High-Titer Packaging Cells Producing Recombinant Retroviruses Resistant to Human Serum

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Novel retroviral protein expression constructs were designed to retain minimal retroviral sequences and to express dominant selectable markers by reinitiation of translation after expression of the viral genes. HT1080 cells were selected as producer cells for their ability to release high-titer viruses that are resistant to inactivation by human serum. Two HT1080-based packaging cell lines which produce Moloney murine leukemia virus cores with envelope glycoproteins of either amphotropic murine leukemia virus (FLYA13 line) or cat endogenous virus RD114 (FLYRD18 line) are described. Direct comparison with previous retroviral packaging systems indicated that 100-fold-higher titers of helper-free recombinant viruses were released by the FLYA13 and FLYRD18 lines.

Some experimental and clinical gene transfer protocols require the design of gene transfer vectors suitable for in vivo gene delivery (19). Retroviral vectors are attractive candidates for such applications because they can provide stable gene transfer and expression (28) and because packaging cells which produce non-replication-competent viruses have been designed (18). However, currently available recombinant retroviruses suffer from a number of drawbacks. First, such vectors are rapidly inactivated by human serum, which makes them inappropriate for many clinical in situ gene transfer protocols. Second, the current split packaging function systems provide limited titers of infectious retroviral vectors (10^5 to 10^7 infectious units [i.u.] per ml). In these cells, the two helper genomes have been introduced by cotransfection with plasmids encoding selectable markers (7, 17, 20). Thus, no direct selection is applied to the packaging genome itself, and helper functions can be lost during passage of the cells in culture (6). Third, while the retroviral vectors prepared from split packaging function cell lines are usually not contaminated by replication-competent retroviruses (RCRs), other types of recombinant retroviruses have been shown to arise spontaneously from such cells (4, 9, 22, 31).

Here, we report the construction of new packaging cell lines designed to overcome these constraints. First, we have previously shown that inactivation by complement in human serum is controlled by the cell line used to produce the virions and by viral envelope determinants (37). We now describe packaging cells that will produce complement-resistant virus using human HT1080 cells and cat virus RD114 envelope. Second, we established direct selection for expression of packaging functions by expressing selectable marker genes after viral genes by reinitiation of translation. Finally, the generation of RCRs involves multistep recombinations between vector plasmids and *gag-pol*

and *env* genomes provided either by the packaging plasmids (4, 9, 22) or by endogenous retroviral sequences (26, 40). The possibility of generation of such RCRs was therefore reduced by decreasing the viral sequences in the helper constructs. The new packaging cell lines were able to transduce helper-free, human complement-resistant retroviral vectors at titers consistently higher than 10^7 i.u./ml.

MATERIALS AND METHODS

Cell lines and viruses. Cell lines A204 (ATCC HTB 82), HeLa (ATCC CCL2), HT1080 (ATCC CCL121), MRC5 (ATCC CCL171), T24 (ATCC HTB 4), Vero (ATCC CCL81), and D17 (ATCC CCL183) were purchased from the American Type Culture Collection. HOS, TE671, and Mv-1-Lu cells and their clones harboring the MFGnlslacZ retroviral vector have been described previously (37). All these cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% fetal calf serum.

EB8 (1), psiCRE, psiCRELLZ, and psiCRIP (7) cells were kindly provided by O. Danos and J. M. Heard. GP+EAM12 (17) cells were kindly provided by A. Bank. These cell lines and also NIH 3T3 murine fibroblasts were grown in DMEM supplemented with 10% newborn calf serum.

Mv-1-Lu, TE671, and HT1080 cells were transfected by the calcium phosphate precipitation method (29) as described elsewhere (1). CeB-transfected Mv-1-Lu, TE671, and HT1080 cells were selected with 3, 6 to 8, and 4 μg of blasticidin S (ICN) per ml, respectively, and blasticidin-resistant colonies were isolated 2 to 3 weeks later. Cells transfected with the various *env* expression plasmids were selected with phleomycin (CAYLA): 50 μg/ml for ALF-transfected cells and 10 μg/ml for AXF-, AF-, and RDF-transfected cells. Phleomycin-resistant colonies were isolated 2 to 3 weeks later.

The production of LacZ pseudotype viruses from replication-competent viruses, amphotropic murine leukemia virus (MLV-A) strain 1504, and cat endogenous virus RD114 was carried out as described previously (37).

Plasmids. The *env* gene of pCRIP (kindly provided by O. Danos and J. M. Heard) (7) was excised by *Hpa*I-*Cla*I digestion. A 500-bp PCR-generated DNA fragment was obtained from pSV2-bsr (kindly provided by F. Hanaoka) (12) as the template and a pair of oligonucleotides (5′-CGGAATTCGGATCCGAGCT CGGCCCAGCCGGCCCACCATGAAAACATTTAACATTTCTC at the 5′ end and 5′-GATCCATCGATAAGCTTGGTGGTAAAACTTTT at the 3′ end) with *Sfi*I and *Cla*I sites, respectively. This fragment was inserted into the *Hpa*I and *Cla*I sites of pCRIP by coligation with an 85-bp *Hpa*I-*Sfi*I DNA fragment isolated from pOXEnv (kindly provided by S. J. Russell) (27), which provides the end of the Moloney murine leukemia virus (MoMLV) *pol* gene. The resulting plasmid, named CeB (Fig. 1), could express the MoMLV *gag-pol* gene as well as the *bsr* selectable marker, conferring resistance to blasticidin S, both driven by the MoMLV 5′ long terminal repeat (LTR) promoter.

A series of *env* expression plasmids were generated by using the 4070A

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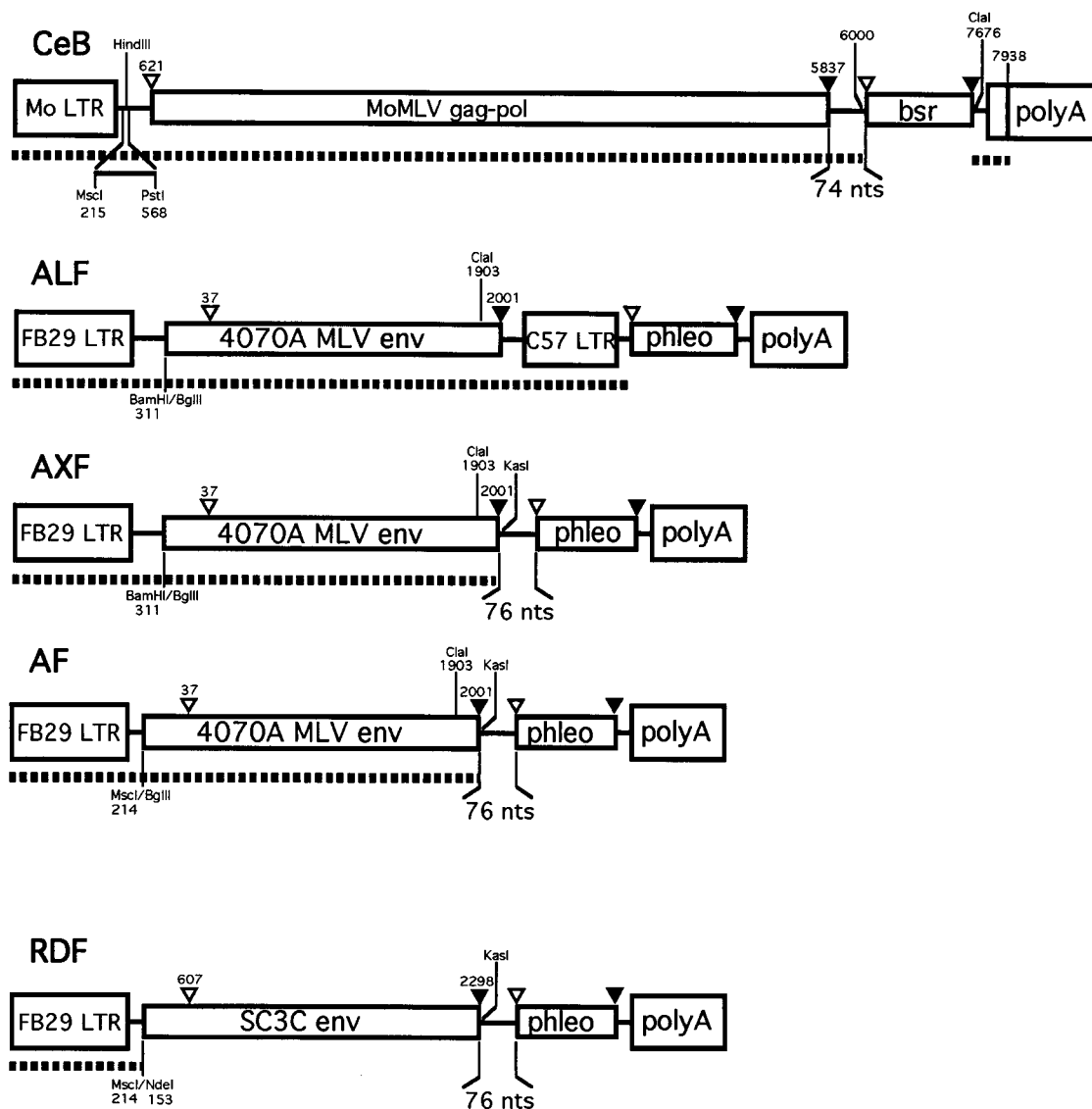
FIG. 1. Schematic diagrams of constructs. Initiation (▽) and termination (▼) codons are shown. The thick dotted line shows MLV-derived sequences. The nucleotide positions of MLV-derived sequences are shown according to Shinnick et al. (33) for CeB (from nt 1 to nt 6000, with deletion of the packaging signal from *Bal*I [nt 215] to *Pst*I [nt 568] and with some further MoMLV sequences from nt 7676 to nt 7938), Perryman et al. (23) (from nt 1 to nt 311 or 214), and Ott et al. (24) (from *Bgl*II to nt 2001) for ALF, AXF, and AF and Perryman et al. (23) (from nt 1 to nt 214) for RDF. Numbering of the RD114 sequence for RDF (from nt 153 to nt 2298) is as reported (EMBL accession number X87829).

MLV-A *env* gene (23) and the FB29 Friend MLV promoter (24). In ALF (Fig. 1), a *Bgl*II-*Cla*I fragment containing the *env* gene was cloned into the *Bam*HI and *Cla*I sites of plasmid FB3LPh (1a), which also contained the C57 Friend MLV LTR driving the expression of the phleomycin resistance (*phleo*) selection marker (8). A 136-bp *env* fragment was generated by PCR with plasmid FB3 (10) as the template and a pair of oligonucleotides, 5'-GCTCTTCGGACCCTGC ATTC at the 5' end (before the *Cla*I site) and 5'-TAGCATGGCGCCCTATGG CTCGTACTCTATAGGC at the 3' end, providing a *Kas*I restriction site immediately after the *env* stop codon. This PCR fragment was digested with *Cla*I and *Kas*I. A DNA fragment containing the FB29 LTR and the MLV-A *env* gene was obtained by *Nde*I-*Cla*I digestion of ALF. The fragments were coligated in *Nde*I-*Kas*I-digested pUT626 (kindly provided by Daniel Drocourt, CAYLA). In the resulting plasmid, named AXF (Fig. 1), the *phleo* selectable marker was expressed from the same mRNA as the *env* gene. A *Bgl*II restriction site was created after the *Msc*I site at position 214 in the FB29 leader by using a commercial linker (Biolabs). An *Nde*I-*Bgl*II fragment containing the FB29 LTR was coinserted with the *Bgl*II-*Cla*I *env* fragment into *Nde*I-*Cla*I-digested AXF plasmid DNA, resulting in plasmid AF (Fig. 1). AF has a 100-bp-larger deletion in the leader region than AXF.

The RD114 *env* gene was first subcloned in plasmid Bluescript KS+ (Stratagene) as a 3-kb *Hin*dIII insert isolated from SC3C, an RD114 infectious DNA clone (kindly provided by S. O'Brien) (25). This *env* gene was sequenced (EMBL accession number X87829). The 5' noncoding sequence upstream of an *Nde*I site was deleted by *Eco*RI and *Nde*I digestion followed by filling-in with Klenow enzyme and self-ligation. From this plasmid, two DNA fragments were obtained: a *Bam*HI-*Nco*I 2.5-kb fragment and a 63-bp PCR-generated DNA fragment (using 5'-CGCCTCATGGCCTTCATTAA at the 5' end [before the *Not*I site] and 5'-TAGCATGGCGCCTCAATCCTGAGCTTCTTCC at the 3' end), providing a *Kas*I restriction site just after the RD114 *env* gene stop codon. The PCR fragment was digested with *Nco*I and *Kas*I. Both fragments were coinserted between the *Bgl*II and *Kas*I sites of AF, and the resulting plasmid was named RDF (Fig. 1).

Plasmid pCRIPAMgag⁻ (7) (kindly provided by O. Danos and J. M. Heard) was used for transfection.

Infection assays. Target cells were seeded in 24-multiwell plates (4 × 10⁴ cells per well) and incubated overnight. Infections were then carried out at 37°C by plating 1 ml of dilutions of viral supernatants in the presence of Polybrene (4 μg/ml; Sigma) on target cells. After 3 h, virus-containing medium was replaced by fresh medium, and infected cells were incubated for 2 days before X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining, performed as previously described (36, 37). Viral titers were determined by counting LacZ-positive colonies as previously described (5). The stability of LacZ pseudotype viruses

TABLE 1. Titer and stability of LacZ pseudotypes[a]

Producer cells	LacZ(MLV-A)		LacZ(RD114)	
	Titer (i.u./ml)	Stability (% of control)	Titer (i.u./ml)	Stability (% of control)
A204	650	<3	1,200	105
HeLa	9	ND[b]	2,000	115
HOS	4,500	6	23,000	86
HT1080	2,000,000	26	400,000	129
MRC-5	450	10	1,000	ND
T24	350	ND	1,200	ND
TE671	15,000	2	90,000	38
Vero	260	ND	90	ND
D17	900	<1	200,000	1
Mv-1-Lu	80,000	1	200,000	120

[a] Titer was determined on TE671 cells. Stability is expressed as the infectivity of human serum-treated viruses as a percentage of that of fetal calf serum-treated viruses.
[b] ND, not done.

in fresh human serum was examined by titrating surviving virus after incubation in a 1:1 mixture of virus harvest in serum-free medium and fresh human serum for 1 h at 37°C as described before (37).

RT assays. The reverse transcriptase (RT) assays were performed either as described previously (37) or with an RT assay kit (Boehringer Mannheim) following the manufacturer's instructions but using $MnCl_2$ (2 mM) instead of $MgCl_2$.

Nucleotide sequence accession number. The EMBL accession number for the sequence of the RD114 *env* gene is X87829.

RESULTS

Screening of producer cell lines. We have shown that viral particles generated with RD114 envelopes are more stable in human serum than virions with MLV-A envelopes and that the producer cell line also controls sensitivity (37). We have also found that viruses produced by certain nonprimate cells are sensitive to human serum because they bear Gal(α1-3)galactosyl sugar on their envelope, which activates complement via "natural" antibodies against this sugar epitope present in human serum (38). We therefore screened a panel of cell lines, mostly of primate origin, for their ability to produce high-titer viruses and for the sensitivity of these virions to human serum. To do this, cells were infected at high multiplicity with LacZ pseudotype viruses of either MLV-A or RD114, and cells producing helper-positive LacZ pseudotype viruses were established. Human HT1080 and TE671 and mink Mv-1-Lu cells were found to release high-titer LacZ(RD114) and LacZ (MLV-A) viruses. The LacZ(MLV-A) pseudotype viruses produced by HT1080 cells were more resistant to human serum than those produced by other cells. The titer of these viruses was only fourfold lower following a 1-h incubation with human serum than in a control incubation with fetal calf serum (Table 1). The LacZ(RD114) pseudotype viruses produced by human cells and mink Mv-1-Lu cells were in general stable in human serum (Table 1). These results suggested that HT1080, TE671, and Mv-1-Lu cells provided the best combination of high LacZ virus titers and resistance to human serum, and they were therefore used for the generation of retroviral packaging cells.

Construction of an improved *gag-pol* expression vector. An MoMLV *gag-pol* expression plasmid, CeB (Fig. 1), was derived from pCRIP (7). Approximately 2 kb of *env* sequence were removed from pCRIP, and the *bsr* selectable marker, conferring resistance to blasticidin S (12), was inserted 74 nucleotides (nt) downstream of the *gag-pol* gene. This 74-nt interval had no

ATG triplets and was thought to provide an optimal distance between the stop codon of the *pol* reading frame and the start codon of the *bsr* gene to allow reinitiation of translation (15). Therefore, *bsr* could only be expressed by reinitiation of translation after the upstream *gag-pol* gene had been expressed. Consequently, after transfection of CeB into Mv-1-Lu/MFGnls LacZ (ML), TE671/MFGnlsLacZ (TEL), or HT1080 cells, blasticidin S-resistant bulk populations and most cell clones expressed high levels of Gag-Pol proteins, as assessed from the RT activity found in cell supernatants (Table 2). Considerably higher RT activities were found in bulk populations of CeB-transfected ML cells than in bulk populations of ML cells stably transfected with the parental pCRIP construct. Similarly, the RT activities of two packaging cell lines generated by using the pCRIPenv⁻ construct, psiCRE cells (7) and EB8 cells (1), were less than that of CeB-transfected clones (Table 2). Indeed, RT activity in CeB-transfected cell supernatants was higher than that of cells chronically infected by replication-competent MLV-A (Table 2).

To rescue infectious LacZ viruses, MLCeB and TELCeB clones were transfected with ALF DNA, a plasmid designed to express the MLV-A *env* gene (Fig. 1). Bulk populations of stable ALF transfectants were isolated, and supernatants were titrated by using TE671 cells as targets. Titers of LacZ viruses were higher than in either MLV-A-infected ML or TEL cells or ALF-transfected EB8 cells (Table 2). These data suggested that CeB was an extremely efficient MLV *gag-pol* expression vector in mink Mv-1-Lu and TE671 cells. We therefore used CeB to derive packaging cells by transfection of HT1080 cells. Forty-one of 49 blasticidin S-resistant colonies had detectable levels of RT; 9 had RT activity higher than that of control MLV-A-infected HT1080 cells (data not shown). Expression of the Gag precursor was confirmed in cell lysates and superna-

TABLE 2. Secreted RT expression

Cells[a]	RT activity[b]	LacZ virus titer[c] (i.u./ml)
ML/MLV-A	1	8×10^4
MLSvB	0.1	<1
MLCRIP (bulk)	0.15	ND[d]
MLCeB (bulk)	1.7	ND
MLCeB1	4.2	1×10^6
MLCeB4	1.6	1×10^6
TEL/MLV-A	3.6	2×10^6
TELCeB6	5.2	4×10^7
HT1080/MLV-A	1.1	1×10^6
HTCeB6	1.9	1×10^6
HTCeB18	2.7	2×10^6
HTCeB22 (FLY)	6.9	5×10^6
HTCeB48	5.5	3×10^6
EB8	0.22	1×10^4
psiCRE-LLZ	1.2	1×10^{5e}

[a] ML, Mv-1-Lu cells harboring a MFGnlslacZ provirus; TEL, TE671 cells harboring a MFGnlslacZ provirus; /MLV-A, cells chronically infected with MLV-A strain 1504; MLSvB, ML cells transfected with plasmid pSV2bsr alone; MLCRIP, ML cells cotransfected with pCRIP and pSV2bsr.
[b] Average of ratios of RT activity relative to that released by ML/MLV-A cells of at least two independent experiments is shown. The standard errors did not exceed 20% of the values.
[c] Titer determined on TE671 cells (except as noted) after polyclonal transfection of a plasmid which expresses MLV-A *env* in MLCeB clones, TELCeB clones, HTCeB clones, and EB8 cells.
[d] ND, not done.
[e] Titer determined on NIH 3T3 cells.

TABLE 3. Titers following *env* construct transfection[a]

Producer cells	*env* source	Titer (i.u./ml)
psiCRIP lacZ 5[b]	pCRIPAMgag⁻	6×10^4
GP+EAM12 lacZ 25[b]	envAM	3×10^5
TELCeB6[c]	ALF	5×10^7
	AXF	2×10^7
	AF	2×10^7
TELCeB6	AF 1	3×10^7
	AF 4	2×10^7
	AF 6	1×10^7
	AF 7	5×10^7
	AF 8	1×10^7
	RDF 2	1×10^6
	RDF 4	3×10^5
	RDF 7	1×10^7
	RDF 8	2×10^6
FLY[d]	AF 1	1×10^1
	AF 4	1.5×10^6
	AF 5	1×10^6
	AF 7	1×10^6
	AF 13	7×10^6
	AF 14	4×10^6
	AF 15	1×10^6
	AF 16	5×10^6
	AF 17	6×10^6
FLYA4 lacZ 3[b]	AF 4	2×10^7
FLY[d]	RDF 1	2.5×10^6
	RDF 2	1×10^7
	RDF 6	5×10^6
	RDF 10	2×10^6
	RDF 11	3×10^6
	RDF 13	1×10^6
	RDF 17	5×10^6
	RDF 18	3×10^7
	RDF 19	6×10^6

[a] Average titers of at least three independent experiments are shown. The standard errors did not exceed 30% of the titer values. Titers were determined on TE671 cells (LacZ virus i.u. per milliliter).
[b] Best MFGnlslacZ producer clones.
[c] Bulk populations of *env* transfectants in TELCeB6 cells.
[d] Titration after bulk infection with helper-free MFGnlslacZ.

tants of these nine HTCeB clones by immunoblotting with antibodies against p30-CA (data not shown). The four clones with the highest expression of Gag proteins (clones 6, 18, 22, and 48) were infected at high multiplicity with helper-free LacZ pseudotypes bearing MLV-A envelopes [MFGnlslacZ (A)] produced by TELCeB6/ALF (Table 3) and then transfected with ALF. Supernatants of bulk, phleomycin-resistant transfectants were assessed for RT activity and LacZ virus titer (Table 2). Clone HTCeB22, named FLY, was found to be the best Gag-Pol producer clone and was used to introduce *env* expression vectors for the generation of packaging cell lines.

Construction of *env* expression vectors. A series of MLV-A *env* expression plasmids were then generated (Fig. 1). In ALF, the *env* gene was inserted between two Friend-MLV LTRs, its expression driven by the FB29 MLV LTR (24). Most of the packaging signal located in the leader region was deleted. This plasmid also expressed the *phleo* selectable marker (8) driven by the 3′ LTR. AXF and AF were then designed following the same strategy used for CeB. These two vectors differed only by the extent of deletion of the packaging signal, AF having vir-

tually no leader sequence. Compared with the pCRIPAMgag⁻ and pCRIPgag-2 *env* plasmids expressed in psiCRIP and psi-CRE packaging cells (7), about 5 kb of *gag-pol* sequence was removed. In addition, the 258-bp retroviral sequence containing the end of the *env* gene and the beginning of U3 found in pCRIPAMgag⁻ and pCRIPgag-2 was also removed. For both the AXF and AF plasmids, the *phleo* selectable marker was inserted downstream of the *env* gene by positioning a 76-nt linker with no ATG codons between the two open reading frames. *phleo* could therefore only be expressed by reinitiation of translation by the same ribosomal unit that had expressed the upstream *env* open reading frame. AF was also used to generate RDF, an RD114 envelope expression plasmid (Fig. 1).

After transfection of the *env* plasmids into TELCeB6 cells (Table 2), bulk populations of phleomycin-resistant colonies were isolated, and their production of LacZ virus was measured (Table 3). ALF gave a titer of 5×10^7 LacZ virus i.u./ml, while the titers with AXF and AF were 2×10^7 LacZ virus i.u./ml (Table 3). Titers of 5×10^7 and 10^7 LacZ virus i.u./ml could be obtained with some AF cell clones and RDF clones, respectively.

As AF has minimal virus-derived sequences and was shown to be the safest construct (see below and Table 4), it and RDF were used to generate packaging lines from FLY cells (clone HTCeB22 [Table 2]). Envelope expression of these clones was assayed by interference to challenge with MFGnlslacZ(A) and MFGnlslacZ(RD) pseudotype viruses produced by TELCeB6/AF-7 and TELCeB6/RDF-7, respectively (Table 3). The cell lines showing the most interference were cross-infected at high multiplicity with these pseudotype viruses to provide MFGnls lacZ proviruses, and supernatants were then titrated on TE671 cells (Table 3). FLY-AF-13 (FLYA13 packaging line) and FLY-RDF-18 (FLYRD18 packaging line) gave the highest productions of LacZ viruses, about 10^7 LacZ virus i.u./ml. The best MFGnlslacZ producer clones, derived from either psi-CRIP cells (7) or GP+EAM12 cells (17), gave approximately 50-fold-lower titers (Table 3). The LacZ virus titers of the FLY-derived lines shown in Table 3 are lower than those of the best TELCeB6-derived lines after transfection of either AF or RDF (Table 3). However, it should be noted that the LacZ provirus expressed in TELCeB6 cells was obtained after clonal selection but was introduced polyclonally in FLY-derived *env*-transfected cell clones. When FLY-AF-4 cells (FLYA4 packaging line), infected with helper-free MFGnlslacZ(RD), were cloned by limiting dilution, the best clones (e.g., FLYA4lacZ3) were found to produce 20 times more infectious viruses than the bulk population, reaching the range of titers obtained with the best TELCeB6-AF clones (Table 3).

Assays for transfer of *gag-pol* and *env* functions. To assay for replication-competent viruses, supernatants were used to infect TEL cells (a clone of TE671 cells harboring an MFGnls lacZ provirus). Infected cells were passaged for 6 days or longer, and their supernatants were used for infection of fresh TE671 cells. No transmission of LacZ viruses could be detected (Table 4), demonstrating that the supernatants of pCRI PAMgag⁻-, ALF-, AXF-, or AF-transfected TELCeB6 cells were helper-free. A similar absence of replication-competent recombinant retroviruses was demonstrated by using the supernatant from a clone of psiCRIP-MFGnlslacZ cells or two clones of FLYA-MFGnlslacZ cells (Table 4).

There have been reports that helper-free retroviral vector stocks may nevertheless contain recombinant retroviruses (replication incompetent) carrying either *gag-pol* or *env* genes (4, 9, 16). To assay for such recombinant retroviruses, we attempted to mobilize an MFGnlslacZ provirus from two indicator cell lines, which could cross-complement potential re-

TABLE 4. Transfer of packaging function

Producer cells	Indicator cells	Input virus[a] (i.u./ml)	No. of 4 expt giving indicated result[b]		
			++	+	−
RCRs					
psiCRIP lacZ 5	TEL	2×10^4	0	0	4
TELCeB6-pCRIPAMgag⁻	TEL	5×10^6	0	0	4
TELCeB6-AXF	TEL	5×10^6	0	0	4
TELCeB6-AF	TEL	5×10^6	0	0	4
FLYA4 lacZ 3	TEL	1×10^7	0	0	4
FLYA4 lacZ 7	TEL	1×10^7	0	0	4
GPRs					
TELCeB6-AF 7	TELMOXF	2×10^7	0	1	3
		2×10^6	0	2	2
		2×10^5	0	2	2
		2×10^4	0	0	4
ERs					
TELCeB6-pCRIPAMgag⁻	TELCeB6	5×10^6	2	1	1
		5×10^5	1	1	2
		5×10^4	0	2	2
TELCeB6-AXF	TELCeB6	5×10^6	0	2	2
		5×10^5	0	1	3
		5×10^4	0	1	3
TELCeB6-AF	TELCeB6	5×10^6	0	1	3
		5×10^5	1	3	0
		5×10^4	0	0	4

[a] Number of LacZ virus i.u. used to infect indicator cells.
[b] Results from four experiments in which indicator cells were exposed to 1 ml of virus in each experiment. The ranges of LacZ virus titers rescued from infected indicator cells are shown for each virus input: ++, >100 LacZ virus i.u./ml; +, 1 to 100 LacZ virus i.u./ml; −, <1 LacZ virus i.u./ml. Titers were determined on TE671 cells for RCRs and ERs and on NIH 3T3 cells for GPRs.

combinant viruses. The TELCeB6 line (Table 2), expressing Gag-Pol proteins, was used as the indicator cell line to test for the presence of *env* recombinant (ER) viruses. The TELMOXF indicator line, expressing MoMLV *env* glycoproteins (obtained by transfection of MOXF, a plasmid expressing the MoMLV *env* gene using the AXF backbone, into TEL cells), was used to detect the presence of *gag-pol* recombinant (GPR) retroviruses. After passaging for 4 to 8 days, the supernatants of the infected indicator cells were used to infect either human TE671 cells or murine NIH 3T3 cells.

TELCeB6 cells transfected with the various *env*-expressing constructs, pCRIPAMgag⁻, AXF, and AF, were compared. Although the supernatants of TELCeB6-AF cells were devoid of replication-competent retroviruses, they were found sporadically to transfer *gag-pol* genomes (Table 4). No GPR viruses could be detected when fewer than 2×10^5 virions were used to infect the indicator cells. Similarly, TELCeB6 indicator cells infected with various helper-free viruses were shown to sporadically release LacZ virions (Table 4). The number depended both on the *env* expression vector used and on the virus input quantity. Compared with LacZ viruses generated by using the pCRIPAMgag⁻ plasmid, the frequency of detection of the ER viruses was lower for supernatants generated by using AXF and AF constructs (Table 4). For the AF construct when fewer than 5×10^5 MFGnlslacZ(A) helper-free virions were used to infect the indicator cells, no ER retroviruses could be detected. From these experiments, it could be estimated that a supernatant produced from TELCeB6-AF cells, containing 10^7 i.u. of MFGnlslacZ retroviral vector, contained less than one

replication-competent virus and about 100 GPR and 100 ER retroviruses.

Complement resistance and helper-free status of LacZ vectors produced by FLYA13 and FLYRD18 cells. In order to confirm the resistance to complement and the absence of replication-competent virus in our best packaging lines, MFGnlslacZ(A) and MFGnlslacZ(RD), harvested from FLYA13 and FLYRD18, respectively, after polyclonal transduction of MF-GnlslacZ (Table 3) were tested for stability in fresh human serum and generation of replication-competent virus. The titers of MFGnlslacZ(RD) from FLYRD18 after 1 h of incubation with three independent samples of fresh human serum were 80 to 120% of those in control incubations without serum, while the titers of MFGnlslacZ(A) from FLYA13 were 50 to 90% of control titers (data not shown). No replication-competent virus was detected in the assay described above (Table 4) when 10^7 i.u. each of MFGnlslacZ(A) and MFGnlslacZ(RD) were tested.

DISCUSSION

We describe novel retroviral packaging cells, called FLY cells, designed for in vivo gene delivery. The retroviral vectors prepared from these cells are not inactivated by human serum and can be obtained at much higher titers, unlike previous vectors.

We found considerable variations between the various cell lines screened for their ability to release type C retroviruses. In addition, few cell lines were able to produce retroviruses that were completely resistant to human serum. By these two criteria, human fibrosarcoma HT1080 cells were selected for the construction of packaging cells. Other studies have shown the importance of endogenous retrovirus expression in the generation of recombinant retroviruses from retroviral packaging lines (26, 40). The copackaging of an endogenous genome and a vector can lead to the emergence of recombinant retroviruses (40). Recombination involves template switching during reverse transcription of such hybrid retroviruses (11), and homologies between the two genomes considerably enhance the frequency of RT jumps (41). Therefore, an ideal packaging cell line should not express endogenous MLV-like (or type C retrovirus-like) retroviral genomes which can be packaged by type C Gag proteins (30, 39). In recent studies (23a), we could not detect expression of type C retroviruses in HT1080 cells by PCR analysis with generic primers (32), suggesting that HT1080-derived FLY packaging cells may be safer in this respect than those generated from NIH 3T3 cells, which are known to express and package sequences related to type C retroviruses (30).

To generate the FLY packaging cell lines, HT1080 cells were transfected with *gag-pol* and *env* expression plasmids designed to optimize viral protein expression. Direct selection for viral gene expression was achieved by expression of a selectable marker gene by reinitiation of translation of the mRNA expressing the viral proteins. This strategy resulted in packaging cell lines capable of producing extremely high-titer viruses. Furthermore, selection for packaging functions can be maintained in these cells. We also eliminated many unnecessary viral sequences from the packaging constructs to reduce the risk of helper virus generation. The final packaging cells did not produce helper virus, in that no RCR could be detected per 10^7 vector particles, although it must be noted that only a small volume of viral supernatant was assayed. However, it is of concern that recombinant retroviruses expressing either MLV Gag-Pol or Env proteins could be detected. Our data demonstrate that retroviral vector stocks are contaminated

with such recombinant viruses at about 1 per 10^5 vector particles. The new FLY packaging cells are safer than, for example, psiCRIP cells, at least for the generation of ER retroviruses in a short-term assay (Table 4), probably because fewer retroviral sequences overlapping the vector were present in our *env* expression plasmid. Few reports have addressed the question of the characterization of recombinant retroviruses (4, 9). It is possible that such viruses could not be detected in previous packaging cell lines because of low overall titers. Recombinant retroviruses are defective in normal cell culture conditions but are likely to evolve to replication-competent viruses if they are allowed to replicate in cells complementing their expression, such as cocultivated packaging cells (3, 4). They may modify other viruses, such as human immunodeficiency virus or endogenous viruses, by phenotypic and genetic mixing in gene therapy recipients. It will be important for the future development of retroviral packaging systems to eradicate recombinant retroviruses, perhaps by removal of viral LTRs from the packaging construct.

Consistent with our previous studies (37), LacZ(RD114) pseudotypes produced from HT1080 cells were more resistant to human complement than LacZ(MLV-A) pseudotypes. We therefore decided to use the RD114 *env* gene to generate recombinant virions with MoMLV cores. We have determined the sequence of the RD114 *env* gene and found it to be very close to that of baboon endogenous virus, a type C retrovirus (2, 13) with an envelope gene displaying similarities to the external part of type D simian retroviruses. RD114 uses the simian retrovirus receptor on human cells (34, 35), making the FLY packaging cells with the RD114 envelope capable of generating virions with different tropisms. The retroviral vectors prepared so far for human gene therapy have used either MLV-A or gibbon ape leukemia virus envelopes, which display many similarities (1) and which use two related cell surface receptors for infection (21). Differences in tissue-specific expression of the MLV-A and the gibbon ape leukemia virus receptors have been reported (14), and it will now be interesting to see whether particular cell types can be recognized by RD114 Env-coated retroviral vectors.

ACKNOWLEDGMENTS

ADDENDUM IN PROOF

The FLYA13 and FLYRD18 cell lines will soon be available for research purposes from the European Collection of Cell Cultures under ECACC accession numbers 95091901 and 95091902, respectively.

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