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The pCL Vector System: Rapid Production of Helper-Free, High-Titer, Recombinant Retroviruses

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We describe the construction and characterization of retroviral vectors and packaging plasmids that produce helper-free retrovirus with titers of 1×10^6 to 5×10^6 within 48 h. These vectors contain the immediate early region of the human cytomegalovirus enhancer-promoter fused to the Moloney murine leukemia virus long terminal repeat at the TATA box in the 5′ U3 region, yielding the pCL promoter. By selecting vectors designed to express genes from one of four promoters (dihydrofolate reductase, Rous sarcoma virus, long terminal repeat, or cytomegalovirus), the pCL system permits the investigator to control the level of gene expression in target cells over a 100-fold range, while maintaining uniformly high titers of virus from transiently transfected producer cells. The pCL packaging plasmids lack a packaging signal ($\Delta\Psi$) and include an added safety modification that renders them self-inactivating through the deletion of the 3′ U3 enhancer. Ecotropic, amphotropic (4070A), and amphotropic–mink cell focus-forming hybrid (10A1) envelope constructions have been prepared and tested, permitting flexible selection of vector pseudotype in accordance with experimental needs. Vector supernatants are free of helper virus and are of sufficiently high titer within 2 days of transient transfection in 293 cells to permit infection of more than 50% of randomly cycling target cells in culture. We demonstrated the efficacy of these vectors by using them to transfer three potent cell cycle control genes (the $p16^{INK4A}$, p53, and Rb1 genes) into human glioblastoma cells.

Production, cloning, and characterization of helper-free retrovirus vectors take about 2 months when conventional murine retrovirus packaging cell lines are used (19). A number of transient-transfection systems have been developed to speed this process. These have permitted the production of useful quantities of virus within 2 days after transfection of 3T3 (21), COS (13), or 293 (6, 24, 27) cells. The pCL vectors have been designed to maximize recombinant-retrovirus titers in a simple, robust, and flexible experimental system. These vectors permit the expression of a number of cell cycle-regulatory proteins that are otherwise difficult to study in retroviruses because of their potent cytostatic or cytocidal effects. The pCL vectors are also well suited for producing retrovirus vectors pseudotyped in viral envelope glycoproteins that may be toxic to human cells when constitutively expressed at high levels, such as the murine leukemia virus (MLV) amphotropic envelope and vesicular stomatitis virus G protein.

Figure 1 illustrates the design features of the pCL expression vectors. The pCL vectors were cotransfected with the pCL-Eco packaging construct (see Fig. 2) into 293 cells by using a modification of the HEPES-buffered saline calcium phosphate method (10). The medium was replaced 18 h after transfection. Supernatants were collected after 2 days and filtered through a 0.45-μm-pore-size filter, and 1 μl to 1 ml was used to infect NIH 3T3 cells in 8 μg of polybrene per ml. One day after infection the cells were split 1 to 20. Two days after infection they were either placed under G418 selection (800 μg/ml) and grown for 10 days or fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (26). Infection and selection in G418-containing medium were by standard methods (19). Titers for Neo-containing vectors were expressed as numbers of G418-resistant CFU per milliliter. Titers for pCLMFG-LacZ were determined by infecting 10^6 cells with 0.1 ml of pCL supernatant in 10 ml of medium. Target cells were fixed and stained 2 days after infection. Multiplicity of infection was calculated as −ln (uninfected fraction of cells), derived from Poisson statistics (ln = $\log_e$). The virus titer was calculated as follows: (multiplicity of infection $\times 10^6$ cells)/0.1 ml, expressed as infectious units per milliliter. Thirty-five to fifty percent of NIH 3T3 cells were typically infected (stained blue) under these conditions.

Figure 2 illustrates the design of the pCL packaging constructs expressing ecotropic, amphotropic, and 10A1 envelope proteins. We found that the principal limitation to the use of existing retrovirus-packaging cell lines (which are all derived from NIH 3T3 cells) for transient-retrovirus production was their poor and variable transfection efficiencies. Transfection efficiency averaged about 0.5%, but varied over a 500-fold range (0.01 to 5%) from experiment to experiment, when identical methods, DNA, and reagents were used (Table 1). We tested transient-virus production in six murine retrovirus-packaging cell lines by transfecting each with the conventional, safety-modified murine retrovirus vector LXSN (20). Supernatants were harvested 2 days after transfection, and titers were determined by infecting NIH 3T3 cells and selecting for G418 resistance. Figure 3 shows that the mean virus titers from transiently transfected packaging cell lines was 0.3×10^3 to 1×10^3 CFU/ml, with large variations that correlated with variations in transfection efficiency.

We next tested the suitability of CV1, COS, and 293 cells for transient-virus production by cotransfecting LXSN and the ecotropic packaging construct SV-Ψ^--E-MLV (13) (Fig. 3). Of

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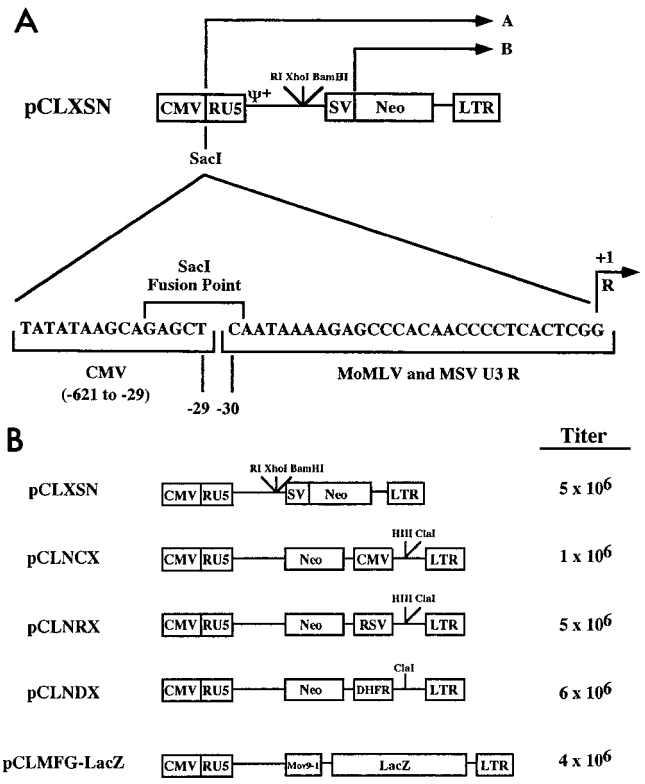

FIG. 1. pCL vector design. (A) Parental vector construction. A 3.1-kb fragment of LXSN (20), spanning from the *Sac*I site in the 5′ LTR near the U3-R junction to the *Acc*I site just beyond the 3′ LTR, was cloned into the 4.5-kb CMV expression construct, CMX-low, at a corresponding *Sac*I site (−29) within the CMV immediate early enhancer-promoter. This produced the parental vector pCLXSN, from which all other pCL vectors were derived. Cloning details including nucleotide sequences are available upon request. SV, simian virus 40; MoMLV, Moloney MLV; MSV, murine sarcoma virus; RI, *Eco*RI. (B) Construction and infectious titers of the five pCL expression vectors. pCLNCX was derived from LNCX (20) by partial digestion with *Sac*I. pCLNRX and pCLNDX were produced by removing the CMV promoter from pCLNCX with *Bam*HI and *Hin*dIII (HIII) and substituting the 0.3-kb Rous sarcoma virus (RSV) LTR and 0.3-kb dihydrofolate reductase (DHFR) promoters, respectively. The possible cloning sites for the second gene, driven by CMV, RSV, or dihydrofolate reductase, are also shown. pCLMFG-LacZ was derived in three subcloning steps from MFG-LacZ (provided by Richard Mulligan). Cloning details are available upon request.

these three cell lines, the adenovirus-transformed, E1A-expressing, human embryonic kidney cell line, 293 (9), consistently showed the highest transfection efficiencies (30 to 50%) (Table 1). Vector titration and immunoprecipitation experiments showed that under typical conditions, viral proteins were produced in a 20-fold stoichiometric excess, and the abundance of packageable viral RNA (and not viral proteins) was the principal determinant of recombinant-retrovirus titer (data not shown). In designing the chimeric pCL promoter, we exploited two natural features of 293 cells: (i) high transfectability and (ii) strong E1A-mediated stimulation of cytomegalovirus (CMV)-programmed transcription. To improve retroviral RNA transcription in 293 cells, we deleted the 5′ enhancer of the Moloney murine sarcoma virus long terminal repeat (LTR) from LXSN (which is inhibited by E1A) and fused it at the TATA box to the enhancer of the human CMV immediate early region (which is stimulated by E1A). The resulting viral RNA is predicted to initiate at or near the +1 position in the R region of the naturally programmed retrovirus RNA. This configuration resulted in transient-retrovirus titers consistently in the range of 2×10^6 to 5×10^6 CFU/ml (Fig. 3). In contrast, the titers of conventional murine retroviral vectors with unmodified 5′ LTRs were 2×10^4 to 4×10^4 CFU/ml when the vectors were transfected into 293 cells (Fig. 3).

We found that pCL virus titers did not drop as we increased the size of the included cDNAs from 0.7 kb (chloramphenicol acetyltransferase gene; 4×10^6) to 3.0 kb (*lacZ*; 6×10^6) (Fig. 1B). The titers of non-Neo-containing vectors, such as pCLMFG-LacZ, were measured by direct infection of randomly cycling cells with pCL supernatant as described above. Early studies revealed the presence of a cytostatic factor(s) in the undiluted supernatants of 293 and COS cells transfected with either vector or control plasmid (pBS) DNA. This factor(s) reduced the number of mitoses in NIH 3T3 cells, changed their morphology to a flatter, more quiescent phenotype, and caused them to accumulate refractile perinuclear granules (unpublished observations). Because murine retrovirus vectors can infect only dividing cells, this factor(s) reduced apparent titers and led to the paradoxical observation that fewer cells were infected when more undiluted supernatant was used for infection. We overcame this problem in two ways. First, by changing the medium 18 h after transfection of 293 cells and collecting virus supernatant 36 to 48 h after transfection, we were able to reduce the cytostatic effects of the medium while maintaining excellent recovery of infectious virus. Second, we avoided the use of virus supernatant more concentrated than a one-to-one (equal volume) mix with the medium of cells to be infected. Under these conditions, typically 65% of randomly cycling human glioblastoma cells and 80 to 90% of NIH 3T3 cells could be infected. We next optimized the cotransfection stoichiometry of the pCL vectors and packaging constructs in 293 cells and found that a combination of 10 μg (2.0 pmol) of vector and 10 μg (1.2 pmol) of packaging construct per 10^6 cells on a 6-cm dish produced optimum titers of infectious retrovirus.

Intracellular amplification of transfected plasmid DNA did not result in further increases in virus titer. A 100-fold amplification of the pCL DNAs (which contain the simian virus 40 origin in the backbone) was achieved by cotransfecting a simian virus 40 large-T-antigen expression construct into 293 cells. This maneuver increased the vector DNA copy number from a basal level of 10,000 copies per cell to an amplified level of 1.1×10^6 copies per cell (quantitated by Southern blot analysis) but did not increase the titer of infectious virus (data not shown). Timed immunoprecipitations after metabolic labeling for 3 h revealed that despite a 100-fold amplification of vector DNA, viral protein production was increased only about 2-fold. In addition, the processing of envelope glycoprotein in amplified cells was delayed. Ecotropic envelope glycoprotein appeared predominantly as a variant form that migrated at a position in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels slightly above the position of normally processed gp70 but below the position of unprocessed gp85 (data not shown). Parallel labeling and immunoprecipitation of unamplified cells showed only normally processed gp70 and p15(E). These observations led us to the conclusion that the glycoprotein biosynthesis and processing of 293 cells cotransfected with large T antigen and the pCL vectors had proceeded to capacity.

We tested for the presence of helper virus in the supernatants of 293 cells transiently transfected with pCL vectors and packaging constructs in four ways: (i) XC assay for ecotropic replication-competent retroviruses (RCR) (25), (ii) S^+L^- assay for amphotropic RCR (18), (iii) *lacZ* marker rescue for ecotropic, amphotropic, and mink cell focus-forming RCR (3, 5), and (iv) SC1 amplification-reverse transcriptase assay for

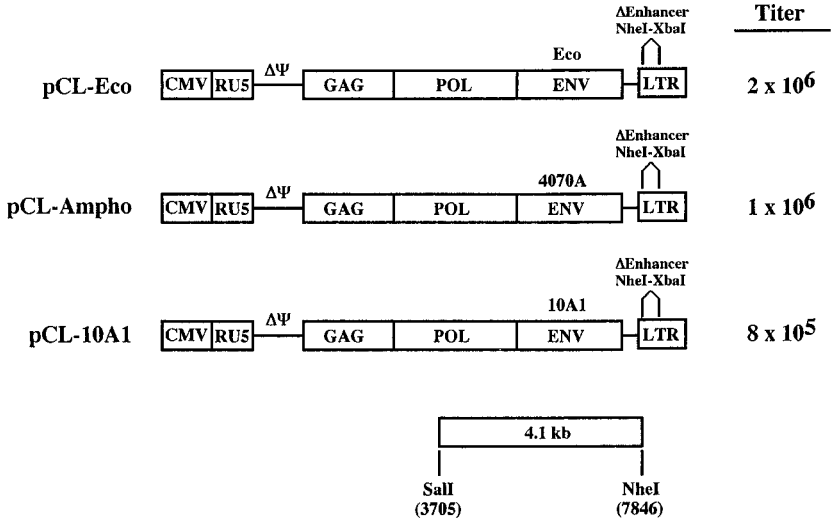

FIG. 2. pCL packaging constructs. A non-SIN version of pCL-Eco was derived from SV-Ψ⁻-E-MLV (13), which was derived from the ΔΨ Moloney MLV genome pMOV-Ψ⁻ (14). It contained a deleted packaging signal from the *Bal*I to the *Pst*I site (212 to 563) and a new *Hin*dIII site that was generated at the point of deletion. pCL-Eco was made SIN by removing the 4.1-kb *Sal*I-to-*Nhe*I (*pol-env*-IN) fragment and subcloning it back into its parent cleaved with *Sal*I and *Xba*I, utilizing the compatible ends generated by *Nhe*I and *Xba*I. This maneuver deleted 267 bp (from 7846 to 8113) in U3, encompassing the entire 3′ LTR enhancer, and rendered the genome transcriptionally inactive upon reverse transcription (30). pCL-Ampho and pCL-10A1 were created by substituting the 4.1-kb *Sal*I-to-*Nhe*I fragment from pAM (18) and pB6-10A1 (23) into the corresponding region of pCL-Eco (non-SIN) cut with *Sal*I and *Xba*I. The pCL packaging constructs were cotransfected into 293 cells with pCLXSN as described in the text. Supernatants were collected after 2 days and used to infect NIH 3T3 cells. Titers were expressed as numbers of G418^r CFU per milliliter.

ecotropic, amphotropic, and mink cell focus-forming RCR detection (3). No helper virus was detected by any of these methods in three independent transfections with the pCL system vectors (Table 2). The sensitivity of the SC1 amplification-reverse transcriptase assay in our hands was 1 to 10 infectious units/ml, as determined by limiting dilution of Moloney MLV in parallel XC assays.

We tested the efficacy of the pCL system by expressing three potent cell cycle-regulatory proteins. We chose $p16^{INK4A}$ (22), Rb1, and p53 for this purpose. Genes with cytostatic or cytocidal phenotypes are particularly difficult to study with conventional retrovirus vectors because the growth-suppressing activity of these genes severely limits the expansion and selection of stable packaging cell lines. We cloned the cDNAs for these three cell cycle-regulatory genes into pCLXSN, produced retrovirus stocks by transiently transfecting 293 cells, and used the resulting recombinant retrovirus supernatants to infect human glioblastoma cells in culture. We then performed immunoprecipitations 2 days after infection (without G418 selection) to test the efficiency of gene expression in the infected human glioblastoma cells. High levels of p53, Rb1, and p16 proteins were expressed in each case (Fig. 4) and resulted in potent cell cycle arrest (4).

The pCL vector system was designed to exploit two natural features of 293 cells: (i) high-level transfectability and (ii) strong E1A-mediated stimulation of CMV-programmed transcription. This configuration maximized virus titers by producing very high levels of packageable RNA in transiently transfected cells, while minimizing promoter interference and permitting the natural reconstitution of the retroviral LTR upon reverse transcription and integration into the genomic DNA of infected cells. Moreover, because 293 cells are of nonmurine origin, the problem of selective packaging and inadvertent transfer of VL30 genomes (present in all murine packaging cells) was avoided. We have shown that the retrovirus titers obtained after transient transfection with this system are 100 times higher than those achieved after transfecting 293 cells with conventional retrovirus vectors and packaging constructs (Fig. 3). Virus titers obtained 48 h after transient transfection were typically 2×10^6 to 5×10^6 CFU/ml. These compared favorably with the best titers observed from stably infected, cloned packaging cell lines, requiring several months of selection and characterization. All four members of the pCL expression vector family (pCLXSN, pCLNCX, pCLNRX, and pCLNDX) (Fig. 1B) have an extended packaging signal (Ψ^+) (1) and were derived from safety-modified retrovirus vectors in which the *gag* open reading frame has been closed by either a point mutation (20) or a frame shift mutation (pCLMFG-LacZ) (21a), thereby minimizing the opportunity for RCR production by recombination with packaging genomes. All three members of the pCL packaging plasmid family (pCL-Eco, pCL-Ampho, and pCL-10A1) (Fig. 2) have been safety engineered by deletion of the packaging signal and the 3′ LTR enhancer. These mutations render the RNAs of pCL helper

TABLE 1. Transient-transfection efficiencies

Cell type (reference)	Efficiency[a] (%)
293 (19)	30–50
Bosc23 (24)	30–50
COS (7)	5–15
CV1 (7)	2–15
Psi-2 (14)	0.1–2
AM12 (16)	0.04–2
CRIP4[b] (5)	0.1–2
E86 (15)	0.2–2
PA317 (17)	0.01–1
CRE (5)	0.01–1

[a] n = 3 to 6. Cells were transfected with RSV-*lacZ* by the HEPES-buffered saline calcium phosphate method (10) and quantitated by fixation and staining with X-Gal (26).

[b] Derived from ΨCRIP by subcloning. Fifty clones were picked and grown in mycophenolic acid and hygromycin, and the clone producing the highest titers of transient virus was selected.

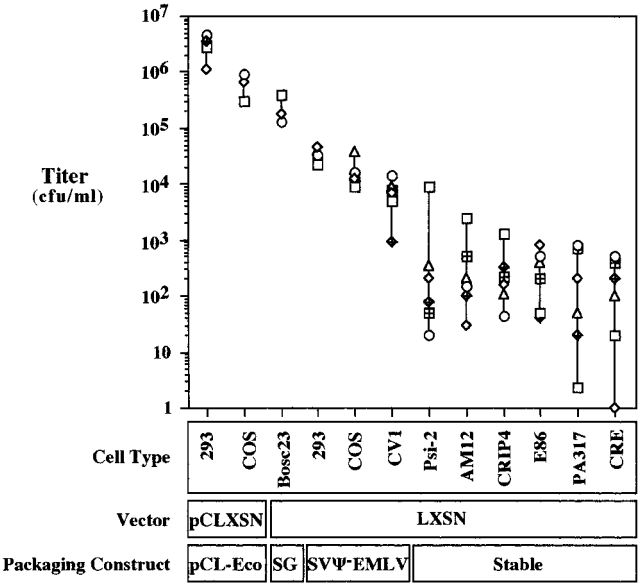

FIG. 3. Transient-retrovirus titers. Six stable murine retrovirus packaging cell lines were tested for transient-virus production by transfection with LXSN (20). Different symbols represent results from separate experiments. Supernatants were collected after 2 days and used to infect NIH 3T3 cells as described in the text. CV1, COS, and 293 cells were tested by cotransfecting LXSN with the packaging construct SV-Ψ⁻-E-MLV (13). Bosc23 is a split-genome (SG), ecotropic murine retrovirus-packaging cell line constructed in 293 cells (24). It was tested by transfection with LXSN. COS and 293 cells were also tested by cotransfection with pCLXSN and pCL-Eco. Titers were expressed as G418^r CFU per milliliter.

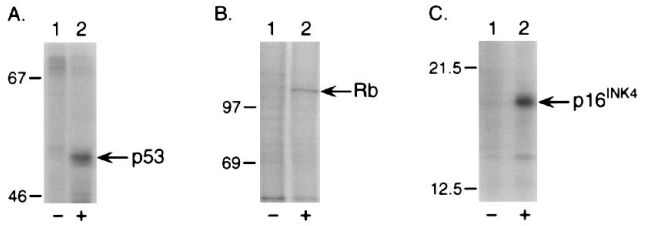

FIG. 4. High-efficiency transfer of cell cycle-regulatory genes into human glioblastoma cells. (A) SF126 human glioblastoma cells were infected with empty pCLXSN vector (lane 1) or pCLXSN-p53 (lane 2). Two days after infection, the cells were labeled for 4 hours with [^{35}S]methionine (150 μCi/ml) and immunoprecipitated with monoclonal antibody 1620 (Oncogene Science). Proteins were resolved by SDS-PAGE and visualized by fluorography overnight. (B) SF126 cells were infected with pCLXSN vector (lane 1) or pCLXSN-pRb (lane 2). Expression was analyzed by immunoprecipitation with monoclonal antibody PMG3-245 (Pharmingen). (C) SF126 cells were infected with pCLXSN (lane 1) or pCLXSN-p16^{INK4A} (lane 2). Expression was analyzed by immunoprecipitation with polyclonal rabbit antiserum to p16^{INK4A} provided by David Beach.

genomes virtually unpackageable and self-inactivating (SIN) (30). The pCL packaging plasmids express high levels of *gag*, *pol*, and *env* proteins with a balanced stoichiometry not easily achieved with either transiently or stably expressed split-genome (nonsplicing) packaging constructs. The choice of vectors expressing ecotropic, amphotropic, or 10A1 envelopes permits great experimental flexibility. All supernatants were free of helper virus when measured by four different methods, including the most sensitive SC1 amplification-reverse transcriptase assay (Table 2). The pCL vector system is well suited for rapid and efficient construction of retrovirus cDNA expression libraries, as it avoids genetic bottlenecks and ping-pong amplification steps that impose limitations on current systems that rely on stable retrovirus packaging cell lines (11, 29). In addition, the high levels of packageable RNA produced by the pCL vectors make them well suited for shortening the time required to produce high-titer, pantropic retrovirus vectors (2), from 2 months to potentially less than a week. The simplicity, reliability, and efficiency of the pCL system make it a convenient and rapid alternative for the production of helper-free, high-titer recombinant retroviruses.

TABLE 2. pCL helper assays

pCLXSN supernatant	Result of assay			
	XC (FFU/ml)[a]	S$^+$L$^-$[b] (FFU/ml)	*lacZ* marker rescue[c]	SC1[d] (cpm/10 μl)
pCL-Eco	<10	ND	Neg.	110
pCL-Ampho	ND[e]	<10	Neg.	125
pCL-10A1	ND	<10	Neg.	92
Mock	<10	<10	Neg.	112
MoMLV[f]	2×10^6	ND	++++	52,144
1504A[g]	ND	5×10^4	+++	1,865

[a] One-milliliter dilutions were used to infect 5×10^4 NIH 3T3 cells, which were cultured 4 days until just confluent, UV irradiated, overlaid with 1×10^6 XC cells, and cultured another 4 days (25). FFU, focus forming units.

[b] One-milliliter dilutions were used to infect 10^5 CCC-81 (cat) cells, cultured 2 days, then overlaid with 10^6 NRK cells, and cultured another 4 days (18).

[c] One-milliliter dilutions were used to infect 1×10^5 TKDL2 3T3 cells (which carry a rescuable *lacZ* provirus) and cultured 2 days, and the supernatant was used to infect 2×10^5 NIH 3T3 cells. Neg., no blue cells; +, relative no. of blue cells.

[d] One milliliter of filtered medium was used to infect 2×10^5 SC1 cells (12). Cells were cultured with three passages over 1 week in the presence of 8 μg polybrene per ml and then assayed for reverse transcriptase activity (3, 8). Results are expressed in counts per minute per 10 μl of culture supernatant.

[e] ND, not done.

[f] Moloney MLV (MoMLV) (ecotropic) helper virus was produced from a clone of SC1 cells (146E7).

[g] Amphotropic MLV helper virus was produced from SC1 cells.

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REFERENCES

1. **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.
2. **Burns, J. C., T. Friedmann, W. Driever, M. Burrascano, and J.-K. Yee.** 1993. VSV-G pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and non-mammalian cells. Proc. Natl. Acad. Sci. USA **90:**8033–8037.
3. **Cornetta, K., N. Nguyen, R. A. Morgan, D. D. Muenchau, J. W. Hartley, R. M. Blaese, and W. F. Anderson.** 1993. Infection of human cell with murine amphotropic replication-competent retroviruses. Hum. Gene Ther. **4:**579–588.
4. **Costanzi, E., B. E. Strauss, R. K. Naviaux, and M. Haas.** Restoration of growth arrest by p16^{INK4A}, p21^{WAF1}, pRb, and p53 is dependent on the integrity of the endogenous cell-cycle control pathways in human glioblastoma cell lines. Submitted for publication.
5. **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.
6. **Finer, M. H., T. J. Dull, L. Qin, D. Farson, and M. R. Roberts.** 1994. *Kat*: a high efficiency retroviral transduction system for primary human T lymphocytes. Blood **83:**43–50.
7. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of

early SV40 mutants. Cell **23:**175–182.
8. **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.
9. **Graham, F. L., J. Smiley, W. C. Russel, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36:**59–72.
10. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52:**456–467.
11. **Gudkov, A. V., A. R. Kazarov, R. Thimmapaya, S. A. Axenovich, I. A. Mazo, and I. B. Roninson.** 1994. Cloning mammalian genes by expression selection of genetic suppressor elements: association of kinesin with drug resistance and cell immortalization. Proc. Natl. Acad. Sci. USA **91:**3744–3748.
12. **Hartley, J. W., and W. P. Rowe.** 1975. Clonal cell lines from a feral mouse embryo which lack host-range restrictions for murine leukemia viruses. Virology **65:**128–134.
13. **Landau, N. R., and D. R. Littman.** 1992. Packaging system for rapid production of murine leukemia virus vectors with variable tropism. J. Virol. **66:**5110–5113.
14. **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.
15. **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.
16. **Markowitz, D., S. Goff, and A. Bank.** 1988. Construction and use of a safe and efficient amphotropic packaging cell line. Virology **167:**400–406.
17. **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.
18. **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.
19. **Miller, A. D., D. G. Miller, J. V. Garcia, and C. Lynch.** 1993. Use of retroviral vectors for gene transfer and expression. Methods Enzymol. **217:**581–599.
20. **Miller, A. D., and G. J. Rosman.** 1989. Improved retroviral vectors for gene transfer and expression. BioTechniques **7:**980–990.
21. **Morgenstern, J. P., and H. Land.** 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary, helper-free packaging cell line. Nucleic Acids Res. **18:**3587–3596.

21a. **Mulligan, R.** Personal communication.

22. **Okamoto, A., D. J. Demetrick, E. A. Spillare, K. Hagiwara, S. P. Hussain, W. P. Bennet, K. Forrester, B. Gerwin, M. Serrano, D. Beach, and C. C. Harris.** 1994. Mutations and altered expression of $p16^{INK4A}$ in human cancer. Proc. Natl. Acad. Sci. USA **91:**11045–11049.
23. **Ott, D., R. Friedrich, and A. Rein.** 1990. Sequence analysis of amphotropic and 10A1 murine leukemia viruses: close relationship to mink cell focus-inducing viruses. J. Virol. **64:**757–766.
24. **Pear, W. S., G. P. Nolan, M. L. Scott, and D. Baltimore.** 1993. Production of high-titer helper-free retroviruses by transient transfection. Proc. Natl. Acad. Sci. USA **90:**8392–8396.
25. **Rowe, W. P., W. E. Pugh, and J. W. Hartley.** 1970. Plaque assay techniques for murine leukemia viruses. Virology **42:**1136–1139.
26. **Sanes, J. R., J. L. R. Rubenstein, and J.-F. Nicolas.** 1986. Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. EMBO J. **5:**3133–3142.
27. **Soneoka, Y., P. M. Cannon, E. E. Ramsdale, J. C. Griffiths, G. Romano, S. M. Kingsman, and A. J. Kingsman.** 1995. A transient three-plasmid expression system for the production of high titer retroviral vectors. Nucleic Acids Res. **23:**628–633.
28. **Svoboda, J.** 1961. The tumorigenic action of Rous sarcoma in rats and the permanent production of Rous virus by the induced rat sarcoma XC. Folia Biol. (Prague) **7:**46–60.
29. **Wong, B. Y., H. Chen, S.-W. Chung, and P. M. C. Wong.** 1994. High-efficiency identification of genes by functional analysis from a retroviral cDNA expression library. J. Virol. **68:**5523–5531.
30. **Yu, S.-F., T. von Rüden, P. W. Kantoff, C. Garber, M. Seiberg, U. Rüther, W. F. Anderson, E. F. Wagner, and E. Gilboa.** 1986. Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells. Proc. Natl. Acad. Sci. USA **83:**3194–3198.