HUMAN GENE THERAPY 7:1405–1413 (August 1, 1996)
Mary Ann Liebert, Inc.

Episomal Vectors Rapidly and Stably Produce High-Titer Recombinant Retrovirus

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ABSTRACT

The nuclear replication and retention functions of the Epstein–Barr virus (EBV) have been utilized here to maintain retroviral constructs episomally within human cell-based retroviral packaging lines. These hybrid EBV/retroviral constructs are capable of producing helper-free recombinant retrovirus as soon as 48 hr and for at least 30 days after transfection into 293T-based ecotropic and/or amphotropic retroviral packaging cells. Viral titers greater than 10^7 TU/ml were obtained after puromycin selection of transfected retroviral packaging cells. This episomal approach to retroviral production circumvents some limitations inherent in transient and chromosomally stable retroviral producer systems, affording reproducibly rapid, large-scale, stable, and high-titer retrovirus production.

OVERVIEW SUMMARY

Producing high-titer retrovirus can be a limiting factor in the successful application of retroviruses for gene transduction. A retroviral producer system capable of both rapid and long-term virus production would circumvent many of the restrictions inherent to chromosomal-stable and/or transient-based production methods. It is demonstrated here that replication and nuclear retention functions of the human Epstein–Barr virus (EBV) can confer stable episomal maintenance to retroviral constructs within human cell-based packaging lines. Such hybrid EBV/retroviral constructs permit both rapid and long-term production of high-titer retrovirus. Furthermore, it is demonstrated that these constructs are not prone to arrangements when maintained episomally in packaging cells and that viral stocks are helper-virus free.

INTRODUCTION

PRODUCTION OF RECOMBINANT RETROVIRUS generally uses eukaryotic cells engineered to stably express the retroviral genes *gag*, *pol*, and *env* (Mann *et al.*, 1983; Watanabe and Temin, 1983; Miller, 1990). To generate recombinant vector, retroviral constructs are transfected into such packaging cells and the expressed viral RNA, by virtue of a packaging signal termed ψ, is incorporated into maturing viral particles. One class of packaging systems is based on chromosomal integration of the retroviral construct DNA or vector within packaging cells and the subsequent identification of cell clones that stably generate high-titer recombinant retrovirus. Once such stable retrovirus producer clones have been identified, they can be repeatedly accessed to produce large volumes of retrovirus. However, creating and selecting such producer clones can take several weeks and must be repeated for each new vector, limiting the turnaround time and versatility of this approach (Miller, 1990; Mulligan, 1993; Pear *et al.*, 1993; Kasahara *et al.*, 1994).

More recently, investigators have developed packaging systems based on human and/or primate cells that utilize the transient expression of retroviral DNA constructs for recombinant virus production (Landau and Littman, 1992; Pear *et al.*, 1993, 1995; Finer *et al.*, 1994). These systems are distinguished by the inherently high transfectability of the packaging line, allowing the production of approximately 10^6 TU/ml of recombinant retrovirus within 48 hr of a transient transfection. In addition to the greatly reduced time and labor input required for transient-based systems, this approach confers a versatility not offered by the chromosomal-stable systems. For instance, transient vector production has been used to rapidly assess the differentiation potential of C/EBP variants (Yeh *et al.*, 1995) and to generate high-titer, high-complexity retrovirus cDNA libraries (Kitamura *et al.*, 1995). However, transient-based methods can be limited by the relatively brief period of vector pro-

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duction following transfection of the packaging line. Furthermore, vector titers can be compromised by low transient transfection efficiencies.

We have used replication and nuclear retention functions of the human Epstein–Barr virus to stably maintain retroviral constructs as DNA episomes within human-based retroviral packaging cells. Establishment of retroviral constructs as stable episomes combines rapid generation of retroviral vector with long-term production capabilities. In addition, several features afforded by the ability to maintain retroviral constructs episomally within packaging lines are exploited here to obtain vector titers higher than readily achieved with conventional production systems.

MATERIALS AND METHODS

Plasmid construction

pBabePuro has been previously described (Morgenstern *et al.*, 1990) and contains long terminal repeat (LTR) and ψ packaging sequences from the Moloney murine leukemia virus (MoMuLV). The puromycin-resistance gene is present within the retroviral backbone and is under the transcriptional control of the SV40 early promoter (Benoist and Chambon, 1981; Fromm and Berg, 1982). pBabePuro(ΔSV40) is a derivative of pBabePuro in which the SV40 early promoter has been removed by digestion with Sal I and Hind III, followed by an end-filling reaction with Klenow fragment and religation of the blunted plasmid. pBabePuro(ΔSV40)A was constructed by the insertion of an adapter (5′-*Bgl* II–*Hin*d III–*Xho* I–*Eco* RI-3′) into a unique *Not* I site present within the backbone of pBabePuro(ΔSV40); the *Not* I site was not reconstructed. p220.2 (gift of M. Calos) contains the Epstein–Barr virus (EBV) EBNA-1 gene, EBV *oriP cis* elements, and the hygromycin-resistance gene in a pBR322 backbone (Dubridge *et al.*, 1987). EBNA-1 is expressed by a cryptic promoter auspiciously present in pBR322 sequences (Heinzel *et al.*, 1988). Proximal to the *oriP* sequences are mRNA termination sequences from the herpes simplex virus type-1 thymidine kinase (*tk*) gene (Dubridge *et al.*, 1987). These sequences are positioned to prevent opposing transcriptional readthrough into the EBV *oriP cis* elements, which, if left unchecked, can suppress *oriP*-mediated replication (Dubridge *et al.*, 1987). PGK-Puro was created by ligating the puromycin resistance gene and SV40 polyadenylation sequences (*Pst* I–*Bam* HI fragment) from the plasmid pPUR into the *Pst* I and *Bam* HI sites of the plasmid PGKNeo-SUT-1 (gift of T. Kitamura, DNAX, Palo Alto, CA). The resulting construct contained the SV40 polyadenylation sequences and the puromycin-resistance gene driven by the phosphoglycerol kinase-1 (PGK-1) promoter. pBabePuro-LacZ (gift of R. Bhatt) is a derivative of pBabePuro in which the *lacZ* gene, obtained from the plasmid pARV-Z (G.P.N. unpublished) (*Dra* I–*Dra* I fragment), has been blunted into the *Sna* BI polylinker site of pBabePuro. pBabeM is a retroviral plasmid (gift of S. Kinoshita, unpublished) constructed using the 5′ LTR and the Ψ packaging sequences from the MFG vector (gift of R.C. Mulligan, unpublished) (*Pst* I–*Bam* HI fragment) and the *lacZ* gene and 3′ LTR of pBabePuro-LacZ. Polylinker sequences that flank the *lacZ* gene of pBabeM were inserted using synthesized oligonucleotides. pBabePuro^{EBV} and LZRS-LacZ(A) were constructed as outlined in Fig. 1.

Cell lines

BOSC23 cells are an ecotropic retroviral packaging cell line constructed from human 293T cells as previously described (Pear *et al.*, 1993). The BING retroviral packaging cell line is the amphotropic equivalent of the BOSC23 system and was constructed utilizing identical reagents with the exception that the *env* gene expressed in this cell line is derived from the 4070A retrovirus (W. Pear, P. Achacoso, and D. Baltimore, and G.P. Nolan, unpublished). Culture conditions for all cell lines utilized in these experiments have been previously described (Pear *et al.*, 1995). Briefly, cells were maintained at 37°C in 5% CO_2 and Dulbecco's modified Eagle Medium with 10% fetal calf serum (FCS) (JRH BIOSCIENCES, Lenexa, KS 66215), 1% glutamine, and 1% penicillin-streptomycin (Life Technologies, Inc., Grand Island, NY).

Establishment of episomally resident retroviral plasmids and vector production

Approximately 2×10^6 BOSC23 and/or BING retroviral packaging cells were transfected with constructs using a modified version of the calcium phosphate co-precipitation protocol (Pear *et al.*, 1995). At 24 hr post-transfection, medium was replaced with 3 ml of fresh medium and the incubation was continued at 32°C and 5% CO_2. For transiently produced vector titers (Pear *et al.*, 1993), supernatant was harvested at 48 hr post-transfection, placed in 1-ml aliquots (total 3 ml of virus for each sample) and frozen at −80°C.

After the initial vector collection at 48 hr post-transfection, all cells were trypsinized and placed into 100-mm tissue culture plates containing fresh medium and puromycin. Puromycin concentrations of 1 μg/ml were used for experiments involving both pBabePuro^{EBV} and LZRS-LacZ(A). Cells were maintained in the selective medium until 48 hr prior to any given collection of retroviral vector. And 48 hr before collecting vector stocks, 2×10^6 cells were placed into 60-mm tissue culture plates and overlaid with puromycin free medium. Then 24 hr before collecting vector stocks, medium was again replaced with 3 ml of fresh puromycin-free medium and cells were placed at 5% CO_2 and 32°C for the production of virus. This procedure was carried out for the production and collection of vector at time points 6, 10, 14, 18, and 30 days post-transfection for pBabePuro^{EBV} and at time points 10 and 28 days post-transfection for LZRS-LacZ(A). Collected vector stocks were placed in 1-ml aliquots and stored at −80°C to await titering at a later time point.

Titer determination

Vector production for the construct pBabePuro^{EBV} was determined using both the ecotropic BOSC23 and the amphotropic BING retroviral packaging cell lines. Frozen vector stocks collected from time points 2, 6, 10, 14, 18, and 30 days post-transfection were serially diluted 10^4-, 10^5-, and 10^6-fold. Diluted vector stocks were overlaid onto 5×10^5 NIH-3T3 cells in 100-mm tissue culture plates. Polybrene was then added to the vector-containing medium to a final concentration of 8 μg/ml. Cells were incubated at 37°C and 5% CO_2 for 24 hr, after which time

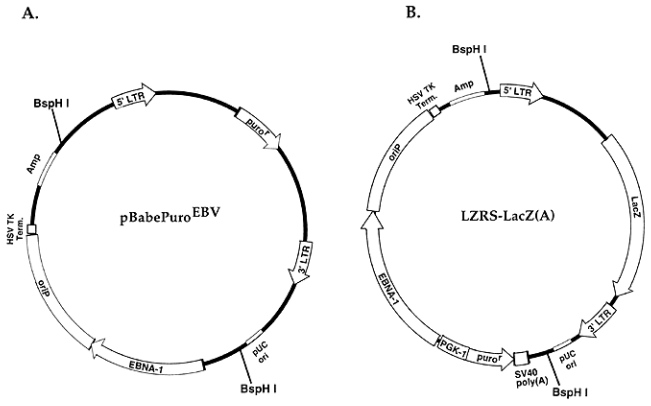

FIG. 1. A. pBabePuro^{EBV} was constructed in a series of steps from p220.2 and the retroviral construct pBabePuro: pBabePuro was digested with *Sal* I and *Hind* III, blunted, and religated to create pBabePuro(ΔSV40). A small adapter (5′ *Bgl* II, *Hind* III, *Xho* I, *Eco* RI-3′[b] was then inserted into the unique *Not* I site of pBabePuro(ΔSV40) to yield pBabePuro(ΔSV40)A. The *Eco* RI–*Bam* HI fragment of p220.2, which contains the EBV *oriP* elements and the EBNA-1 gene, was inserted into the *Bgl* II and *Eco* RI adapter sites of pBabePuro(ΔSV40)A to create pBabePuro^{EBV}. B. The construction of LZRS-LacZ(A) was as follows: an adapter containing *Eco* RI and *Bgl* II sites was inserted into the *Eco* RI site of pBabePuro^{EBV}. The *Eco* RI–*Bam* HI fragment of PGKPuro, which contained the PGK-1 promoter, the puromycin resistance gene, and SV40 polyadenylation sequences, was then placed into the *Eco* RI and *Bam* HI sites of the adapter to create pREPP(A). The transcriptional orientation of the PGKPuro *Eco* RI–*Bam* HI fragment is opposite that of the EBNA-1 gene. *Bsp* HI digestion of pREPP(A) and pBabeM-lacZ, followed by ligation of the appropriate fragments, yielded LZRS-LacZ(A).

the medium was replaced with fresh medium and cells were incubated under identical conditions for an additional 24 hr. At 48 hr post-infection culture medium was replaced with 15 ml of medium that had been supplemented with puromycin to a final concentration of 2.5 μg/ml. After 5 days of puromycin selection, the medium was carefully replaced with 15 ml of fresh, puromycin-containing medium (final concentration, 2.5 μg/ml) and resistant cell populations were allowed to grow for an additional 8 days. Colony numbers were determined by ethanol fixation and methylene blue staining. Titer (transducing units/ml) was calculated as: (number of colonies) × (dilution of infecting retrovirus)/(total volume of diluted vector overlaid onto cells).

LZRS-LacZ(A) was tested for vector production capabilities using BOSC23 cells. Frozen vector stocks collected from transfected BOSC23 cells at 2, 10, and 28 days post-transfection were thawed, serially diluted 50-, 100-, and 1,000-fold, supplemented with 8 μg/ml Polybrene, and used to infect 5×10^5 NIH-3T3 cells. Infected cells were assayed by FACS-Gal analysis at 48 hr post-infection (Nolan *et al.*, 1988; Krasnow *et al.*, 1991). Viral titers were determined by applying the following formula: (percent cells positive by FACS-Gal analysis) × (number of cells at time of infection) × (dilution of infecting retrovirus)/(total volume of diluted vector overlaid onto cells) = Transducing units/ml.

Helper virus assays

NIH-3T3 BAG cells (Price *et al.*, 1987), which contain stable proviral copies of a *lacZ* recombinant retrovirus, were infected with vector stocks produced at days 2, 6, 10, 14, 18, and 30 post-transfection of BOSC23 cells with pBabePuro^{EBV}. Infected NIH-3T3 BAG cells were trypsinized, diluted 1:5, and replated at days 2, 5, and 8 post-infection. Medium was collected 10 days after the initial infection, supplemented with Polybrene (8 μg/ml), passed through a 0.45-μm syringe filter, and overlaid onto NIH-3T3 cells. And 48 hr later, cells were fixed and assayed for β-galactosidase (β-Gal) activity by X-Gal staining to test for the presence of helper-conferring activities (Krasnow *et al.*, 1991).

Sensitivity of the helper-virus rescue assay was determined by serially diluting replication-competent Moloney viral stocks and performing rescue assays with a total of 3 ml of diluted virus as described above. Starting titers of Mo-MuLV stocks

were determined by parallel infections of 2×10^5 NIH-3T3 cells with serially diluted virus, followed by FACS analysis of Env surface expression at 24 hr post-infection. Sensitivity was calculated as: (titer of replication-competent Mo-MuLV stock)/(last serial dilution of replication-competent Mo-MuLV stock at which *lacZ* rescue occurred).

Episome stability analysis

BOSC23 cells that had been transfected with pBabePuro^{EBV} and maintained in puromycin selection were harvested at days 6, 10, 14, and 30 and Hirt extractions were performed (Hirt, 1967). Isolated episomal DNA was subjected to digestion with *Dpn* I (to remove DNA that was not replicated in human cells) and subsequently electroporated into *Escherichia coli* Stable II cells (GIBCO-BRL, Grand Island, NY). Ten individual colonies were selected from each time point and inoculated into 5-ml liquid LB/AMP cultures; plasmid DNA was isolated after 16 hr of culture. Isolated plasmid DNA was then analyzed for potential gross rearrangements by digestion with *Kpn* I and *Bam* HI.

Southern blot analysis was conducted on BOSC23 cells that had been selected for episomal LZRS-lacZ(A) and on NIH-3T3 cells infected with retrovirus produced from these packaging cells. BOSC23 cells were lipofected with LZRS-lacZ(A) utilizing lipofectAMINE according to the manufacturer's protocol (GIBCO-BRL, Grand Island, NY). Lipofected BOSC23 cells were selected in puromycin-selective medium as described above and passaged in culture for 30 days. After collecting virus at day 30, genomic DNA was isolated from 7×10^6 BOSC23 cells by standard methods and digested with either *Nhe* I or *Bsp* HI for Southern blot analysis. Vector stocks produced at day 30 were used to infect 8×10^5 NIH-3T3 cells as described above. When the population of infected NIH-3T3 cells had expanded to 1.5×10^7 cells, genomic DNA was isolated by standard methods and digested with *Nhe* I for Southern blot analysis. All digested genomic samples were separated on an 0.8% agarose gel, subjected to acid hydrolysis, transferred to Hybond-N$^+$ membrane (Amersham), UV cross-linked, and probed with the products of a randomly primed reaction (*redi*prime DNA labeling system, Amersham) using the gel-purified *Eco* RI fragment of *lacZ* from LZRS-lacZ(A). Membranes were hybridized overnight, washed twice with 0.1% SDS and 0.1 × SSC at 42°C, and exposed to film for 4 days.

RESULTS

Conventional recombinant retroviral construct DNA persists in an extrachromosomal form only transiently after transfection into retroviral packaging cell lines. Unless construct DNA is stably integrated into the host cell genome, production of recombinant vector is limited to the first 4–6 days after transfection of retroviral constructs into the packaging line. Here, we show that imparting episomal maintenance functions to retroviral constructs can extend the production capabilities of retroviral packaging systems without requiring the selection of individual stable clones. We have utilized two elements from the Epstein–Barr virus, *oriP* and EBNA-1, to confer stable episomal maintenance capabilities to retroviral constructs. Because stable episomal establishment of EBV-based constructs occurs at an efficiency approaching the transient transfection rate (Dubridge *et al.*, 1987; M. Calos, personal communication), it was possible to rapidly generate a population packaging cells stably producing high titers of helper-free recombinant retrovirus vector.

EBV-based retroviral constructs

Two prototype vector plasmids were constructed (Fig. 1). The first of these constructs, designated pBabePuro^{EBV}, was constructed in a series of steps from the retroviral plasmid pBabePuro and the EBV-based plasmid p220.2. The second construct, LZRS-LacZ(A), differs organizationally from pBabePuro^{EBV} in that the puromycin-resistance gene, now under the control of the phosphoglycerol kinase-I promoter, has been moved into nonretroviral portions of the plasmid. This reorganization continued to allow rapid selection of transduced packaging cells while freeing potential subcloning space within retroviral portions of the hybrid constructs. In addition, 5′ LTR and Ψ packaging sequences present within the LZRS-LacZ(A) vector construct were derived from the MFG series of retroviral vector plasmids.

Long-term virus production

Initial experiments utilizing the pBabePuro^{EBV} construct tested the short- and long-term ability of these EBV-based retroviral constructs to produce retroviral vector. pBabePuro^{EBV} was transfected into both the ecotropic BOSC23 and the amphotropic BING retroviral packaging cell lines. Two days after transfection, vector stocks were harvested and frozen. Cells were then replated into puromycin-containing medium to select for episomally resident plasmids. The presence of EBNA-1/*oriP* in the backbone of the pBabePuro^{EBV} construct conferred stable puromycin resistance in a high proportion of the transfected cell population. In contrast, puromycin resistance in pBabePuro(ΔSV40)A transfected packaging cells dropped rapidly after 6 days, indicating a loss of vector production likely due to its inability to be actively partitioned or maintained as an episome. Vector produced from cells containing stably-maintained and selected episomes was collected on days 6, 10, 14, 18, and 30 post-transfection. Infection of NIH-3T3 cells demonstrated the ability of these hybrid constructs to produce high-titer retroviral vector as soon as 48 hr and for as long as 30 days after transfection (Fig. 2). Vector titers were typically in the range of 10^7 TU/ml at 2 days post-transfection; however, in some instances puromycin selection of packaging cells containing pBabePuro^{EBV} resulted in titers as high as 10^8 TU/ml.

The construct LZRS-LacZ(A) was tested for retroviral vector production ability after transfection into BOSC23 cells. After infection of NIH-3T3 cells, vector titers were determined by FACS-Gal analysis (Fig. 3). This vector construct performed in a manner similar to pBabePuro^{EBV}, producing high-titer vector at all tested time points up to 28 days. Titers achieved with LZRS-LacZ(A) were estimated to be in the range of 3×10^6 TU/ml.

Helper-free vector production and episomal stability

An important feature of retroviral packaging systems is their ability to produce recombinant retroviral vector without the gen-

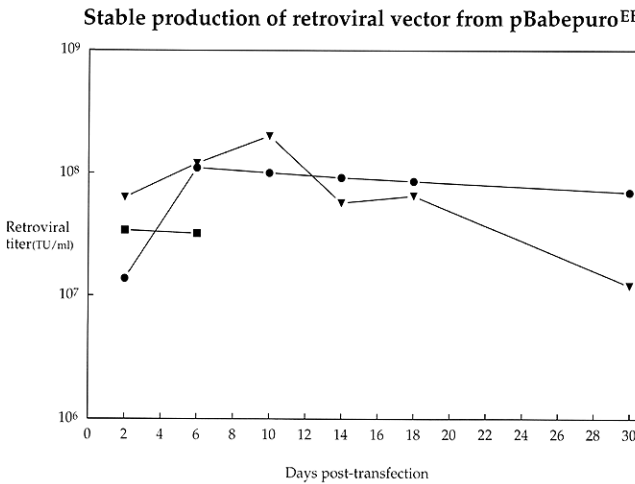

FIG. 2. The hybrid construct pBabePuro^{EBV} was tested for vector production in both the ecotropic BOSC23 and the amphotropic BING retroviral packaging lines. Frozen vector stocks from time points 2, 6, 10, 14, 18, and 30 days post-transfection were serially diluted 10^2-, 10^5-, and 10^6-fold and overlaid onto approximately 5×10^5 NIH-3T3 cells as per Materials and Methods. Infected cells were selected with 2.5 μg/ml puromycin, and resistant colonies were fixed, stained with methylene blue, and counted at 13 days post-infection. All transfections, selections, dilutions, and infections utilizing pBabepuro^{EBV} were performed in duplicate and data presented in this figure represents the calculated titer averaged from a 10^6-fold dilution/infection series. Symbols: (●) Vector titer from amphotropic BING retroviral packaging line transfected with the hybrid construct pBabePuro^{EBV}; (▼) vector titer from ecotropic BOSC23 retroviral packaging line transfected with the hybrid construct pBabePuro^{EBV}; (■) vector titer from ecotropic BOSC23 retroviral packaging line transfected with the control retroviral construct, pBabePuro(ΔSV40)A, which contained no EBV sequences. Titer could not be determined for pBabePuro(ΔSV40)A after day 6 due to the death of greater than 99% of the producer cell population after puromycin selection.

eration of replication-competent helper virus. Retrovirus produced from pBabePuro^{EBV} was tested for the presence of helper virus activities by infecting NIH-3T3 BAG cells and subsequently assaying for the ability of supernatant from these cells to transfer the *lacZ* phenotype to naive NIH-3T3 cells. Recombinant vector stocks were free of replication-competent retrovirus at all time points tested, from days 2 to 30, as assayed by BAG cell rescue experiments. Sensitivity of the BAG cell rescue assay was determined by utilizing serially diluted replication-competent Moloney retrovirus. Mo-MuLV viral stocks with a titer of 1.2×10^7 TU/ml allowed BAG cell rescue of *lacZ* provirus at serial dilutions of up to 10^7, demonstrating this assay could detect as few as 1.2 helper viruses per milliliter of supernatant (data not shown).

Hirt extraction of pBabePuro^{EBV}-transfected packaging cells at days 6, 10, 14, and 30 and electroporation into *E. coli* Stable II cells demonstrated that these episomal constructs were replicated in mammalian cells as episomes and could be rescued (data not shown). The restriction analysis of vector construct DNA isolated from 40 individual bacterial colonies indicated that no gross rearrangements occurred within these hybrid constructs at any of the time points after transfection. Restriction analysis of representative samples from each time point is shown in Fig. 4.

EBV/retroviral episomes, as well as virus generated from these constructs, were further analyzed by Southern blot analysis (Fig. 5). Figure 5 demonstrates that no detectable rearrangements occur after 30 days of episomal maintenance in LZRS-lacZ(A)-lipofected BOSC23 cells or in NIH-3T3 cells infected with virus produced at 30 days post-lipofection of BOSC23 cells.

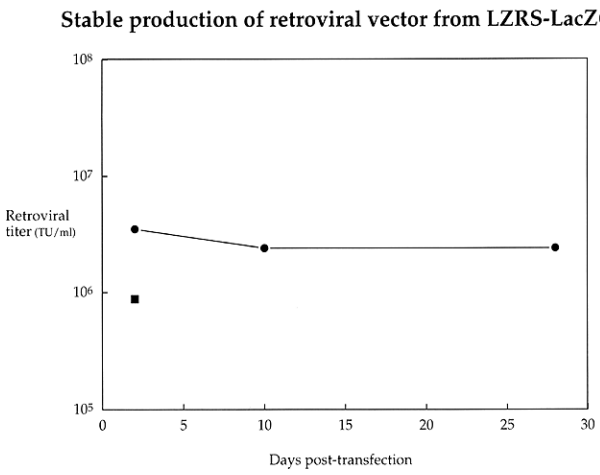

FIG. 3. LZRS-LacZ(A) was tested for vector production using the ecotropic BOSC23 retroviral packaging cell line. Vector from 2, 10, and 28 days post-transfection was serially diluted 50-, 100-, and 1,000-fold, and used to infect NIH-3T3 cells. Infected cells were assayed by FACS-Gal analysis 48 hr after transduction. Data presented in this figure represents a single, representative series of 10^6-fold dilutions/infections. Symbols: (●) Vector titer from ecotropic BOSC23 packaging line transfected with the retroviral/EBV hybrid construct LZRS-LacZ(A); (■) vector titer from ecotropic BOSC23 packaging line transfected with the control retroviral construct pBabeM-LacZ. Titer could not be determined for pBabeM-LacZ after day 2 due to the death of greater than 99% of the producer cell population after puromycin selection.

DISCUSSION

The production of recombinant retroviruses for academic and/or clinical research has been hampered by a number of technical limitations. The most limiting aspects of recombinant retroviral vector production have been manifested in the areas of titer capabilities, time and labor requirements, and the ability to readily produce large volumes of retrovirus vector for extended time periods. Although some retroviral production systems have overcome certain of these limitations, none are capable of individually and efficiently surmounting all of these restrictions. We have demonstrated that EBV-based retroviral constructs are an effective step toward meeting these key production needs, enabling both rapid and long-term production of high-titer retroviral vector stocks.

Three requirements must be met if circular plasmid DNA is to be stably maintained as an episome within dividing cells (Biamonti *et al.*, 1985; Reisman *et al.*, 1985; Yates *et al.*, 1988; Krysan *et al.*, 1989). First, the DNA construct must replicate; second, the construct must have an nuclear retention function; and, third, there must be efficient partitioning of replicated construct DNA into daughter cells. The human EBV, which is a member of the Herpesviridae family, contains two elements capable of cooperating to provide replication, nuclear retention, and partitioning: *oriP* and the EBNA-1 gene. *oriP* consists of two sets of sequence motifs—a region of dyad symmetry and a series of 20 direct repeats. EBNA-1 binds to both sets of motifs within *oriP*, initiating once per cell cycle replication from the region of dyad symmetry and promoting nuclear retention and partitioning through interactions at the direct repeats (Yates *et al.*, 1984; Rawlins *et al.*, 1985; Reisman and Sugden, 1986; Krysan *et al.*, 1989). The *oriP* dyad, the family of direct repeats, and EBNA-1 can stably maintain episomes at copy a number of 5–20 per cell (Lupton and Levine, 1985; Yates *et al.*, 1985; Dubridge *et al.*, 1987). In the absence of selection, episomal constructs are lost at a low, but constant, rate (Reisman *et al.*, 1985; Haase *et al.*, 1989; Krysan and Calos, 1993).

The first of our prototype constructs, pBabePuro^{EBV}, utilized *oriP* and EBNA-1 DNA sequences to impart episomal replication and retention functions to a retroviral vector construct. pBabePuro^{EBV} produced high-titer vector as soon as 48 hr and for at least 30 days after transfection into the ecotropic BOSC23

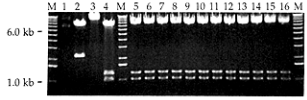

FIG. 4. Hirt extractions were performed on BOSC23 cells at 6, 10, 14, and 30 days post-transfection with pBabePuro^{EBV}. Isolated vector DNA was subjected to *Dpn* I digestion and electroporated into *E. coli* stable II cells. Plasmid DNA was isolated from colonies derived from each time point and analyzed by digestion with *Kpn* I and *Bam* HI. Lanes 1–4, Control plasmid; lane 1, uncut pBabePuro^{EBV}; lane 2, *Kpn* I digestion of pBabePuroEBV; lane 3, *Bam* HI digestion of pBabePuro^{EBV}; lane 4, *Kpn* I and *Bam* HI digestion of pBabePuroEBV; lanes 5–7, *Kpn* I and *Bam* HI digestion of isolated vector DNA derived from day 6 post-transfection of BOSC23 cells; lanes 8–10, *Kpn* I and *Bam* HI digestion of isolated vector DNA derived from day 10 post-transfection of BOSC23 cells; lanes 11–13, *Kpn* I and *Bam* HI digestion of isolated vector DNA derived from day 14 post-transfection of BOSC23 cells; lanes 14–16, *Kpn* I and *Bam* HI digestion of isolated vector DNA derived from day 30 post-transfection of BOSC23 cells. M indicates 1-kb DNA marker.

and/or amphotropic BING retroviral packaging cell lines. Despite the fact that the initial transfections of the BOSC23 and BING cells were efficient (75–85%, data not shown), stable selection of transduced cells resulted in significant increases in viral titers estimated to be as high as 10^6 TU/ml. In some instances, vector titers were increased approximately 6- to 11-fold after selection. Such increases in vector production after selection cannot be explained by the increase in the percentage of cells carrying retroviral vector construct, because transient transfection rates were consistently high and producer cell numbers were controlled throughout the experiments. Several possibilities could account for the unexpected increase in vector titers from our hybrid constructs after selection of stable cells. First, it is possible that the transfection procedure itself may adversely affect transient retroviral vector production. Therefore, stable retroviral vector production for time periods longer than possible with the transient transfection of conventional constructs may allow vector to be generated under more optimal conditions for producer cells. Second, because the puromycin-resistance gene present in the pBabePuro^{EBV} construct is under the transcriptional control of the viral LTR, growth of producer cells in selective media is based directly on retroviral LTR expression. This could create a scenario in which the selection pressure results in a population of cells and/or episomal constructs with strong LTR transcription, leading to producer populations capable of generating higher titers of retrovirus.

A second construct, LZRS-LacZ(A), provided a more useful genetic organization for production of vector and also tested the general applicability of the EBV episomal production concept. In LZRS-LacZ(A), the puromycin-resistance gene has been relocated from within the retroviral genomic region to a nonretroviral portion of the plasmid backbone. This reorganization continued to allow rapid and stable selection of transduced packaging cells while freeing potential subcloning space within the transduced regions of the retrovirus. This construct demonstrated episomal maintenance and long-term vector production in a manner similar to that of pBabePuro^{EBV}. However, vector production from LZRS-LacZ(A) was less efficient than pBabePuro^{EBV}. The observed differences in vector production between the two construct designs might be explained by the proximity and orientation of the puromycin-resistance gene relative to the viral 3′ LTR in LZRS-LacZ(A). For example, opposing transcription from the retroviral LTR(s) may directly compromise expression from the downstream puromycin-resistance gene, creating conditions that favor puromycin expression at the expense of retroviral transcription when cells are grown in selective medium. Also, in the absence of selection pressures favoring LTR expression, epigenetic shutdown of

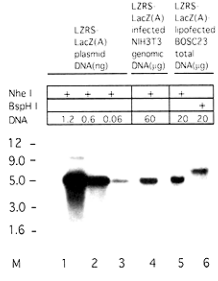

FIG. 5. Southern blot analysis of NIH-3T3 cells infected with retroviral vector produced at day 30 post-lipofection of BOSC23 cells with LZRS-LacZ(A) (lane 4). Southern Blot analysis of LZRS-LacZ(A) lipofected BOSC23 cells after 30 days of puromycin selection (lanes 5 and 6). Samples were probed with the products of a randomly primed Klenow polymerase reaction utilizing the *Eco* RI fragment from LZRS-LacZ(A) as the starting template. Lanes 1–3, Control plasmid; lane 1, *Nhe* I digestion of 1.2 ng of LZRS-LacZ(A) plasmid DNA; lane 2, *Nhe* I digestion of 0.6 ng of LZRS-LacZ(A); lane 3, *Nhe* I digestion of 0.06 ng of LZRS-LacZ(A) plasmid DNA; lane 4, *Nhe* I digestion of 60 μg of genomic DNA from LZRS-LacZ(A)-infected NIH-3T3 cells; lane 5, *Nhe* I digestion of 20 μg of total DNA (genomic and episomal) from LZRS-LacZ(A) lipofected BOSC23 cells; lane 6, *Bsp* HI digestion of 20 μg of total DNA (genomic and episomal) from LZRS-LacZ(A) lipofected BOSC23 cells. Data in Lanes 1–4 were obtained from a single Southern blot (intervening lanes between control plasmid and NIH-3T3 genomic DNA were removed for figure clarity). Data presented in lanes 5–6 were obtained from an single independent Southern blot under identical conditions. M represents the position of 1-kb ladder fragments. *Nhe* I cleaves within the U3 region of both retroviral LTRs, releasing a 5080-bp fragment from LZRS-LacZ(A)-derived sequences. *Bsp* HI cleaves at sites 5′ and 3′ to the retroviral LTR within puc-derived plasmid sequences, yielding a 6,708-bp restriction fragment.

retroviral LTRs could lead to a loss of recombinant vector RNA production. Thus, repositioning or modifying the transcriptional regulatory elements of the puromycin-resistance gene might circumvent these problems and result in vector capabilities similar to pBabePuro[EBV].

Many research and clinical applications depend on retroviruses as a tool for gene transduction (Dai *et al.*, 1992; Krauss, 1992). As outlined, technical limitations inherent in retroviral production systems can be a limiting factor in application of this valuable technology. Although highly transfectable cell lines based on human 293T or primate COS-7 cells have demonstrated successful short-term vector production, transient transfection efficiencies can vary—this can be problematic for vector production. Conferring episomal maintenance on retroviral constructs can ensure a large population of stably expressing producer cells in a very short period of time, even if the initial transient transfection efficiencies were less than desirable. These hybrid EBV/retroviral constructs should greatly improve the production capabilities and versatility of human cell line-based retroviral packaging systems. Furthermore, episomal production strategies similar to those described here may prove useful when applied to other recombinant vector production systems.

ACKNOWLEDGMENTS

We thank Philip Achacoso, Chaitanya Pai, and Edward Huynh for technical assistance. We are indebted to members of the Nolan laboratory for helpful suggestions. We thank Michelle Calos and members of her laboratory for useful discussion and gifts of plasmids. This work was supported by grants from the National Institutes of Health (grant AI35304), Systemix, Inc., and a gift from Tularik, Inc. T.K. is supported by a NIH training grant and G.P.N. is a Scholar of the Leukemia Society of America and a recipient of the Burrough's Wellcome New Investigator Award in Pharmacology.

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Received for publication October 3, 1995; accepted after revision April 4, 1996.