JOURNAL OF VIROLOGY, June 1992, p. 3725–3732
0022-538X/92/063725-08$02.00/0

Vol. 66, No. 6

A New Retrovirus Packaging Cell for Gene Transfer Constructed from Amplified Long Terminal Repeat-Free Chimeric Proviral Genes

YOSHIYUKI TAKAHARA,[1]* KUMIKO HAMADA,[1] AND DAVID E. HOUSMAN[2]

Central Research Laboratories, Ajinomoto Co., Ltd., Kawasaki-ku, Kawasaki 210, Japan,[1] and Center for Cancer Research, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139[2]

Received 31 January 1990/Accepted 28 February 1992

The retroviral gene transfer system is a powerful tool for somatic gene therapy. A retroviral stock with a high viral titer and lacking replication-competent virus (RCV) is desirable for this type of gene transfer. To fulfill these requirements, we made a new packaging cell line, designated ampli-GPE. To reduce the homology between proviral DNA in the packaging cell and retroviral vector, the *gag-pol* and *env* genes of Moloney murine leukemia virus were separated onto two different plasmids, pGP-KV and pENV-KV, respectively, in which the 5′ long terminal repeat and the 3′ long terminal repeat had been replaced by the mouse metallothionein I promoter or the human β-globin gene containing the polyadenylation site as control units for the *gag-pol* and *env* genes. In addition, these plasmids contained 69% of the bovine papillomavirus gene for gene amplification to obtain production of virus at a high titer. NIH 3T3 clones containing approximately 20 to 50 copies of the *gag-pol* and *env* genes were selected and designated ampli-GPE. When ampli-GPE was transfected with the N2 vector or pZipNeoSV(DHFR) derived from pZipNeoSV(X)1, we established clones producing titers of 5×10^6 and 1×10^6 CFU/ml, respectively. There was no sign of RCV generation in any virus-producing cells from ampli-GPE. However, virus-producing cells derived from Ψ2 cells transfected with N2 did generate RCV. Thus, we showed that ampli-GPE, possessing the minimum complement of proviral genes, has potential for the development of a gene transfer system.

Several studies have recently demonstrated that a foreign gene can be transferred into murine (2, 10, 11, 13, 15, 23, 24, 27, 28, 40), canine (16, 38), primate (4, 22), or human (25) hematopoietic progenitors and stem cells by using retroviral vectors. This technology holds promise for potential somatic cell genetic therapy, or it could be a powerful tool for analyzing the dynamics of somatic cells such as hematopoietic cells.

Hematopoietic stem cells are an important target of somatic gene therapy. Lemischka et al. (26) reported that only a few stem cell clones account for the majority of mature hematopoietic cells. Therefore, uninfected stem cell clones could compete against the gene-transferred clones in vivo. On the other hand, infection of hematopoietic progenitor cells depends on the viral titer of virus-producing cells (17). Therefore, one requirement for transfer of genes to stem cells is the production of viral vectors at a titer high enough for gene transfer to the majority of stem cells.

Packaging cells, such as Ψ2 (31), ΨAM (9), and PA12 (34), which contain recombinant mutant proviruses and have a *cis*-active deficiency in the packaging of genomic RNA, have been used to generate helper virus-free retroviral vectors. However, generation of replication-competent virus (RCV) in some defective retrovirus stocks has been reported (3, 5, 9, 12, 31, 33, 35). When proviral DNA or RNA in packaging cells is given a packaging sequence by recombination with retroviral vector DNA or RNA, RCV is generated. This recombination is thought to be due mainly to copackaging of the retroviral RNA and defective proviral RNA in the viral particle. To avoid the generation of RCV, improved packaging cells have been developed recently (5, 12, 32, 33). The strategy of the improvement is to reduce the homology between the retroviral vectors and proviral DNA of packaging cells and/or separation of the *gag pol* DNA element from the *env* DNA element to reduce the chance of recombination. Homologous sequences between retroviral vectors and proviral DNA are the 5′ long terminal repeat (LTR), the 3′ LTR, and part of the *gag* sequence in some cases. The *gag* sequence is known to enhance the packaging of retroviral vector mRNA in the viral particle to yield a high titer of virus (1, 3). The 3′ LTR of proviral DNA is replaced with simian virus 40 poly(A) in these new helper cells, PA317 (33), GP+E-86 (32), and ΨCRIP and ΨCRE (12). However, when the 5′ LTR of proviral DNA of packaging cells is deleted or replaced with another promoter, it is difficult to obtain a high viral titer (5, 33).

We intended to minimize the proviral gene in packaging cells without reducing its ability to package retroviral vectors, and to increase the viral titer by amplification of the proviral gene in packaging cells. The new packaging cells contained two kinds of recombinant chimeric proviral gene in the BMGNeo vector, which was developed for overproduction of cytokine genes (21). It was possible to remove both the 5′ LTR and the 3′ LTR from the proviral gene by application of the BMGNeo vector. Instead of the 5′ LTR, the mouse metallothionein I promoter (14, 18) drives the *gag-pol* element or the *env* element separately. The 3′ LTR is replaced with a rabbit β-globin unit containing its poly(A) additional sequence. Furthermore, the *gag-pol* element or the *env* element is amplified separately by the bovine papillomavirus (BPV) unit (6). This expression and amplification system for the *gag-pol* and *env* genes produces a viral titer high enough for gene transfer to somatic cells and avoids the generation of RCV.

MATERIALS AND METHODS

Cell culture and selection for transfection. NIH 3T3 cells were maintained in Dulbecco's modified Eagle medium

* Corresponding author.

(DMEM) containing penicillin, streptomycin, and 10% fetal calf serum (FCS). Twenty-four hours after 2.5×10^5 cells were seeded on a 60-mm dish, the culture was cotransfected with 5 μg of pGP-KV (see Results) and 5 μg of pENV-KV (see Results) by the procedure of Chen and Okayama (8). After overnight culture with DNA, the cells were washed and cultured for 2 days. The transfected cell monolayers were treated with trypsin, and 1×10^4 to 1×10^5 cells were dispersed on a 100-mm dish in DMEM containing penicillin, streptomycin, 10% FCS, and 250 μg of G418 per ml. The culture fluids were changed every 3 or 4 days to fresh medium containing the same supplements. Colonies were isolated with cloning cylinders after 14 days. The isolated clones were cultured on 24-well plates until the cells became confluent, and reverse transcriptase (RTase) in the culture fluids was assayed. Packaging cells were recloned as follows. Cells were cultured in DMEM containing the same supplements on 96-well plates at a dilution rate of one cell per three wells. After 1 week of culture, 50 μl of fresh DMEM with the same supplements was added to the plates, and the cells were cultured for one more week. Clones with high RTase activity were transferred to 24-well plates. A proportion of the grown cells were frozen, and the rest were subjected to DNA preparation.

ampli-GPE packaging cells (see Results) were maintained in DMEM containing penicillin, streptomycin, 10% FCS, and 250 μg of G418 per ml. Twenty-four hours after 2.5×10^5 cells were seeded on a 60-mm dish, the culture was cotransfected with 5 μg of pZipNeoSV(DHFR) or N2 vector (1) and 0.5 μg of pSV2-*gpt* (36) according to the procedure of Chen and Okayama (8). After overnight culture with DNA, the cells were washed and cultured for 2 days. The transfected cells were treated with trypsin, and 1×10^4 to 1×10^5 cells were dispersed on a 100-mm dish in the selection medium, i.e., DMEM containing penicillin, streptomycin, 10% FCS, G418 (250 μg/ml), xanthine (250 μg/ml), hypoxanthine (15 μg/ml), L-glutamine (150 μg/ml), thymidine (10 μg/ml), aminopterin (2 μg/ml), and mycophenolic acid (25 μg/ml). The culture fluids were changed to fresh medium containing the same supplements. Colonies were isolated with cloning cylinders after 2 to 3 weeks. The selected clones were cultured in selection medium on 24-well plates until the cells became confluent, and the viral titers of the culture fluids were determined. The clones producing viruses were recloned in the same way as for packaging cells with selection medium. The viral titer of culture fluids on 24-well plates was assayed.

Ψ2 packaging cells were maintained in DMEM containing penicillin, streptomycin, and 10% FCS. Ψ2 cells were transfected in the same way as for ampli-GPE, except that only pZipNeoSV(DHFR) was used for transfection, and DMEM containing penicillin, streptomycin, 10% FCS, and 250 μg of G418 per ml was used as the selection medium.

Virus assay. Culture fluids from virus-producing cells were centrifuged at 3,000 × *g* for 15 min or filtered with a 0.45-μm-pore-size membrane (Millex-HA; Millipore). The virus was assayed immediately or frozen at −80°C for subsequent assay of virus carrying the selectable marker. Recipient NIH 3T3 cells were seeded at 5×10^5/60-mm dish on day 1. On day 2, the medium was changed to 1 ml of fresh medium containing 8 μg of Polybrene, and aliquots of test virus were added. On day 4, the medium was changed to one containing 250 μg of G418 per ml. The medium was changed to one containing 250 μg of G418 per ml every 3 or 4 days. Colonies were counted on day 14.

Assay of RCV. Plasmid N2 (5 μg) was transfected into NIH 3T3 cells by the method described above. Transfectants were selected in medium containing G418 (250 μg/ml). Selected clones were tested for generation of RCV containing N2 by infection with supernatants of the Moloney murine leukemia virus-producing cell line. One selected clone was designated N2-5. Packaging activity was assayed as follows. NIH 3T3 or N2-5 cells were infected with sample solution as in the viral assay described above. After 3 to 8 days of culture of the infected cells, the culture supernatants were stored at −80°C and the cells were maintained by passage every 4 days in 10% FCS-DMEM without G418 for 20 to 30 days. The culture supernatants were stored at −80°C. Viral titer of the stored supernatants was assayed as for the viral assay described above.

RTase assay. RTase assay was done by the procedure of Goff et al. (19). Autoradiographs of dot blots were used for screening the packaging cell clones.

DNA extraction and hybridization procedures. Total cellular DNA and low-molecular-weight DNA were obtained from cloned cultures of the transfectants as described by Maniatis et al. (30) and Hirt (20), respectively. The Hirt supernatants were extracted once with phenol and once with chloroform and then treated with ethanol to precipitate the DNA. Total cellular DNA or Hirt DNA (5 μg) was cleaved with restriction endonucleases, subjected to electrophoresis on a 0.7% agarose gel, transferred to a nitrocellulose filter, and annealed with a ^{32}P-labeled nick-translated probe containing *gag-pol*, *env*, or BPV sequences. The *gag-pol* probe was a fragment of about 3.8 kbp from pMOVΨ$^-$ (31) obtained by digestion with *Xho*I and *Nde*I. The *env* probe was a fragment of about 2.4 kbp from pENV-KV obtained by digestion with *Xho*I. The BPV probe was a fragment of about 4.8 kbp from the BMGNeo vector (21) obtained by digestion with *Cla*I and *Not*I.

Vectors. Plasmids pZipNeoSV(X)1 and pMOVΨ$^-$ were kindly provided by R. C. Mulligan. pZipNeoSV(DHFR) plasmids were prepared as follows. Dihydrofolate reductase (DHFR) cDNA was obtained from pSV2-*dhfr* (39) by digestion with *Hin*dIII and *Bgl*II. This DHFR fragment was blunt ended with Klenow DNA polymerase and ligated with a *Bam*HI linker. After cleavage with *Bam*HI, the DHFR fragment was inserted into the *Bam*HI site of pZipNeoSV(X)1 (7). The N2 vector was kindly provided by E. Gilboa. The BMGNeo vector was kindly supplied by H. Karasuyama.

RESULTS

Construction of chimeric retroviral DNA. The fragments containing the *gag-pol* region or the *env* region were prepared from pMOVΨ$^-$ (31). Plasmid pMOVΨ$^-$ was digested with *Eco*RI and *Sca*I to prepare the fragment containing the *gag-pol* region. This fragment contained the sequence from the initiation codon of *gag* to the termination codon of *pol*. We prepared the fragment containing the sequence from the initiation codon to the termination codon of *env* from pMOVΨ$^-$ by *Xba*I digestion. A *gag-pol* fragment or *env* from pMOVΨ$^-$ prepared by *Xba*I digestion was inserted into a BMGNeo vector (21). The *gag-pol* fragment was blunt ended with Klenow fragment DNA polymerase. Also, the BMGNeo vector cleaved with *Xho*I was blunt ended with Klenow fragment DNA polymerase. Then the blunt-ended *gag-pol* fragment and the blunt-ended BMGNeo vector were joined with ligase. The BMGNeo vector inserted with the *gag-pol* fragment was designated pGP-KV. The *env* fragment was blunt ended with Klenow fragment DNA polymer-

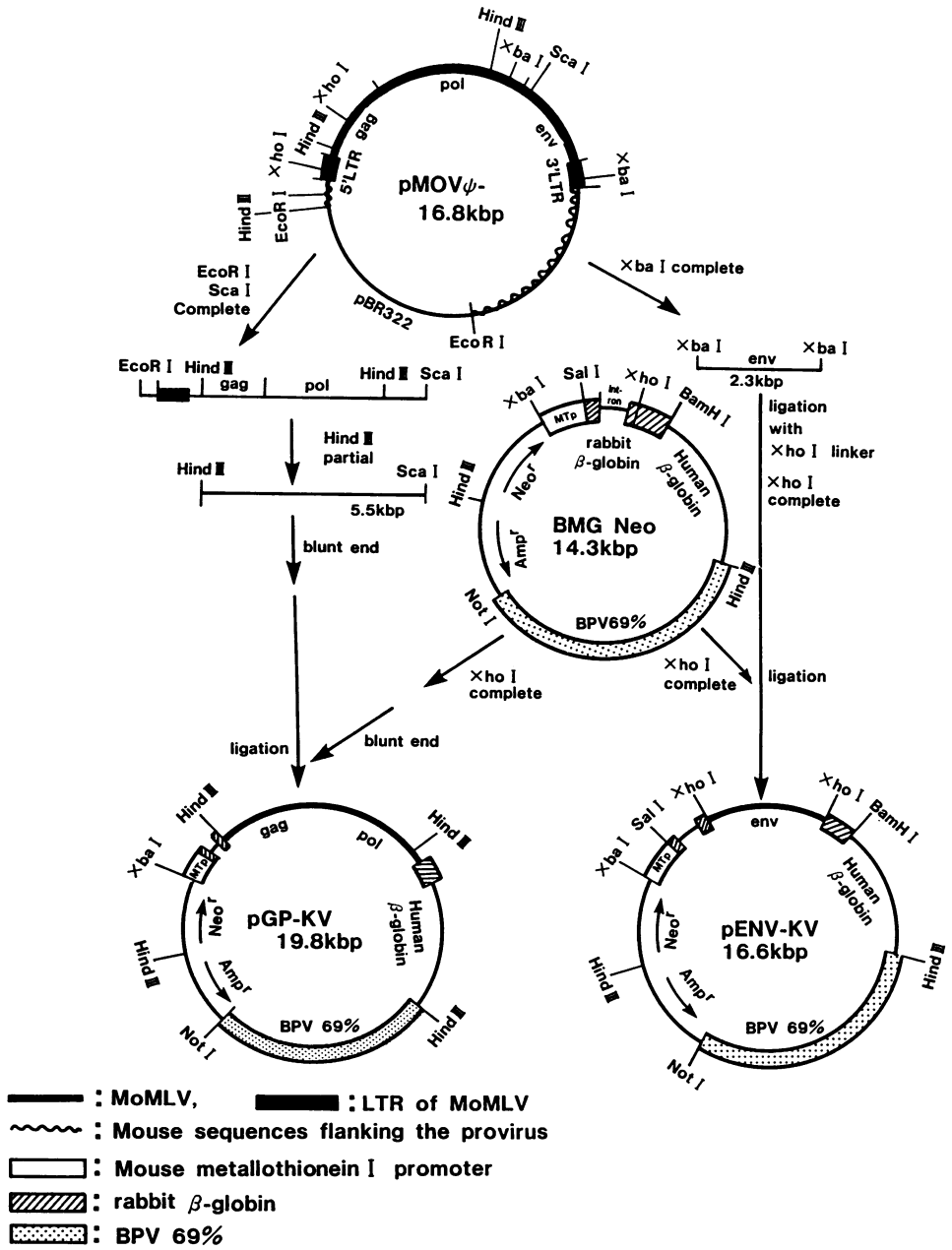

FIG. 1. Construction of pGP-KV and pENV-KV. A *gag-pol* fragment from pMOVΨ⁻ was inserted into the *Xho*I site of a BMGNeo vector by blunt-end ligation. An *env* fragment from pMOVΨ⁻ was inserted into the *Xho*I site of the BMGNeo vector with *Xho*I linkers. For details, see text. Amp^r, ampicillin resistance; Neo^r, neomycin resistance; MTp, MT promoter; MoMLV, Moloney murine leukemia virus.

ase and then ligated with *Xho*I linker [pd(CCTCGAGG)]. The *Xho*I site of the *env* fragment was made by *Xho*I digestion. Then the *env* fragment was ligated with the BMGNeo vector cleaved with *Xho*I to give pENV-KV. The process of construction is summarized in Fig. 1.

Selection of packaging cells. NIH 3T3 cells were cotransfected with pGP-KV and pENV-KV and then cultured in medium containing G418 for about 2 weeks. Approximately 200 clones were selected in 24-well plates and cultured until the cells became confluent. Then the RTase of the culture fluids was assayed. Clones with high RTase activity were chosen and expanded in 100-mm dishes to prepare total cellular DNA. This DNA was digested with *Hin*dIII and analyzed by Southern blotting. Seven clones which had high copy numbers of both the *gag-pol* and *env* genes were selected on the basis of their hybridization patterns with the *env* and *gag-pol* probes. These clones were recloned in 96-well plates, and the RTase activity of these culture fluids was assayed. Thirty clones showing high RTase activity were selected, and the total cellular DNA and Hirt DNA were prepared from the same cultured cells. Nine clones containing the *gag-pol* and *env* genes showing high copy

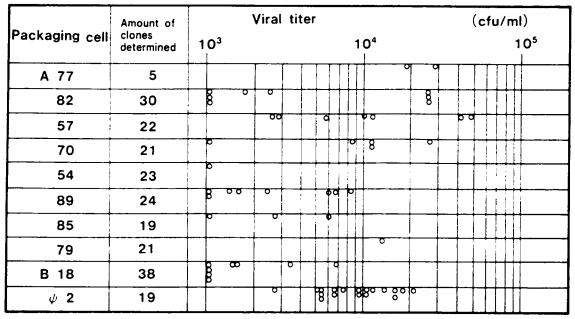

FIG. 2. Titers of virus-producing cells from packaging cells transfected with pZipNeoSV(DHFR). Clones from ampli-GPE transfected with pZipNeoSV(DHFR) and pSV2-*gpt* and clones from Ψ2 cells transfected with pZipNeoSV(DHFR) were transferred to 24-well plates and then cultured until the cells became confluent. The viral titers of the culture fluids were then assayed.

numbers by Southern blot analysis were chosen. These packaging cells containing pGP-KV and pENV-KV were designated ampli-GPE.

Viral production by selected packaging cell clones. pZipNeoSV(DHFR) was cotransfected into nine selected packaging cell clones with pSV2-*gpt* (36) as a selection marker. These transfected cells were cultured for 2 to 3 weeks in hypoxanthine-aminopterin-thymidine selection medium containing mycophenolic acid and G418. Colonies were selected and cultured in 24-well plates. The viral titer of the supernatants was assayed when the cells became confluent. We prepared virus-producing cells from Ψ2 cells following the same procedure. Ψ2 packaging cells were transfected with pZipNeoSV(DHFR). These transfected cells were selected in G418 and cultured in 24-well plates. The viral titer of the supernatants was assayed when the cells became confluent. The results for the first cloned cells obtained from ampli-GPE or Ψ2 cells are summarized in Fig. 2. Nine clones of the first cloned cells producing viruses at a high titer were recloned in 96-well plates. The growing clones were transferred to 24-well plates, and the viral titer of the culture fluids was assayed. The efficiency of cloning virus-producing cells from A-57 was relatively higher than that of cloning

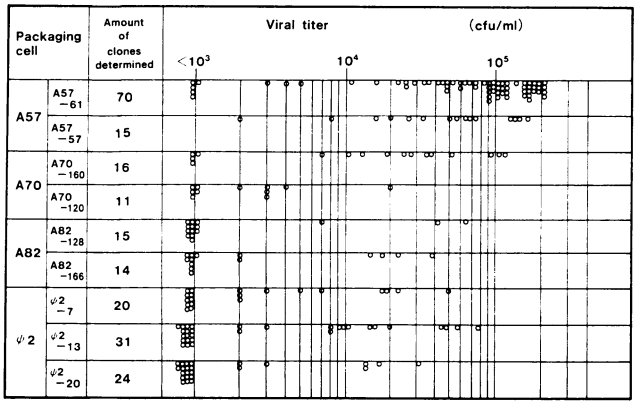

FIG. 3. Titers of recloned virus-producing cells obtained from packaging cells transfected with pZipNeoSV(DHFR). High-titer viral producers selected as described in the legend to Fig. 2 were recloned in 96-well plates and then cultured until the cells became confluent. The viral titers of the culture fluids were then assayed.

TABLE 1. Titers of virus-producing cell lines selected from packaging cells containing the pZipNeoSV(DHFR) vector

Packaging cell line	Virus-producing cell line		Viral titer (10^5 CFU/ml)
	First clone	Recloned	
A-57	A-57-57	A-57-57-3	6
		A-57-57-6	4
		A-57-57-11	7
	A-57-61	A-57-61-14	9
		A-57-61-17	9
		A-57-61-41	7
A-70	A-70-160	A-70-160-5	4
		A-70-160-8	11
		A-70-160-10	6
Ψ2	Ψ2-7	Ψ2-7-4	0.5
		Ψ2-7-12	2
		Ψ2-7-15	0.4
	Ψ2-13	Ψ2-13-13	2
		Ψ2-13-29	0.7
		Ψ2-13-37	0.9
	Ψ2/pZipNeoSV(X)1[a]		0.4

[a] Standard viral stock from Ψ2/pZipNeosV(X)1 was stored at −80°C.

from other ampli-GPE packaging cell lines. The results for the second cloned cells are summarized in Fig. 3. We were able to obtain virus-producing clones at a higher titer from the packaging cell lines A-57, A-70, and A-82 than from Ψ2 cells.

Selected clones were transferred to 5 ml of DMEM containing 10% FCS with or without G418 at 250 μg/ml in a 50-ml flask (Falcon 3013). After 1 month of culture, 5×10^5 cells of each clone were transferred to 5 ml of DMEM containing 10% FCS and 250 μg of G418 per ml in a 50-ml flask. Viral titers of culture fluids were assayed at days 3 and 4. The maximum titers of the selected clones are shown in Table 1. The viral titer of each virus-producing cell clone after 1 month of culture was similar to that of each respective clone which was transferred before long-term culture and cultured in a 50-ml flask under the same conditions (data not shown). Also, the viral titers of clones after 1 month of culture with or without G418 were approximately equal (data not shown). The viral titer of virus-producing cells from ampli-GPE was relatively higher than that of cells from Ψ2.

Viral production by selected packaging cells with a retroviral vector containing part of the *gag*-coding gene. A retroviral vector containing part of the *gag* sequence, such as N2, has been reported to be capable of producing virus at a higher titer than a retroviral vector without a *gag* sequence (1, 3). ampli-GPE or Ψ2 cells were transfected with N2, and the transfectants were isolated. The viral titers of the transfectants are shown in Fig. 4. Clones producing viruses at high titer were recloned, and the titers of the recloned viral

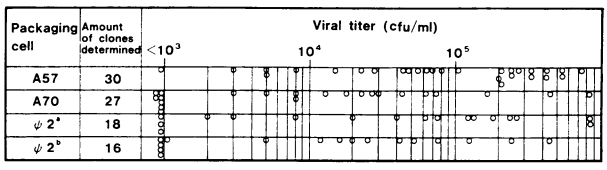

FIG. 4. Titer of virus-producing cells obtained from packaging cells transfected with N2. Clones from ampli-GPE or Ψ2 cells with N2 and pSV2-*gpt* and clones from Ψ2 cells transfected with N2 were transferred to 24-well plates and then cultured until the cells became confluent. The viral titers of the culture fluids were then assayed.

TABLE 2. Titers of virus-producing cell lines recloned from packaging cells containing the N2 vector

Packaging cell line	Virus-producing cell line		Viral titer (10^5 CFU/ml)
	First clone	Recloned	
A-57	57N14	57N14-9	31
		57N14-12	17
		57N14-28	46
		57N14-29	15
		57N14-30	11
A-70	70N11	70N11-12	23
		70N11-32	8
		70N11-41	6
Ψ2	Ψ2NS19	Ψ2NS19-3	19
		Ψ2NS19-6	12
		Ψ2NS19-11	17
		Ψ2NS19-12	22
		Ψ2NS19-13	21
		Ψ2NS19-14	18
		Ψ2NS19-15	16
		Ψ2NS19-16	15
		Ψ2NS19-19	19
	Ψ2/pZipNeoSV(X)1[a]		0.4

[a] Standard viral stock from Ψ2/pZipNeoSV(X)1 was stored at −80°C.

producers are listed in Table 2. Transfectants or recloned viral producers were assayed as described in the preceding section. It is clear that the transfectants with N2 produced more virus than the transfectants with pZipNeoSV(DHFR). ampli-GPE with N2 had the same titer as Ψ2 cells with N2, unlike the ampli-GPE containing pZipNeoSV(DHFR).

Analysis of proviral DNA in packaging cells. Total cellular DNA and Hirt DNA from the selected packaging cells A-57, A-70, and A-82 were prepared. As controls, DNA from Ψ2 and NIH 3T3 cells was also prepared. This DNA was digested with *Hin*dIII or *Bam*HI and then analyzed by Southern blotting. ^{32}P-labeled *gag-pol*, *env*, and BPV probes (Fig. 5) were used for hybridization. The sizes of the expected hybridized bands, if intact copies of pGP-KV and pENV-KV were present, are indicated in Table 3. As shown in Fig. 6, bands of the expected size were the main bands in the ampli-GPE packaging cell lines A-70 and A-82, although there were other additional hybridizing fragments of various sizes and low intensity. The Southern blot patterns of Hirt DNA from A-70 or A-82 showed almost the same patterns as those of total cellular DNA (data not shown). Other bands with sizes varying from those of the expected bands were evident in the hybridization patterns of digested total cellular

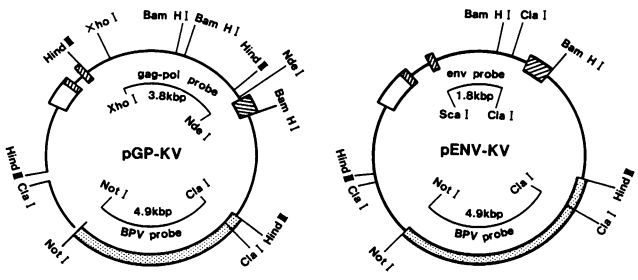

FIG. 5. Probes for hybridization with DNA from packaging cells. The *gag-pol* probe was prepared from pMOVΨ$^-$ by digestion with *Xho*I. The *env* probe was prepared from pENV-KV by digestion with *Xho*I. The BPV probe was prepared from the BMGNeo vector by digestion with *Cla*I and *Not*I.

TABLE 3. Expected fragments from pGP-KV or pENV-KV after digestion with *Hin*dIII or *Bam*HI hybridizing with the *gag-pol*, *env*, or BPV probe

Probe[a]	Fragment (kbp)			
	pGP-KV[b]		pENV-KV[b]	
	*Hin*dIII	*Bam*HI	*Hin*dIII	*Bam*HI
gag-pol	4.2	0.3		
	4.3	2.9		
		16.3		
env			8.8	2.1
				13.7
BPV	7.7	16.3	7.7	13.7

[a] See Fig. 5.
[b] See Fig. 1.

DNA from A-57. It is not known whether these represent rearranged plasmids or integrated species. In the Southern blot patterns of Hirt DNA from A-57, the intensity of some bands was lower than that of total cellular DNA, but the intensity of the other bands was the same as that of total cellular DNA (data not shown). This analysis gave inconclu-

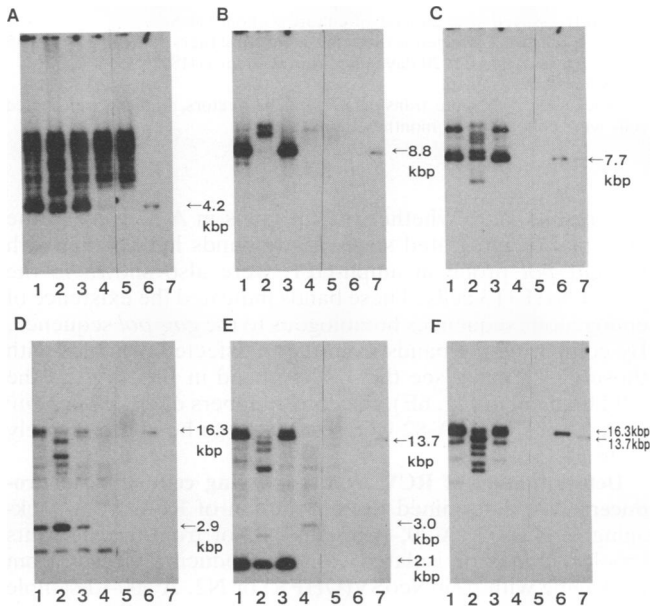

FIG. 6. Detection of pGP-KV and pENV-KV sequence in ampli-GPE packaging cell lines. Total cellular DNA was digested with a restriction enzyme, separated on a 0.7% agarose gel, and transferred to a nitrocellulose filter. The filter was hybridized with a nick-translated ^{32}P-labeled probe. (A) Total cellular DNAs from three ampli-GPE clones, Ψ2 cells, and NIH 3T3 cells were digested with *Hin*dIII. The filter was hybridized with the ^{32}P-labeled *gag-pol* probe. (B) The same filter as that in panel A was dehybridized and then hybridized with the ^{32}P-labeled *env* probe. (C) The same filter as that in panel A was dehybridized and then hybridized with the ^{32}P-labeled BPV probe. (D) Total cellular DNA from the same clones as those in panel A was digested with *Bam*HI. The filter was hybridized with the ^{32}P-labeled *gag-pol* probe. (F) The same filter as that in panel D was dehybridized and then hybridized with the ^{32}P-labeled *env* probe. (G) The same filter as that in panel D was dehybridized and then hybridized with the ^{32}P-labeled BPV probe. Lanes: 1, A-82; 2, A-57; 3, A-70; 4, Ψ2 cells; 5, NIH 3T3 cells; 6, pGP-KV; 7, pENV-KV.

TABLE 4. Generation of RCV from virus-producing cells

Packaging cell line	Virus-producing cells	Viral titer (10^5 CFU/ml)	Generation of RCV (CFU/ml)	
			Expt 1[a]	Expt 2[b]
A-57	Mixture of cells Transfected with:			
	N2 vector[d]	0.5	ND[c]	>2
	57N10	7	>2	>2
	57N14	5	>2	>2
	57N14-9	31	>2	>2
	57N14-28	46	>2	>2
A-70	Mixture of cells Transfected with:			
	N2 vector[d]	0.2	ND	>2
	70N11	8	>2	>2
	70N15	5	>2	>2
	70N11-12	23	>2	>2
	70N11-41	6	>2	>2
Ψ2	Mixture of cells Transfected with:			
	N2 vector[d]	4	ND	2×10^5
	NS4	9	8×10^4	2×10^4
	NS6	1	>2	1×10^4
	NS14	2	>2	>2
	NS19	8	>2	>2
	NS19-16	15	3×10^4	9×10^3

[a] 3T3 cells were infected with samples, and viral titers of the infected 3T3 cells were assayed after 20 days of culture without G418.

[b] N2-5 cells were infected with samples, and viral titers of the infected N2-5 cells were assayed after 20 days of culture without G418.

[c] ND, not determined.

[d] Packaging cells were transfected with N2 vectors, and the transfected cells were cultured for 1 month without G418.

sive results as to whether the plasmids in A-57 were in the episomal or integrated state. Many bands hybridizing with the *gag-pol* probe in ampli-GPE were also shown in the parent NIH 3T3 cells. These bands indicated the existence of endogenous sequences homologous to the *gag-pol* sequence. By comparing the bands from the transfected plasmids with those of Ψ2 cells (see the 4.2-kbp band in Fig. 6A and the 3.0-kbp band in Fig. 6E), the copy numbers of *gag-pol* or *env* in A-57, A-70, or A-82 were estimated to be approximately 20 to 50.

Determination of RCV from packaging cells or viral producers. We determined the production of RCV from packaging cells A-57, A-70, A-82, and Ψ2 or from transfectants (pooled clones or isolated clones) producing viruses from packaging with pZipNeoSV(DHFR) or N2. A 500-μl sample of 4-day-cultured supernatants of packaging cells or virus-producing cells was applied to NIH 3T3 cells or N2-5 cells in 60-mm plates in the same manner as for the assay of viral titer. Supernatants of infected NIH 3T3 or N2-5 cells cultured for a short (3 to 8 days) or long (20 to 30 days) period in medium without G418 were used for secondary infection of NIH 3T3 cells. The secondarily infected cells were cultured in medium containing G418 to determine the production of virus containing the neomycin resistance gene. Once RCV had been generated in packaging cells or viral producers, RCV would be expected to spread and amplify in long-term cultures of infected NIH 3T3 or N2-5 cells. If the generated RCV had no functional neomycin resistance gene, infection of N2-5 by RCV resulted in generation of RCV containing N2 vector which had the neomycin resistance gene. The titers of the long-term-cultured supernatants are listed in Table 4. RCV were not found in any of the culture fluids of ampli-GPE itself or ampli-GPE with pZipNeoSV(D-HFR) or N2, nor were they generated from Ψ2 cells or Ψ2 cells with pZipNeoSV(DHFR). However, a mixture of Ψ2 cells transfected with N2 and some isolated clones of Ψ2 cells transfected with N2 produced RCV. The titers of the long-term-cultured supernatants were about 100- to 1,000-fold higher than that of each respective short-term cultured supernatant when RCV existed in them (data not shown). This suggests that RCV is amplified in the long-term culture.

DISCUSSION

A retroviral gene transfer system consists of two components. One component is a retroviral vector made from proviral DNA. The *gag-pol* and *env* genes in proviral DNA are replaced with the desired gene for transfer, but some sequences of proviral DNA remain in the vector. The 5′ LTR, the 3′ LTR, and the packaging signal which are essential for this system remain in the vector. Substitution of the LTR has not yet been successful because the multiple functional mechanisms of the LTR are not entirely understood. Also, some vectors have a part of the *gag* sequence which is reported to increase viral production markedly because of effective packaging of viral mRNA in viral virions (1, 3). The second component is a packaging cell producing viral virions for packaging the retroviral vector. The packaging cell contains proviral DNA for which the packaging signal has been deleted. These two kinds of proviral DNA have homologous sequences, the 5′ LTR and 3′ LTR. Some vectors also have homology in the *gag* sequence. Greater homology results in more frequent recombination. Virus-producing cells consisting of a vector containing part of the *gag* sequence have been reported to generate RCV at high frequency (5, 33). As in this study, Ψ2 cells containing the N2 vector produced RCV. To reduce such recombinations, new types of packaging cells have been reported (5, 12, 32, 33). PA317 was constructed with a proviral gene from which the 5′ LTR was partially deleted and the 3′ LTR was replaced with the simian virus 40 poly(A) site (33). Clone 32 has two separate proviral DNAs, one with the *gag-pol* gene and the other with the *env* gene from which the 5′ LTRs have been replaced with the metallothionein I promoter, leaving the 3′ LTR (5). Clones GP+E (32) and ΨCRIP and ΨCRE (12) were constructed from two separate plasmids, one of which has only functional *gag-pol* DNA and the other which has only functional *env* DNA. Their 3′ LTRs have been replaced with a simian virus 40 poly(A) additional sequence. They have a virus-producing ability comparable to that of Ψ2, ΨAM, or PA12 cells. Removal of both the 5′ LTR and 3′ LTR of the proviral DNA in the packaging cells has not been reported.

Our strategy to prevent the generation of RCV and for high viral production is as follows. (i) Remove both the 5′ LTR and 3′ LTR to reduce the homology between the retroviral vector and proviral DNA in the packaging cells. (ii) Separate the *gag-pol* unit from the *env* unit to reduce the frequency of recombination. (iii) Amplify the *gag-pol* or *env* DNA with the BPV unit to yield a high viral titer. We chose the BMGNeo vector to fulfill these requirements. Combination of the metallothionein I promoter with the BPV unit effectively expressed the inserted gene and amplified it. Combination of the rabbit β-globin intron, human β-globin sequence, and rabbit β-globin polyadenylation site stabilized the episomal state of the amplified vector gene. High production of lymphokines, interleukin-2 (IL-2), IL-3, IL-4, or IL-5 has been reported with this vector (21). We inserted the

gag-pol and *env* genes into the *Xho*I site of the BMGNeo vector. BMGNeo vectors made transfectants in which the *gag-pol* or *env* gene was amplified 20 to 50 times in a stable state. We were able to make virus-producing cells that made 5×10^5 to 1×10^6 CFU/ml using this packaging cell and pZipNeoSV(DHFR). This high viral production did not change after 1 month of passage of virus-producing cells. This stable viral production suggests that the amplified *gag-pol* and *env* genes are stable. ampli-GPE transfected with the N2 vector was able to produce defective viruses at 5×10^6 CFU/ml without any sign of RCV generation. Bosselman et al. (5) reported clone 32, a packaging cell line containing chimeric proviral DNA using the metallothionein I promoter instead of the 5′ LTR, which generated virus-producing cells with a maximum titer of about 2.6×10^4 CFU/ml. ampli-GPE, in which the 5′ LTR and 3′ LTR were substituted, has been able to generate virus-producing cells at 5×10^6 CFU/ml. Therefore, gene amplification in ampli-GPE may contribute to the high viral production by ampli-GPE. However, this effect of gene amplification has not been clearly observed for viral production with the N2 vector. One explanation may be that multiple infection of Ψ2 cells with RCV containing the N2 vector results in multiplication of the N2 gene in the virus-producing cells, contributing to the high titer.

The titer of retroviral vectors has been reported to depend markedly on the assay used (29). As in this report, when the cells were split 1:10 in selective medium 2 days after infection, this assay gave about a two- to five-fold-higher apparent titer than our standard assay (data not shown). Therefore, although viral titer cannot be exactly compared with the titer reported, ampli-GPE can give virus-producing cells with the same titer as other reported safe packaging cells (12, 32, 33).

Because the neomycin resistance gene is already present in these packaging cells, cotransfection with another marker plasmid such as pSV2-*gpt* must be done for selection of virus producers with a retrovirus vector containing the neomycin resistance gene, which is usually used in retroviral vectors which have no other selection marker. The intricacy involved in producing ampli-GPE is a disadvantage in comparison with other reported packaging cells. However, the frequency with which high virus producers are obtained is sufficient for practical use. Also when pMZhyg-1 (41), containing the gene for hygromycin B resistance, has been used, clones have produced viruses at the same titer as the virus producer from ampli-GPE transfected with pZipNeoSV(DHFR) (data not shown).

To obtain a higher titer with ampli-GPE, we inserted the DHFR gene into pZipNeoSV(X)1. Amplification of the viral vector gene by stress with methotrexate (37) would be expected to provide a higher titer without generation of RCV. However, we did not try amplification of the viral vector in this study because we simply intended to clarify the effect of amplification of the proviral DNA, isolated from the effect of amplification of the vector gene.

ACKNOWLEDGMENTS

We thank Richard C. Mulligan for supplying pZipNeoSV(X)1, pMOVΨ⁻ and Ψ2/pZipNeoSV(X)1 and for helpful discussions. We are grateful to H. Karasuyama and E. Gilboa for supplying the BMGNeo and N2 vectors, respectively, and also to Phillip A. Sharp and Kunitada Shimotono for helpful discussions.

REFERENCES

1. **Armentano, D., S.-F. Yu, P. W. Kantoff, T. von Ruden, W. F. Anderson, and E. Gilboa.** 1987. Effect of internal viral sequences on the utility of retroviral vectors. J. Virol. **61**:1647–1650.
2. **Bender, M. A., R. E. Gelinas, and A. D. Miller.** 1989. A majority of mice show long-term expression of a human beta-globin gene after retrovirus transfer into hematopoietic stem cells. Mol. Cell. Biol. **9**:1426–1434.
3. **Bender, M. A., T. D. Palmer, R. E. Gelinas, and A. D. Miller.** 1987. Evidence that the packaging signal of Moloney murine leukemia virus extends into the *gag* region. J. Virol. **61**:1639–1646.
4. **Bodine, D. M., K. T. McDonagh, S. J. Brandt, P. A. Ney, B. Agricola, E. Byrne, and A. W. Nienhuis.** 1990. Development of a high-titer retrovirus producer cell line capable of gene transfer into rhesus monkey hematopoietic stem cells. Proc. Natl. Acad. Sci. USA **87**:3738–3742.
5. **Bosselman, R. A., R.-Y. Hsu, J. Bruszewski, S. Hu, F. Martin, and M. Nicolson.** 1987. Replication-defective chimeric helper proviruses and factors affecting generation of competent virus: expression of Moloney murine leukemia virus structural genes via the metallothionein promoter. Mol. Cell. Biol. **7**:1797–1806.
6. **Campo, M. S.** 1985. Bovine papillomavirus DNA: a eukaryotic cloning vector, p. 213–238. *In* D. M. Glover (ed.), DNA cloning, vol. 2. IRL Press, Oxford.
7. **Cepko, C. L., B. E. Roberts, and R. C. Mulligan.** 1984. Construction and applications of a highly transmissible murine retrovirus shuttle vector. Cell **37**:1053–1062.
8. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7**:2745–2752.
9. **Cone, R. D., and R. C. Mulligan.** 1984. High-efficiency gene transfer into mammalian cells: generation of helper-free recombinant retrovirus with broad mammalian host range. Proc. Natl. Acad. Sci. USA **81**:6349–6353.
10. **Corey, C. A., A. D. DeSilva, C. A. Holland, and D. A. Williams.** 1990. Serial transplantation of methotrexate-resistant bone marrow: protection of murine recipients from drug toxicity by progeny of transduced stem cells. Blood **75**:337–343.
11. **Correll, P. H., J. K. Fink, R. O. Brady, L. K. Perry, and S. Karlsson.** 1989. Production of human glucocerebrosidase in mice after retroviral gene transfer into multipotential hematopoietic progenitor cells. Proc. Natl. Acad. Sci. USA **86**:8912–8916.
12. **Danos, O., and R. C. Mulligan.** 1988. Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. Proc. Natl. Acad. Sci. USA **85**:6460–6464.
13. **Dick, J. E., M. C. Magli, D. Huszar, R. A. Phillips, and A. Bernstein.** 1985. Introduction of a selectable gene into primitive stem cells capable of long-term reconstitution of the hemopoietic system of W/W^v mice. Cell **42**:71–79.
14. **Durnam, D. M., F. Perrin, F. Gannon, and R. D. Palmiter.** 1980. Isolation and characterization of mouse metallothionein-I gene. Proc. Natl. Acad. Sci. USA **77**:6511–6515.
15. **Dzierzak, E. A., T. Papayannopoulou, and R. C. Mulligan.** 1988. Lineage-specific expression of a human beta-globin gene in murine bone marrow transplant recipients reconstituted with retrovirus-transduced stem cells. Nature (London) **331**:35–41.
16. **Eglitis, M. A., P. W. Kantoff, J. D. Jolly, J. B. Jones, W. F. Anderson, and C. D. Lothrop, Jr.** 1988. Gene transfer into hematopoietic progenitor cells from normal and cyclic hematopoietic dogs using retroviral vectors. Blood **71**:717–722.
17. **Gilboa, E., M. A. Eglitis, P. W. Kantoff, and W. F. Anderson.** 1986. Transfer and expression of cloned genes using retroviral vectors. BioTechniques **4**:504–512.
18. **Glanville, N., D. M. Durnam, and R. D. Palmiter.** 1981. Structure of mouse metallothionein-I gene and its mRNA. Nature (London) **292**:267–269.
19. **Goff, S., P. Traktman, and D. Baltimore.** 1981. Isolation and properties of Moloney murine leukemia virus mutants: use of a rapid assay for release of virion reverse transcriptase. J. Virol. **38**:239–248.
20. **Hirt, B.** 1967. Selective extraction of polyoma DNA from

infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
21. **Karasuyama, H., and F. Melchers.** 1988. Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4, or 5, using modified cDNA expression vectors. Eur. J. Immunol. **18**:97–104.
22. **Kantoff, P. W., A. P. Gillio, J. R. McLachlin, C. Bordignon, M. A. Eglitis, N. A. Kernan, R. C. Moen, D. B. Kohn, S.-F. Yu, E. Karson, S. Karlsson, J. A. Zwiebel, E. Gilboa, R. M. Blaese, A. Nienhuis, R. J. O'Reilly, and W. F. Anderson.** 1987. Expression of human adenosine deaminase in nonhuman primates after retrovirus-mediated gene transfer. J. Exp. Med. **166**:219–234.
23. **Karlsson, S., D. M. Bodine, L. Perry, T. Papayannopoulou, and A. W. Nienhuis.** 1988. Expression of human beta-globin gene following retroviral-mediated transfer into multipotential hematopoietic progenitors of mice. Proc. Natl. Acad. Sci. USA **85**:6062–6066.
24. **Keller, G., C. Paige, E. Gilboa, and E. F. Wagner.** 1985. Expression of a foreign gene in myeloid and lymphoid cells derived from multipotent haematopoietic precursors. Nature (London) **318**:149–154.
25. **Laneuville, P., W. Chang, S. Kamel-Reid, A. A. Fauser, and J. E. Dick.** 1988. High-efficiency gene transfer and expression in normal human hematopoietic cells with retrovirus vectors. Blood **71**:811–814.
26. **Lemischka, I. R., D. H. Raulet, and R. C. Mulligan.** 1986. Developmental potential and dynamic behavior of hematopoietic stem cells. Cell **45**:917–927.
27. **Lim, B., J. F. Apperley, S. H. Orkin, and D. A. Williams.** 1989. Long-term expression of human adenosine deaminase in mice transplanted with retrovirus-infected hematopoietic stem cells. Proc. Natl. Acad. Sci. USA **86**:8892–8896.
28. **Lim, B., D. A. Williams, and S. H. Orkin.** 1987. Retrovirus-mediated gene transfer of human adenosine deaminase: expression of functional enzyme in murine hematopoietic stem cells in vivo. Mol. Cell. Biol. **7**:3459–3465.
29. **Lynch, C. M., and A. D. Miller.** 1991. Production of high-titer helper virus-free retroviral vectors by cocultivation of packaging cells with different host ranges. J. Virol. **65**:3887–3890.
30. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Mann, R., R. C. Mulligan, and D. Baltimore.** 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell **33**:153–159.
32. **Markowitz, D., S. Goff, and A. Bank.** 1988. A safe packaging line for gene transfer: separating viral genes on two different plasmids. J. Virol. **62**:1120–1124.
33. **Miller, A. D., and C. Buttimore.** 1986. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol. Cell. Biol. **6**:2895–2902.
34. **Miller, A. D., M.-F. Law, and I. M. Verma.** 1985. Generation of helper-free amphotropic retroviruses that transduce a dominant-acting, methotrexate-resistant dihydrofolate reductase gene. Mol. Cell. Biol. **5**:431–437.
35. **Miller, A. D., D. R. Trauber, and C. Buttimore.** 1986. Factors involved in the production of helper virus-free retrovirus vectors. Somatic Cell. Mol. Genet. **12**:175–183.
36. **Mulligan, R. C., and P. Berg.** 1981. Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase. Proc. Natl. Acad. Sci. USA **78**:2027–2076.
37. **Schimke, R. T. (ed.).** 1982. Gene amplification. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Stead, R. B., W. W. Kwok, R. Storb, and A. D. Miller.** 1988. Canine model for gene therapy: inefficient gene expression in dogs reconstituted with autologous marrow infected with retroviral vectors. Blood **71**:742–747.
39. **Subramani, S., R. C. Mulligan, and P. Berg.** 1981. Expression of the mouse dihydrofolate reductase complementary deoxyribonucleic acid in simian virus 40 vectors. Mol. Cell. Biol. **1**:854–864.
40. **Williams, D. A., I. R. Lemischka, D. G. Nathan, and R. C. Mulligan.** 1984. Introduction of new genetic material into pluripotent haematopoietic stem cells of the mouse. Nature (London) **310**:476–480.
41. **Yang, Z., A. J. Korman, J. Cooper, D. Pious, R. S. Accolla, R. C. Mulligan, and J. L. Strominger.** 1987. Expression of HLA-DR antigen in human class II mutant B-cell lines by double infection with retrovirus vectors. Mol. Cell. Biol. **7**: 3923–3928.