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Gene Transfer to Human Cells Using Retrovirus Vectors Produced by a New Polytropic Packaging Cell Line

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We report here the construction of a new packaging cell line, called MPAC, that packages defective retroviral vectors in viral particles with envelope proteins derived from a Moloney mink cell focus-inducing (MCF) polytropic virus. We characterized the tropism of MPAC-packaged retroviral vectors and show that some human cell lines can be infected with these vectors while others cannot. In addition, we show that some human cells fully support MCF virus replication while others either partially or fully restrict MCF virus replication.

The utility of retroviral vectors for transferring genes to cells is determined by the presence or absence of cell surface receptors for the virus on the target cell and by postpenetration events that affect viral replication. Receptor interference studies show that ecotropic, amphotropic, xenotropic, and polytropic murine leukemia viruses each utilize distinct cell surface receptors (6, 7, 29–31, 34). Packaging cell lines that package defective retroviral vectors into ecotropic (23) and amphotropic (8, 24, 25) envelope proteins have been developed and extensively characterized by using murine leukemia viruses. Although construction of xenotropic virus packaging (12) and Friend polytropic virus packaging (26) cell lines have been reported, they have not been extensively characterized or utilized in the published literature. Murine leukemia virus-based packaging cell lines have been used for tracking the differentiation of hematopoietic lineages (9, 13, 18, 21, 27, 33, 35), identifying viral receptors on specific cell types (12, 26, 37), and gene transfer (8, 19, 22–25, 32, 36). To aid in our studies of retroviral pathogenesis by mink cell focus-inducing (MCF) viruses, we generated a cell line that packages retroviral vectors in MCF virus envelope proteins and characterized the ability of MCF virus envelope packaged retroviral vectors to infect human cells.

In order to generate an MCF virus envelope packaging cell line, we constructed a virus-derived genome that expresses MCF virus envelope glycoproteins, produces all the retroviral proteins necessary for retroviral assembly, and yet is defective in packaging its own genome. We used the plasmid pPAM3, constructed by Miller and Buttimore, to form the backbone of the defective viral genome (24) (Fig. 1A). A 6.1-kb fragment containing most of the gene for the MCF virus envelope (including all of gp70-SU and most of p15E-TM) as well as the polymerase gene (*pol*), a portion of the capsid gene (p30-CA) and all of the nucleocapsid gene (p10-NC) was isolated from pMo-MCF$_{1-1}$ (4) and used to replace the amphotropic envelope gene in pPAM3. The resulting plasmid was named pMPAC. The integrity of the clone was verified by digestion with *Hin*dIII, *Eco*RI, and *Pst*I restriction enzymes. pMPAC retains the 5′ long terminal repeat (LTR), the splice donor site, the p15-matrix gene, the p12 gene, the 5′ portion of the p30-CA gene, the 3′ portion of the envelope p15E-TM gene, and the poly(A) addition site from pPAM3. The 3′ portion of p30-CA, all of p10-NC, the polymerase gene, the envelope (gp70) gene, and the 5′ portion of the p15E-TM gene, are derived from pMo-MCF$_{1-1}$ as shown in Fig. 1A.

To generate the packaging cell line, the defective virus-derived genome of pMPAC was excised from the plasmid vector and cotransfected (19 μg) with pSV2his (1 μg) into NIH 3T3 cells. Transfected cells were selected with 5 mM histidinol. After 10 days of selection with histidinol, isolated histidinol-resistant colonies were clearly visible on the plates. Thirty of these colonies were isolated with cloning cylinders and expanded into separate cultures.

To identify cell lines producing high titers of retroviral vectors, a reverse transcriptase (RT) assay was performed on media (supernatant) collected from confluent cultures. Three lines (MPAC-91, MPAC-95, and MPAC-2433) that produced high levels of RT ($>10^5$ cpm/ml) were transfected with pLXSH, a retroviral vector containing a hygromycin resistance (Hygro^r) gene (25a). Transfected cells were selected with hygromycin (70 μg/ml), cloned, and expanded. A viral stock was isolated from each transfected cell line and titers were determined on NIH 3T3 cells. One clone of the packaging line, MPAC-91, consistently produced titers of virus higher than those produced by other lines (0.5×10^6 to 1×10^6 Hygro^r CFU/ml). We separately transfected other defective retroviral vectors that contained selectable or identifiable markers, including pBAG (containing the beta-galactosidase [β-Gal] gene) (28), pDAP (containing an alkaline phosphatase gene) (10), and pZIP-SV(X)Neo (containing a neomycin resistance gene which confers resistance to G418) (5), into our packaging cell lines. We have grown BAG(MPAC-91) cells in the Cellmax artificial capillary cell culture system and generated MPAC-packaged pBAG viral stocks with titers of 5×10^6 CFU/ml. Therefore, the MPAC cell line functions as a packaging line and can package a variety of retroviral vectors into retroviral particles.

To determine if the MPAC cell line produced replication-competent viruses, two tests were performed. Two 100-mm-diameter plates of Mv-1-Lu cells were infected with virus harvested from ZIP(MPAC-91) cells (multiplicity of infection [MOI] = 1.2). As a control, two parallel plates were infected with the replication-competent virus MCF 247 (3 ml/plate). The cells were cultured separately for 4 weeks at 1×10^6 to 4×10^6 cells per dish. Supernatants (1-ml aliquots) were harvested

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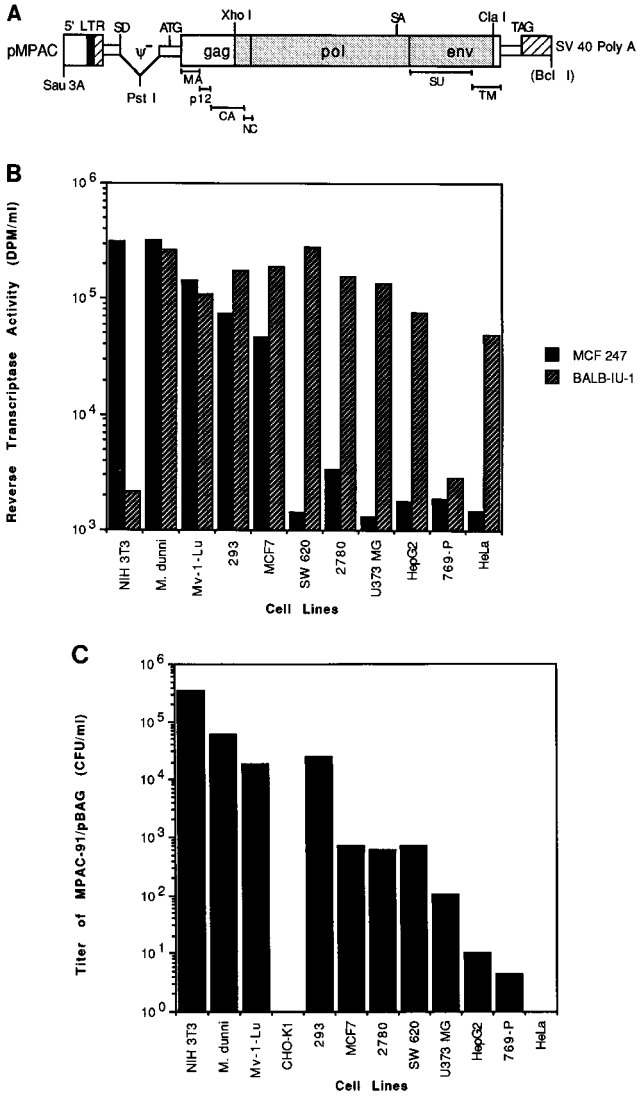

FIG. 1. Construction and characterization of an MCF-virus packaging line. (A) Insert in the plasmid pMPAC. In white are the regions of the genome derived from pPAM3, including the 5′ LTR, the splice donor (SD), the start site (ATG), a portion of the *gag* and *env* genes, the termination site (TAG), and the simian virus 40 (SV 40) poly(A) signal (hatched boxes). The restriction enzyme sites (*Xho*I and *Cla*I) shared between pPAM3 and pMo-MCF_{1-1} were used to introduce the envelope gene as well as other sequences derived from pMo-MCF_{1-1} (4). Sequences derived from pMo-MCF_{1-1} are stippled. (B) The RT activity of media collected from infected cells 4 weeks after infection with MCF 247 or BALB-IU-1 was determined in duplicate as previously described (2, 3). The total number of disintegrations per minute per milliliter of supernatant minus the background is plotted. To determine the background, one aliquot of the RT assay mix was spotted onto filters, dried, washed three times in 5% trichloroacetic acid, dried with 95% ethanol, and counted with scintillation fluid on a Beckman scintillation counter. This background ranged from 0.9×10^3 to 1.0×10^3 dpm, depending on the experiment, and was subtracted from all samples. In addition, the background for each uninfected cell line was determined by assaying 1 ml of supernatant from uninfected cells for RT activity. This background ranged from 1.4×10^3 to 4.4×10^3 dpm, depending on the cell line and the experiment. (C) The titer of BAG(MPAC-91) was determined by separately infecting the indicated cell lines with 10-fold serial dilutions of BAG(MPAC-91). Infections were performed as described previously (2). After 48 h, cells were fixed with 2% paraformaldehyde–2% gluteraldehyde in phosphate-buffered saline and stained for β-Gal activity for 24 h with a solution containing 1 mg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml, 5 mM potassium ferric cyanide, 5 mM potassium ferrous cyanide, and 2 mM magnesium chloride in phosphate-buffered saline. Monolayers were scored microscopically (with a Zeiss Axiovert microscope) as positive when the cytoplasm of the cell was uniformly blue. Uninfected cells of the same type were stained and used as negative controls. BAG(MPAC-91)-infected NIH 3T3 cells were used as a positive control.

from each infection and assayed for RT activity. RT assays of the ZIP(MPAC-91) infections were consistently negative, while cultures infected with MCF 247 were consistently positive. In a second test, NIH 3T3 and *Mus dunni* cells were infected with BAG(MPAC-91) virus (MOI = 1). The cells were cultured for 2 weeks as described above. After culture, supernatant was harvested from the cultures and used for an RT assay (1 ml) or to infect fresh NIH 3T3 and *M. dunni* cells (3 ml each). Two days after infection, the NIH 3T3 and *M. dunni* cells were fixed and stained for β-Gal activity. No blue cells were detected, and the RT assays were negative. These results suggest that the generation of replication-competent virus in the MPAC line is a relatively rare event.

To determine the tropism of MPAC-91-packaged vectors, the ability of BAG(MPAC-91) to infect cell lines of different species was compared to that of MCF 247, the prototypical MCF virus (15–17) (Table 1). In every cell line tested, when β-Gal-positive cells were detected after BAG(MPAC-91) infection, RT activity was detected in parallel cultures infected with MCF 247. Cell lines that became β-Gal positive were consistently able to support MCF virus replication and to produce RT activity, suggesting that the cells could support both MCF virus receptor-mediated infection and replication of the virus. We conclude that the tropism of BAG(MPAC-91) was identical to that of MCF 247 in every cell line tested.

Reports on replication of MCF viruses in human cells are relatively rare. Fischinger et al. (11) reported that HIX, a biological MCF isolate, could infect human embryonic muscle skin cells. However, the titer of the virus was not reported. Cloyd et al. (7) tested 11 different MCF isolates and reported that 9 did not replicate in a human embryonic lung cell line (IMR-90) and that 2 replicated 2,000 to 5,000 times less efficiently than in a mink cell line (Mv-1-Lu). Hartley et al. (15) tested nine MCF strains and reported that none infected a human rhabdosarcoma line (RD). Studies by Sommerfelt and Weiss (34) established that there are seven distinct receptor groups for C-type and D-type retroviruses on human cells, but polytropic MCF viruses were not examined. Therefore, we decided to explore the ability of MCF virus envelope-packaged vectors to infect human cells, since published reports of MCF virus infection of human cells needed to be extended and expanded.

We separately infected six additional human cell lines with MCF 247 and a xenotropic virus, BALB-IU-1 (14), as well as two mouse and one mink cell lines as controls. After at least eight passages and 3 to 4 weeks of culture, supernatants were collected and RT levels were measured (Fig. 1B). Three human cell lines (293, MCF7, and 2780) supported both MCF 247 and BALB-IU-1 replication, as indicated by an RT value at least fivefold over background. Using the same criterion, four human cell lines (SW620, U-373 MG, HepG2, and HeLa) supported BALB-IU-1 replication but restricted the replication of MCF 247. The human cell line 769-P restricted replication of both MCF 247 and BALB-IU-1. These data were confirmed and extended by infecting cultures of the same cell lines with BAG(MPAC-91) and, 48 h later, staining to detect the expression of β-Gal (Fig. 1C). HeLa and CHO-K1 cells as well as uninfected cells of each type were included as negative controls. The results of this experiment confirm the results with replication-competent MCF 247 and suggest that the restriction in replication of MCF 247 in some cell lines (e.g., SW620) is more apparent with replication-competent virus than with the defective vector. One explanation for this could be differential transcription of the vector and the virus in infected cells. This is possible, since the LTR and other control elements of

TABLE 1. Tropism of MCF viruses

Cell line	Species	Cell type	RT activity (dpm/ml) of cells infected with MCF 247[a]	β-Gal activity (CFU/ml) of cells infected with BAG(MPAC-91)[b]
NIH 3T3	Mouse	Fibroblast	3.2×10^5	1.2×10^5
M. dunni	Mouse	Tail fibroblast	3.2×10^5	6.2×10^4
CHO K1	Hamster	Ovary	<1	<1
7-2.9-20[c]	Hamster-mouse hybrid	Ovary epithelial	NT[d]	1.0×10^5
Mv-1-Lu	Mink	Lung epithelial	1.4×10^5	1.9×10^4
293	Human	Embryonal kidney	7.5×10^4	6.6×10^3
HeLa	Human	Ovary epithelial	<1	<1

[a] Cells were infected with replication-competent MCF 247 and then assayed for RT activity after 4 weeks of culture. The values are the averages for supernatant from three separate experiments.
[b] Cells were infected with serial dilutions of BAG(MPAC-91) supernatant for 24 h. Two days later, the cells were fixed and stained for β-Gal activity. Clusters of four or more blue cells were scored as a CFU. The values are the averages from three separate experiments.
[c] Contains a portion of mouse chromosome 1 and the MCF virus receptor gene (20).
[d] NT, not tested.

MCF 247 and pBAG, the defective vector used in these experiments, differ.

To confirm that the cell lines that clearly support MCF virus replication (293 and MCF7) were human, these lines were reacted with an anti-human fluorescein isothiocyanate (FITC)-labeled monoclonal antibody against HLA class I antigens (clone W6/32; Sigma) and examined with a confocal microscope as described previously (1). When 100 cells from each line were examined, both human cell lines reacted positively with the monoclonal antibody, whereas mouse NIH 3T3 cells did not (data not shown). In addition, both 293 and MCF7 cells were obtained from the American Type Culture Collection and the experiment was repeated quantitatively with a Zeiss fluorescent microscope (FITC-positive cells/total cells scored; for NIH 3T3, 0/220; for 293, 298/299; for MCF7, 542/555). Therefore, more than 99% of the cells of each cell line were identified as human by virtue of their reaction with the antibody.

The 293 and MCF7 cell lines from the American Type Culture Collection were also infected with BAG(MPAC-91) virus or replication-competent MCF 247. The results of that experiment were similar to those shown in Fig. 1B and C. The percentages of both 293 and MCF7 cells that had β-Gal activity exceeded the percentage of cells that were FITC negative in the previous experiment, demonstrating that the documented infection of the human cell lines did not result from contamination of the human cells with a minor population of murine cells. We conclude that 293 and MCF7 are human cell lines that support infection by BAG(MPAC-91) and replication of MCF 247.

To determine if these results could be extended to primary human cells, four primary cell cultures (human foreskin fibroblasts [HFF], monocytes, renal epithelial cells, and renal mesangial cells) were infected with BAG(MPAC-91) vector at an MOI of 1. Two days later, the cells were fixed and stained for β-Gal activity. None of the four primary cell cultures examined stained positively for β-Gal activity. Although the cells exhibited a normal morphology and were readily infectible with other viruses (e.g., HFF supported cytomegalovirus infection and monocytes and renal cells supported human immunodeficiency virus infection [data not shown]), they did not stain positively for β-Gal activity. These data show that these primary cells did not support BAG(MPAC-91) infection (Table 2). However, we cannot rule out the possibility that other human primary cells, under certain conditions, might be able to support BAG(MPAC-91) infection.

We also characterized the interference properties of BALB-IU-1 and MCF 247 in two of the human lines capable of supporting replication of both viruses and, in addition, in a mouse cell line, *M. dunni*, and a mink cell line, Mv-1-Lu. Previous interference data showed that xenotropic and MCF viruses form separate interference groups in inbred mouse cell lines (e.g., NIH 3T3) whereas xenotropic and MCF viruses form one interference group in wild mouse lines and in some cell lines of other species (11, 15, 30). Additional data suggest that interference groups may vary when the same viruses are

TABLE 2. Characterization of the ability of cell lines to support MCF virus replication

MCF virus replication support	Primary cell type	Established cell line (description)
Fully supportive[a]		293 (primary embryonal kidney) MCF7 (breast adenocarcinoma) 2780 (ovarian carcinoma)
Restricted[b]		SW620 (colon adenocarcinoma) U-373 MG (glioblastoma, astrocytoma) HepG2 (hepatocellular carcinoma) 769-P (renal cell adenocarcinoma)
Nonsupportive[c]	HFF Monocyte Renal epithelial Renal mesangial	C8166 (T cell) CEM (T lymphoblastoid) G401 (Wilms' tumor) H9 (T-cell lymphoma) HeLa (epitheloid cervical carcinoma) HL-60 (promyelocytic leukemia) K-562 (chronic myelogenous leukemia) Mes-SA (muscle sarcoma) Raji (Burkitt lymphoma) T84 (colon carcinoma) U-937 (histiocytic lymphoma)

[a] Defined as having RT values of more than fivefold over background and a BAG(MPAC-91) titer of >100 CFU/ml.
[b] Defined as having RT values between background and fivefold over background and a BAG(MPAC-91) titer between 1 and 100 CFU/ml.
[c] Defined as having RT values equal to background or a BAG(MPAC-91) titer of <1 CFU/ml.

TABLE 3. MCF 247 or BALB-IU-1 infection interferes with BAG(MPAC-91) infection

Target cell line	Species	Titer[a] of BAG(MPAC-91) in cells treated with:		
		No virus	MCF 247	BALB-IU-1
M. dunni	Mouse	6.2×10^4	0	0
Mv-1-Lu	Mink	1.9×10^4	4.6	0
293	Human	6.7×10^3	0	0
MCF7	Human	4.0×10^2	0	0

[a] Expressed as β-Gal-positive CFU per milliliter (MOI = 1). Cells were either mock infected or chronically infected with the indicated virus 4 weeks earlier. Results are averages of the values obtained for three identical wells from a single experiment.

tested in different cell types (6). We measured the titer of BAG(MPAC-91) on xenotropic virus-infected and uninfected 293, MCF7, *M. dunni*, and Mv-1-Lu cells (Table 3). In each case, infection with BALB-IU-1 interfered with infection by BAG(MPAC-91), suggesting that polytropic and xenotropic viruses use the same receptor on the surface of these cells.

In conclusion, we have generated a packaging cell line capable of packaging defective retroviral vectors in a polytropic MCF virus envelope. We demonstrate that vectors packaged in the line have the same tropism as MCF 247 and can efficiently infect several human cell lines in vitro. In addition, polytropic and xenotropic viruses form a single interference group in the human cell lines 293 and MCF7, suggesting that both viruses use a single receptor on these cells. These conclusions suggest that the MPAC-91 cell line will be useful in studies of the biology, entry, and pathogenesis of MCF viruses.

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