r	Neo^r		
1 (Hy)	1.2×10^5	0		3.85
2 (Hy)	2.6×10^4	2.2×10^2	120	4
3 (Hy)	1.5×10^6	1.5×10^4	100	4
4 (Hy)	9.0×10^5	2.8×10^3	320	4
5 (Neo)	0	3.8×10^4		3.3
6 (Neo)	0	9.5×10^5		2.8
7 (Neo)	0	1.3×10^4		8.3

[a] Virus was harvested from Hygro^r (Hy) or Neo^r (Neo) D17 cell clones, and titers were determined as described in the legend to Fig. 4A. kbp, Kilobase pairs.

provirus, there was very little accumulation of spliced mRNA (Fig. 5, lanes 9 and 10). The ratio of unspliced to spliced JD216HyNeo mRNA was 80:1. Accumulation of spliced JD216NeoHy mRNA was more efficient than accumulation of JD216HyNeo mRNA.

Some mutations occur during a single round of virus replication. To ascertain whether mutations appeared after a single round of viral replication, we established 14 helper cell clones, each usually containing a single parental JD216HyNeo provirus, and we analyzed the virus produced by these clones (Fig. 6A). It should be noted that, in going from the provirus in the helper cell to the provirus in the D17 cell, the virus underwent a single round of replication (see Discussion). The Hygro^r/Neo^r TU titer ratios were high (average, 204) for all of the helper cell virus stocks (Table 3). To test whether mutant proviruses were contained in the Neo^r D17 cells, the D17 cells were superinfected with Rev-A, and virus was harvested and analyzed (Fig. 6A). The average Hygro^r/Neo^r TU titer ratio obtained was 4.2 (Table 3). There was an average change of 50-fold. Therefore, the virus mutated in a single round of replication. The mutant virus produced by the Neo^r D17 cells did not show size heterogeneity but was parental in size (Fig. 6B). This is in contrast to the experiments illustrated in Fig. 3 and 4, in which the progeny virus showed size heterogeneity. However, it must be noted that the experiments shown in Fig. 3 and 4 did not separate the virus replication process from the transfection process.

To prevent any possible virus spread during the growth of the cell clones, we repeated this experiment with the same cells except that we grew the Hygro^r helper cell clones (with parental proviruses) in the presence of antibody against Rev-A proteins (see Discussion). The antibody was then removed. Virus was collected after 3 or 4 days and analyzed as illustrated in Fig. 6A. The average Hygro^r/Neo^r TU titer ratio of the virus stocks was 200 (data not shown). This ratio was in close agreement with our previous results (Table 3).

We examined the steady-state mRNA levels in a few Neo^r D17 cell clones harboring mutant proviruses producing parental-size virus. The results obtained with one clone are shown in Fig. 5, lanes 12 and 13. As can be seen, there was efficient accumulation of the expected spliced mRNA, as well as accumulation of spliced mRNA using an alternative splice site, as evidenced by the protected fragment running between the 560-base (unspliced) and 430-base (expected spliced) bands. Accumulation of spliced JD216HyNeo was also efficient in the other cell clones examined. These results indicate that the mutations occurring after a single round of

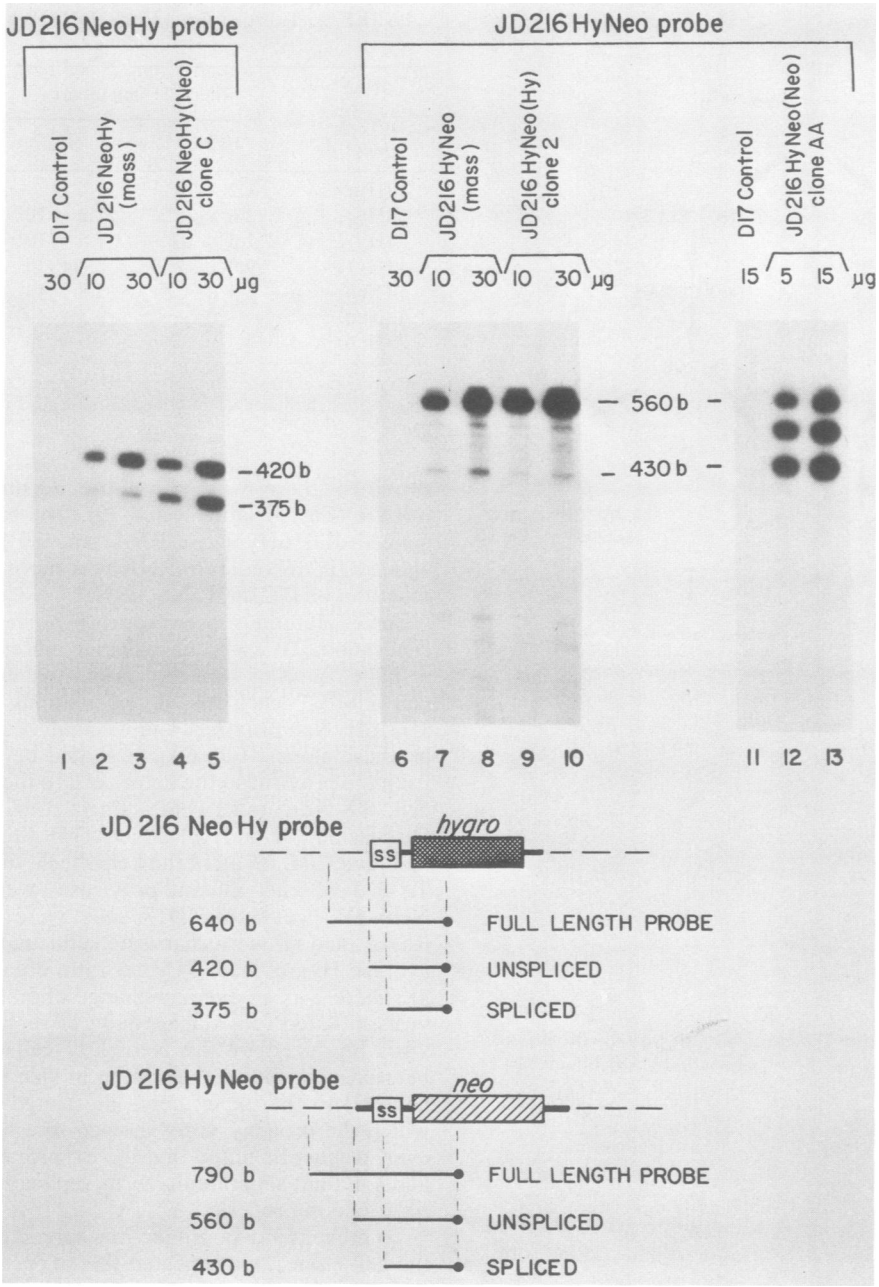

FIG. 5. S1 nuclease mapping to determine the steady-state levels of spliced JD216NeoHy and JD216HyNeo mRNAs. The sources of the cells for RNA isolation were the following: uninfected D17 cells (lanes 1, 6, and 11), D17 cells infected with JD216NeoHy (lanes 2 and 3) or JD216HyNeo (lanes 7 and 8) where these virus stocks were obtained as shown in Fig. 2, or D17 cell clones each containing a single JD216NeoHy (lanes 4 and 5) or JD216HyNeo (lanes 9, 10, 12, and 13) provirus. mass, The RNA was isolated from a mass population of infected D17 cells. The D17 cell clone designated JD216NeoHy(Neo) clone C (lanes 4 and 5) was derived in the same way as clones 5, 6, and 7 in Fig. 4 except that vector pJD216NeoHy was used. JD216HyNeo(Hy) clone 2 (lanes 9 and 10) was the same as clone 2 in Fig. 4. JD216HyNeo(Neo) clone AA (lanes 12 and 13) was a Neo^r D17 cell clone picked at stage I as shown in Fig. 6A. S1 nuclease mapping was performed with the probes diagramed. The horizontal dashed lines represent pUC12 sequences, and the closed circles refer to the ^{32}P end-labeled sites. D17 control, the RNA used was from uninfected D17 cells. The numbers at the top of the lanes refer to the micrograms of RNA used in each reaction. The ratio of unspliced to spliced mRNA was determined by gel scanning densitometry.

viral replication were splicing mutations resulting in efficient accumulation of spliced JD216HyNeo mRNA.

DISCUSSION

We constructed an SNV-based retroviral vector, pJD214, that contained less than 1 kb of virus sequences and had a number of unique cloning sites. To test whether pJD214 contained all the sequences required in *cis* for viral replication, we constructed pJD214Hy into which the *hygro* gene was inserted and pJD214Neo into which the *neo* gene was inserted (Fig. 1). Even though the length of the viral RNA for pJD214Hy was only 1,850 bases and that for pJD214Neo was only 2,250 bases (full-length SNV RNA is 7.7 kb), we were

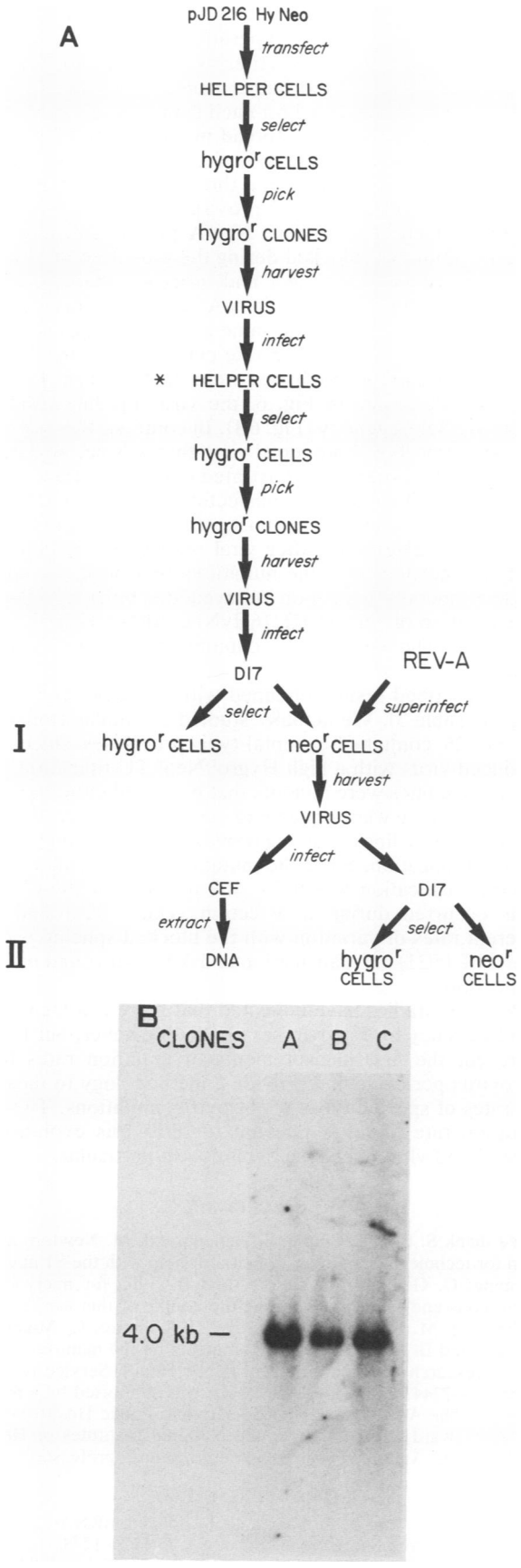

TABLE 3. Titer ratios obtained with virus stocks from helper clones

Clone	Hygro^r/Neo^r TU titer ratio[a] at step:	
	I	II
A	250	10
B	333	15
C	333	10
D	60	10
E	80	3
F	111	3
G	125	1.5
H	250	2
I	320	2
J	50	0.3
K	333	1
L	67	0.2
M	117	0.2
N	429	0.3

[a] Titers were determined as indicated in Fig. 6A. The range of titers obtained with virus from the clones was 1×10^4 to 2×10^5 Hygro^r TU/ml. The average ratio in step I was 204, whereas that in step II was 4.2.

able to generate virus stocks with titers of approximately 10^6 TU/ml with each vector (Fig. 2). Therefore, pJD214 contained all the sequences required in *cis* for viral replication.

Splicing vectors are vectors from which it is theoretically possible to express two genes from a single LTR promoter, one from unspliced mRNA and the other from spliced mRNA. To study SNV-based splicing vectors, we used the *hygro* and *neo* genes. In our splicing vector with the *neo* gene 5′ and expressed from unspliced mRNA and the *hygro* gene 3′ and expressed from spliced mRNA (pJD216NeoHy [Fig. 1]), the level of expression of both genes was almost the same (Table 1). However, when the genes were switched so that the *hygro* gene was 5′ and the *neo* gene was 3′ (pJD216HyNeo [Fig. 1]), the Hygro^r TU titer was 50 times higher than the Neo^r TU titer (Table 1).

S1 nuclease mapping (Fig. 5) showed that accumulation of spliced JD216NeoHy mRNA was more efficient than accumulation of spliced JD216HyNeo mRNA. We believe this difference explains why JD216NeoHy had a Hygro^r TU titer about the same as the Neo^r TU titer and JD216HyNeo had a Hygro^r TU titer 50 times greater than the Neo^r TU titer. To form a Hygro^r or a Neo^r colony, expression of *hygro* or *neo* must surpass a threshold below which cells die and above which cells survive during selection. It is possible for either gene to be expressed at a level below the survival threshold so that a colony will not form. For JD216NeoHy both *neo* and *hygro* were expressed at high enough levels that selection for either gene resulted in rescue of an infected cell. For JD216HyNeo, *hygro* was expressed at high enough levels to rescue infected cells during selection for the *hygro* gene, but *neo* was not expressed at sufficient levels to rescue cells during selection for the *neo* gene. We believe this result occurred because the level of accumulation of spliced

FIG. 6. Mutations occurring after a single round of virus replication. (A) Flow diagram of the experimental procedure. The asterisk (*) indicates that the helper cells were infected with a low virus concentration, so that a single provirus would usually reside in an infected helper cell. (B) Analysis of the unintegrated JD216HyNeo viral DNA obtained with the virus stocks of clones A, B, and C used to obtain titers at stage II (Table 3). A ^{32}P-labeled *neo* probe was used.

JD216HyNeo mRNA was too low for the effective expression of *neo* and rescue of infected cells.

The Neo^r D17 cells originally infected with JD216HyNeo virus contained variant proviruses. We showed that, when virus was harvested from Neo^r D17 cells originally infected with JD216HyNeo, the progeny virus was different from the parental virus (Table 1). The progeny virus had a Hygro^r/Neo^r TU titer ratio of 3, while the parental virus had a Hygro^r/Neo^r TU titer ratio of 50. The progeny virus also showed size heterogeneity (Fig. 3B, lanes 3 and 5) including deleted and recombinant forms of the virus. Six of seven individual Neo^r D17 cell clones, each with a single provirus (three of which are shown in Fig. 4), were variants in which the *neo* gene but not the *hygro* gene was expressed. This result indicates that, because *neo* expression from parental JD216HyNeo was below the survival threshold, selection for *neo* resulted in recovery of cells containing variant proviruses in which *neo* was well expressed. Therefore, if a splicing vector is used to introduce a gene into an organism and expression of the marker gene (which is selected during generation of the virus stocks) is below the threshold for cell survival, it is likely that variant proviruses will be introduced into the organism.

In the experiments outlined in Fig. 3 and 4, the JD216HyNeo variants could have been generated during either transfection or virus replication. To separate mutations occurring during virus replication from variants occurring during transfection, we carried out the experiment illustrated in Fig. 6A. Helper cell clones, each usually containing a single parental JD216HyNeo provirus, were established by infection with helper-free virus. Virus from the clones was then analyzed. The average Hygro^r/Neo^r TU titer ratio for virus from 14 helper cell clones was 204 (Table 3, footnote *a*). Neo^r D17 cells resulting from infection with these viruses contained proviruses that yielded virus with a Hygro^r/Neo^r TU titer ratio of 4.2. There was an average change of 50-fold. Therefore, the Neo^r D17 cells contained mutant proviruses. Since the average Hygro^r/Neo^r TU titer was 204 and the Neo^r D17 cells contained mutant provirus, the mutation frequency was approximately 0.5% (1/204). This mutation frequency is a minimum estimate of the total number of mutations since mutations that did not lead to increased *neo* expression were not scored. It should also be noted that many different types of mutations (point mutations, deletions, etc.) could lead to better expression, so this mutation rate is not a measure of the mutation rate for a particular type of mutation, such as a point mutation.

In going from the provirus in the helper cell to the provirus in the D17 cell, a single round of virus replication occurred. The helper cell provirus was transcribed, the viral RNA was packaged, the virus was released, the virus infected the D17 cell and was uncoated, the viral RNA was reverse transcribed, the DNA copy was inserted into the D17 cell genome (provirus), and the D17 cell replicated with concomitant provirus replication. This process involved one round of RNA transcription, one round of reverse transcription, and multiple rounds of cell replication, that is, one round of virus replication and multiple rounds of provirus replication. Provirus replication is not very error prone (13), so the mutant proviruses found in the Neo^r D17 cells occurred after a single round of virus replication. Therefore, the mutation rate per cycle of virus replication was 0.5% (which was the same as the mutation frequency).

It might be agrued that multiple rounds of infection occurred in the helper cells or in the infected D17 cells. We believe this was not a problem because the helper cells expressed viral envelope proteins setting up viral interference that blocked effective superinfection of the helper cells (interference decreased virus titer by a factor of 100 [data not shown]) and because the helper cells yielded virus stocks free of helper virus (tested for each clone [data not shown]), so there could be no virus spread in the infected D17 cells. Furthermore, the experiment shown in Fig. 6A was repeated with the same cells except that the helper cell clones (each with a single JD216HyNeo provirus) were grown in the presence of antibody against Rev-A proteins, so that any superinfection was blocked during the growth of the clones. The antibody was removed, and virus was harvested and analyzed as illustrated in Fig. 6A (data not shown). The mutation frequency was the same as before, supporting our conclusion that the mutation rate per cycle was 0.5%.

Although mutant proviruses were contained in the Neo^r D17 cells described in Fig. 6, the virus produced did not show size heterogeneity (Fig. 6B). In contrast, the Neo^r D17 cells shown in Fig. 3 produced virus that was heterogeneous in size. In the experiment illustrated in Fig. 3, variants could have arisen either during transfection or during viral replication. In the experiment illustrated in Fig. 6, mutations could have arisen only after viral replication. S1 nuclease mapping indicates that the mutations that occurred after a single round of replication relieved the block in efficient accumulation of spliced JD216HyNeo mRNA (Fig. 5, lanes 12 and 13). The nature of these mutations is presently under study.

We described results obtained with 14 helper cell clones (Fig. 6, Table 3). We actually studied 31 similar clones. Of the 31, 26 contained parental-type proviruses since they produced virus with a high Hygro^r/Neo^r TU titer ratio. The other five clones were mutants that produced either no virus or virus that we were not able to classify. The procedure for obtaining these lines involved transfection and a single round of viral replication. Since the mutation rate per single round of virus replication was 0.5%, the majority of these mutations occurred during transfection. Thus, we found two different rates of variation with the blocked splicing vector, over 10% (5/31) per transfection and 0.5% per round of virus replication.

Previous studies have indicated that there is a high mutation frequency for retroviruses (3, 23). However, our results represent the first measurements of mutation rates for a retrovirus per cycle and provide a methodology to measure the rates of specific types of retrovirus mutations. The high mutation rate found is relevant to retrovirus evolution in general and viral oncogene evolution in particular.

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