Infectious Epstein–Barr Virus Vectors for Episomal Gene Therapy

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Introduction

The Epstein–Barr virus (EBV) is a B-lymphotropic γ-herpesvirus with a 172 kb double-stranded DNA genome. The virus primarily infects human B-lymphocytes by specific binding of the viral protein gp350/220 to the receptor (CD21) present on the B cell surface followed by receptor-mediated endocytosis. A lytic infection begins with viral DNA, RNA, and protein synthesis, followed by the assembly of viral proteins and lysis of the host cells. Alternatively, the more common latent, nonlytic infection can occur, in which the virus establishes itself in the nucleus as a large circular extrachromosomal replicating plasmid known as an episome that is maintained through subsequent cell division.

Ever since it was shown that a vector carrying the EBV latent replication origin (OriP) and the viral nuclear antigen gene (EBNA1) was sufficient for plasmid replication in lymphoblastoid cell lines (LCLs), investigators have tried to develop EBV-based vectors for gene transfer.1–4 In our laboratory, we have previously developed a human artificial episomal chromosome (HAEC) system based on the EBV OriP for propagation and stable maintenance of large DNA inserts ranging from 60 to 330 kb as circular minichromosomes in human cells.5,6 Such a large cloning capacity of an EBV-based vector is ideal for gene therapy and led us to develop infectious viral vectors for delivery of therapeutic genes as HAECs into human cells.

Gene therapy based on HAECs has the following advantages: first, the autonomous replication of HAECs reduces the potential pathological risks associated with “uncontrolled” chromosomal integration occurring with retroviral and adenovirus-associated viral systems. Second, an episomal vector may also avoid the variability

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in expression frequently observed with integrated sequences. Third, autonomous replication is expected to increase the long-term persistence of a transferred gene in cells in comparison to nonreplicating epichromosomal systems based on adenoviruses and herpes simplex virus-1 (HSV-1). Fourth, gene therapy requiring the stable maintenance of large DNA molecules in target cells may be more effective following an episomal strategy than the rather inefficient process of chromosomal integration. Finally, the large cloning capacity of HAECs allows for delivery of multiple therapeutic genes in tandem or large human genes with their native regulatory sequences. This is particularly beneficial for designing vectors with a multigenic strategy to attack polygenic disorders such as cancers, or vectors with large therapeutic genes that require their own regulatory elements to be functional.

Infectious EBV vectors developed in our laboratory deliver such therapeutic HAECs into target cells by transduction. Such a transduction strategy is based on a recombinant amplicon carrying a transgene expression cassette and the minimal viral cis elements required for replication (i.e., the origins for latent and lytic replication, OriP and OriLyt) and packaging (i.e., terminal repeat sequences, TR) as infectious particles (referred to as miniEBV) (Fig. 1A). The miniEBV vector is dependent on the nontransforming packaging cell line HH514 (P3HR1 EBV strain)7 for providing in trans the lytic replication and packaging functions to process and package the recombinant amplicon DNA. When miniEBV virions are produced and purified from this helper cell line, they are mixed with the endogenous P3HR1 EBV (Fig. 1B), which is replication-competent and transforming-incompetent.7,8

Because of the natural B lymphotropism of EBV in humans, such miniEBV vectors naturally target inherited B-cell diseases and B-cell lymphomas. Infectious miniEBV virions were shown not only to express reporter genes such as lacZ9 and GFP10 in the infected human disease B cells, but also to correct phenotypically the defects in LCLs established from Fanconi anemia and Lesch–Nyhan patients.11,12 It was demonstrated that this infectious miniEBV was able to carry inserts in the range of 140–160 kb and was also episomal in the infected B cells.9,12

A rapidly emerging technology for the treatment of various cancers involves the delivery of suicide genes to the affected cells, followed by prodrug treatment that induces cells expressing suicide gene products to undergo cell death.13,14 We have

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Fig. 1. MiniEBV-based vector and helper-dependent packaging system. (A) The structure of miniEBV amplicon. The shaded regions are minimal cis elements required for episomal replication (OriP), amplification (OriLyt), and packaging of the vector (TR). OriP, plasmid origin of replication; OriLyt, lytic replication origin; TR, terminal repeat sequences. "C" indicates the complementary strand in this vector relative to its presence in the native EBV genome. Hygromycin B resistance gene (HygR) and ampicillin resistance gene (amp) were engineered for selection in mammalian and prokaryotic cells, respectively. Two restriction sites (HindIII and BamHI) used for cloning of the transgene expression cassette are indicated. The transgenes could be a reporter gene such as lacZ or GFP, a therapeutic gene for an inherited disease, and a suicide gene for cancer therapy. (B) Helper-dependent miniEBV packaging and delivery of transgenes by miniEBV infection. An octameric miniEBV genome that is packaged into virions is shown. The black bar stands for a transgene cassette. LCLs, B lymphoblastoid cell lines; wtEBV, the helper P3HR1 or the endogenous EBV associated with LCLs or lymphoma cells, or both. Two possible applications of infectious miniEBV vectors are shown: (I) treatment of inherited human diseases that required prolonged expression of the transgene to correct the defective phenotype of disease cells (indicated by shaded LCLs); (II) treatment of cancers using suicide strategy to kill the malignant cells (indicated by a cross on lymphoma cells).

developed such infectious miniEBV-based suicide vectors for possible use in gene therapy of B lymphomas: an infectious, recombinant minEBV carrying the HSV-1 thymidine kinase (TK) gene expression cassette has been shown to successfully transduce both EBV positive and negative lymphoma cells. Moreover, on treatment

with the prodrug ganciclovir (GCV), the growth inhibition of the miniEBV/TK-transduced lymphoma cells cultured \emph{in vitro} or implanted in severe combined immunodeficiency (SCID) mice has been observed.\textsuperscript{15}

\textbf{Methods}

\textit{Generation and Titration of MiniEBV Vector Stocks}

\textit{Transfection of Helper Cell Line HH514 with MiniEBV Amplicon DNA.} HH514 cells were grown in RPMI-1640 with 10\% fetal bovine serum (FBS) and supplemented with 1\% L-glutamine and 0.1\% penicillin/streptomycin. Five million exponentially grown HH514 cells and 20 \(\mu\)g of miniEBV amplicon DNA were mixed in 0.3 ml of complete growth medium in a 0.4 cm electroporation cuvette (Bio-Rad) and incubated on ice for 10 min. Electroporation was carried out at 200 V and 960 \(\mu\)F with a Bio-Rad Gene Pulser. After electroporation, cells were incubated on ice for another 10 min, then seeded into 10 ml of complete growth medium, and incubated at 37\(^{\circ}\)/5\% CO\(_2\). Three days after electroporation, hygromycin B (Boehringer-Mannheim Products) was added to a final concentration of 200 \(\mu\)g/ml to select for stable cell transformants.

\textit{Preparation of MiniEBV (and EBV) Virions.} The stable HH514 cell transformants were cultured in a T-300 flask to near confluence (2 - 4 \(\times\) 10\(^6\) /ml) in a total volume of 500 ml. The lytic phase of EBV was induced by adding to the medium 20 ng/ml 12-O-tetradecanoylphorbol 13-acetate (TPA, Sigma) and 1 mM n-butyric acid (sodium butyrate, Sigma). Five days later, supernatants were collected by centrifugation at 6000g for 10 min at 4\(^{\circ}\). The cell pellet was resuspended with 10 ml complete growth medium and subjected to three cycles of freeze/thaw process in a dry ice/ethanol bath and a 37\(^{\circ}\) water bath. Cell debris was spun down at 6000g for 10 min at 4\(^{\circ}\) and discarded. The supernatants were recovered, combined with previous collection, and treated with DNase I (20 U/ml, Sigma) for 30 min at room temperature to destroy nonpackaged DNA. The DNase I reaction was stopped with addition of 20 mM EDTA (pH 8.0) and 0.1\% of sodium azide to the medium. The supernatants were then filtered through a 0.45-\(\mu\)m filter (Corning) to eliminate all cell debris. Virions were pelleted by centrifugation at 12,500g for 2 hr at 4\(^{\circ}\) and resuspended in 2.5 ml RPMI-1640 without serum. Virions can be used for titration or infection assay directly.

The virus suspension can be aliquotted in glass vials, then quickly frozen in a dry ice/ethanol bath and stored at \(-80^{\circ}\). To minimize the loss of viral titer, we emphasize the use of glass vials other than plastic tubes and low temperature (\(-80^{\circ}\)) for storage. When removing the virus stock from the freezer for use, we recommend rapid thawing in a 37\(^{\circ}\) water bath with swirling of the tube to avoid localized warming. Repeated freezing and thawing of the virus stock should also be avoided.

Determination of Physical Titer of MiniEBV Vectors. In the absence of a lysis plaque- or colony-transforming assay for the infectious nontransforming miniEBV virions, we used the number of physical particles (i.e., physical titer) as a measure of the MOI (multiplicity of infection). Packaged DNA was isolated from virions by proteinase K digestion at 42°C for 1 hr in a solution containing 100 μg/ml proteinase K, 0.2% SDS, 8 mM EDTA (pH 8.0), and 50 mM NaCl, followed by phenol extraction and ethanol precipitation. The purified DNA was subjected to pulse-field gel electrophoresis (PFGE). PFGE was carried out on a CHEF apparatus in 1% agarose and 0.5x TBE at 200 V for 20 hr at a switching time of 15 sec. After electrophoresis, DNA was transferred from agarose gels to nitrocellulose membranes and hybridized with radioactively labeled probes specific for miniEBV or helper EBV. After hybridization, radioactive DNA signals were detected by autoradiography. The yield of the viral vector was established by using a copy number standard of plasmid DNA carrying miniEBV- and EBV-specific sequences. Because of the concatemeric nature of packaged miniEBV DNA (Fig. 1B), the miniEBV signal was normalized to the monomeric level. An average of 4–6 × 10^5 recombinant miniEBV particles were produced per milliliter of virus suspension.12

Gene Transduction in Human Disease B Cells with Infectious MiniEBV

We tested the potential of infectious miniEBV vectors for stable transfer of a therapeutic gene in LCLs from a Fanconi anemia group C patient (HSC536) and an HRPT-deficient Lesch–Nyhan patient (RJK853), and in an EBV-positive lymphoma-derived cell line (Raji).11,12 Five million exponentially grown cells were harvested by centrifugation at 500g for 5 min and resuspended in 1 ml of fresh RPMI-1640 without serum. Virus particles were added at an MOI of 10 into cells, and the mixture was incubated at 37°C/5% CO₂ for 1 to 2 hr. After incubation, the cells were seeded into 10 ml complete growth medium and incubated at 37°C/5% CO₂. Three days after infection, hygromycin B was added to the cells (final concentration 200 μg/ml) to select stable transformants for analysis of the intranuclear fate of the miniEBV DNA described below. To determine the transduction efficiency of miniEBV on target cells, a miniEBV carrying the reporter gene (lacZ or GFP) was produced from HH514 cells and used for infection of target cells. Cells were subsequently subjected to X-Gal staining (for lacZ expression)11,12 or flow cytometry (for GFP expression, Fig. 2).

Intranuclear Fate of MiniEBV DNA in Transduced Cells

Episomal Maintenance of MiniEBV DNA in Stably Transduced Cells. Approximately 2 months after hygromycin B selection, the potential for episomal maintenance of the transduced miniEBV DNAs was examined in the stable transformants by agarose gel electrophoresis. Episomal DNA was extracted from stably
transduced cells as previously described with modifications.\textsuperscript{11,12} Cells ($1 \times 10^8$) were collected by centrifugation at 500g for 5 min, washed once with 2 ml of phosphate-buffered saline (PBS), and then mixed thoroughly with 10 ml of lysis solution (50 mM NaCl, 8 mM EDTA, 1% SDS, pH 12.45) by vigorous vortex for 2 min. The mixture was incubated at 30° for 30 min. Two milliliters of 1 M Tris-Cl (pH 7.0) was added and mixed by gently swirling the tube, followed by addition of 1.32 ml of 5 M NaCl and 0.12 ml of 10 mg/ml proteinase K. The mixture was incubated at 37° overnight. The lysate was then extracted three times with 13 ml of phenol saturated with 0.2 M NaCl and 0.2 M Tris-Cl (pH 8.0) and one time with equal volume of chloroform by gently swirling the tube (vigorous vortex will break the large episomes and thus should be avoided). After centrifugation, the aqueous phase was collected, mixed with 30 ml of ethanol, and incubated at $-20^\circ$ overnight. The episomal DNA was spun down and resuspended in 50 μl of 10 mM Tris-Cl (pH 8.5). The episomal DNA was then separated on a 0.85% agarose gel that was run in 1× TBE at 5.4 V/cm for 10 to 11 hr at 4°. The samples were transferred to nylon membranes for hybridization with a probe specific for miniEBV or EBV. As shown in Fig. 3A, the supercoiled episomal DNA that is close to natural EBV genome can be found between linear DNA (running ahead) and nicked DNA (running behind or retained in wells). MiniEBV DNAs were maintained as 160–180 kb circular DNA molecules in stably transduced cells.\textsuperscript{11,12}
FIG. 3. Intranuclear fate of the transduced miniEBV DNA. (A) Episomal maintenance of miniEBV DNA in transduced cells. Infectious miniEBV (pH210) (J. Wang, S. Banerjee, and J. M. Vos, unpublished data) was produced from the nontransforming helper cell line HH514 and used for infection of EBV-positive lymphoma line Raji and EBV-negative Burkitt’s lymphoma line DG-75. Episomes were extracted and hybridized with a probe specific for miniEBV (hygromycin resistance gene, HygR), or a probe specific for EBV (BamHI W). The signals in lanes 5 and 6 reflect the EBV from both the helper HH514/P3HR1 and the endogenous Raji/EBV, whereas the signal in lane 8 indicates the helper HH514/P3HR1 EBV only. (B) Chromosomal association, but not integration of the miniEBV episomal DNA. The B lymphoma Raji cells were stably transduced with miniEBV (pH265). Interphase and metaphase spreads from uninfected (top) or infected (bottom) cells were prepared and hybridized with a Fluor-12-dUTP labeled probe using FISH technique to detect miniEBV (pH265) only. [This figure is adapted with permission from T.-Q. Sun, E. Livanos, and J.-M. H. Vos, Gene Ther. 3, 1081 (1996).]

Chromosomal Association, but Not Integration of MiniEBV Episomal DNA. To distinguish integrated from episomal miniEBV DNA, fluorescence in situ hybridization (FISH) with vector-specific probes was performed on metaphase spreads of the miniEBV-transduced cells. Integrated miniEBV DNA is expected to result in double, closely spaced, symmetrical signals localized on both sister chromatids while episomal miniEBV DNA will generate single FISH signals.

Preparation of interphase and metaphase spreads was described previously. Ten milliliters of miniEBV-transduced cells (1 x 10⁶/ml) was cultured in complete RPMI-1640 medium containing 200 μg/ml hygromycin B for 2 to 3 days. Two hours prior to cell harvest, 0.1 ml of coccemid solution was added (final concentration 0.1 μg/ml). Cells were then harvested by centrifugation at 500g for

5 min and resuspended thoroughly in 10 ml of prewarmed 0.075 M KCl, followed by incubation at 37° for 15 min. Cells were spun down at 500g for 5 min and supernatant removed. Ten milliliters of fresh fixative (1 part of acetic acid and 3 parts of methanol) were added dropwise and mixed thoroughly with cells. This fixation process was repeated with two more changes of fresh fixative. Cells in suspension were dropped onto clean glass slides in a humid environment to promote spreading of chromosomes. Slides were then air dried overnight at room temperature and stored at -80° with desiccant until further use.

Prior to hybridization, prepared slides were incubated at 60° for 3 hr to promote further drying and harden metaphase spreads. Metaphase chromosomes on prepared slides were denatured by incubation at 70° for 2 min in 70% formamide/2x SSC, followed by dehydration through a series of cold 70%, 90%, and 100% ethanol (10 min each) at room temperature. The slides were air-dried and subjected to hybridization as previously described. The miniEBV-specific probe was labeled with Fluor-12-dUTP by Prime-It-Fluor Kit (Stratagene) to detect the miniEBV DNA only. A total of 50 ng of labeled miniEBV probe was prehybridized for 30 to 60 min at 37° with 10–1000 ng of Cot-1 DNA (GIBCO BRL) and 500 ng of human placental DNA (Sigma, sonicated to less than 500 bp) in a solution containing 2x SSC (0.3 M sodium citrate buffer), 1% BSA, and 10% dextran sulfate (Sigma). Hybridization was performed at 37° in a humidified chamber for at least 18 hr by covering the samples on slides with the solution containing the probe described above. Posthybridization washes were performed by rinsing the sample in 50% formamide/2x SSC at 37° and 50° for 30 min each.

Samples were directly visualized at 1000 x magnification on an Olympus IMT-2 microscope (NY, US) equipped with epifluorescence for fluorescein detection. Color pictures were photographed using Kodak Ultra Gold film (400 ASA) (Kodak, NY, US). As shown in Fig. 3B, some of the miniEBV-specific FISH signals appeared to be associated with human chromosomes while others were located some distance from them. The quantitative analysis by scanning of 100 metaphase signals revealed that the vast majority (approximately 99%) of the FISH signals was detected as single nonintegrated episomes. In addition, an average number of miniEBV episomes was estimated to be 3 to 6 copies per nucleus in the corrected HSC536 cells from Fanconi anemia patients and one to two copies per nucleus in the corrected RJK851 cells from Lesch–Nyhan patients.

Long-Term Transgene Expression and Phenotypic Correction in Human Disease Cells

To demonstrate that stable expression of a transgene is possible from the miniEBV episomal DNA and that therapeutic effects of the transgene could also

be achieved, we transduced miniEBV vectors carrying FACC cDNA and HPRT cDNA into B-lymphoblastoid cells from an FA-C patient and an HPRT-deficient Lesch–Nyhan patient, respectively. The expression of the transgene from stably transduced cells was examined by Northern blot analysis and functional complementation analysis.

**Northern Blot Analysis of Transgene Expression.** After miniEBV infection, cells were selected and maintained in the presence of 200 μg/ml hygromycin B for more than 2 months. Messenger RNA was isolated from cells by the PolyATtract System 1000 (Promega, WI, US) and the concentration determined by spectrophotometry (OD$_{260}$). The mRNA (7 μg) was separated on a 1.5% agarose formaldehyde gel at 5 V/cm for 4 hr and transferred to a nylon membrane. The Northern blot was hybridized with the transgene cDNA and β-actin (as internal loading control) probes. An example is given showing that human HPRT mRNA was detected in the miniEBV-transformed Lesch–Nyhan cells, but absent from nontransformed cells (Fig. 4A).

**Functional Complementation Assay in Human Disease Cells.** The therapeutic effects of the expressed transgenes detected by Northern blot analysis described above were further confirmed by phenotypic correction of a FA-C cell defect by measuring hypersensitivity to diepoxybutane exposure, and of an HPRT-deficient

![Graph](image-url)
cell type by growing cells in hypoxanthine–aminopterin–thymidine (HAT) medium.\textsuperscript{11,12} We take the latter as an example. Lymphoblastoid cells growing actively in complete RPMI-1640 medium were counted, pelleted, and resuspended at 2 × 10^5 cells/ml in fresh medium supplemented with 1 × HAT (GIBCO BRL). Cells were then seeded into 24-well plates (2 × 10^5 cells per ml per well). Living cells were counted at day 3, 4, and 5 after seeding by trypan blue exclusion. As shown in Fig. 4B, the uninfected cells died quickly under HAT selection, whereas the miniEBV-transduced cells grew like the normal HPRT(+) HSC93 cells. This indicates that normal cellular HPRT function had been restored in the majority of the Lesch–Nyhan cell clones stably transduced by the episomal miniEBV. It is therefore concluded that functional human HPRT mRNA and protein were produced from the large HAECs transduced into the human disease B cells by miniEBV infection.\textsuperscript{11}

**Suicide Strategy for Gene Therapy of B Lymphomas with Infectious MiniEBV**

We have designed an infectious, recombinant miniEBV/TK for delivering and expressing HSV-1 TK in both EBV positive and negative lymphoma cells.\textsuperscript{15} The effects of this infectious miniEBV-based suicide vector on the prodrug-mediated destruction of B lymphoma cells have been manifested by growth inhibition of the transduced cells cultured \textit{in vitro} or preimplanted \textit{in vivo} in a SCID mouse model.

**In Vitro Cytotoxicity Assay.** A total of 1 × 10^6 miniEBV/TK-transduced or nontransduced lymphoma cells were plated in a well of 12-well culture dish in 1 ml of complete RPMI-1640 medium with varying concentrations (0–400 μM) of the prodrug GCV (InvivoGen Products). On day 3, 1 ml of fresh medium containing corresponding concentration of the prodrug in each well was added. Viable and nonviable cells were counted on day 5 by trypan blue exclusion. As shown in Fig. 5A, both EBV positive and negative lymphoma cell lines transduced with miniEBV/TK were remarkably sensitive to low concentrations of GCV, whereas the nontransduced cells were resistant to high concentrations of GCV. These results demonstrate the feasibility of using infectious miniEBV carrying HSV-1 TK to effectively eliminate EBV positive as well as negative B lymphomas \textit{in vitro} at low concentrations of the prodrug GCV (i.e., 20–40 μM).

**In Vivo Tumor Growth Inhibition Assay.** As a first step in testing the applicability of this system for treating human B cell derived tumors \textit{in vivo}, we analyzed the ability of this infectious suicide gene delivery vector to inhibit the growth of the stably transduced B lymphoma cells in a SCID mouse model. Lymphoma cells stably transduced with miniEBV/TK were injected subcutaneously (1 × 10^7 cells) into the inguinal region of SCID mice. GCV was injected into the same site every other day at 20 mg/kg in half of the mice, while PBS was injected every other day in the other half mice until tumor burden required euthanasia. Tumor growth was monitored and tumor volume recorded over a 3-week period. As shown in Fig. 5B,
Fig. 5. Targeted destruction of human B cell lymphomas by infectious miniEBV carrying the HSV-1 thymidine kinase gene (pH217, J. Wang, S. Banerjee, and J. M. Vos, unpublished data). (A) Inhibition of growth in vitro of human B lymphoma cells stably transduced by the miniEBV. One million EBV-positive (Raji) and EBV-negative (DG-75) lymphoma cells were cultured in the presence of varying concentrations of GCV (0, 0.1, 10, 20, 40, 80, 200, and 400 μM), and viable cells were counted by trypan blue exclusion 5 days after incubation. (B) Inhibition of human B cell tumor growth in vivo by infectious miniEBV(pH217). EBV-positive Raji or EBV-negative DG-75 cells (1 × 10⁷) stably transduced with miniEBV/pH217 were injected subcutaneously into the inguinal region of SCID mice. GCV (20 mg/kg) was injected at the initial site of tumor cell delivery every other day in half of the mice, and PBS was injected every other day in the other half of the mice until tumor burden required euthanasia. Tumor growth was monitored and volume recorded over a 3-week period.

mice receiving GCV had no detectable tumor even 3 weeks after inoculation, whereas mice receiving PBS had substantial tumor growth by visual inspection and tumor necropsy assay.¹⁵

Summary and Prospects

The development of infectious EBV vectors for therapeutic purposes is still at an early stage. The B lymphotropism of EBV suggests that it may be particularly well adapted for the treatment of diseases involving circulating and/or diffusible gene products. Thus, inherited recessive monogenic disorders of serum proteins such as blood clotting factors (e.g., hemophilia), hormones such as insulin (diabetes), or enzymes such as glucocerebrosidase (Gaucher disease), α₁-antitrypsin (inherited emphysema), and β-glucuronidase (Sly syndrome) may be suitable candidates for EBV-based gene therapy. In addition, EBV may also be useful for treatment of acquired diseases such as cancer (especially B-cell lymphomas) and infectious diseases.
However, several potential difficulties will have to be overcome before EBV can be safely and effectively used in human clinical trials. The current helper-dependent packaging cell line produces miniEBV vectors mixed with the helper virus that has the potential for transcriptional activation and production of oncogenic LMP1 and/or EBNA3C. In addition, a recombination event could potentially occur between the miniEBV amplicon DNA and the helper P3HR1 genome. These considerations may not be critical in suicide strategy for gene therapy of cancers, because any cancer cell coinfected with the helper P3HR1 EBV and miniEBV carrying suicide gene(s) will eventually be ablated with the administration of the prodrug. However, gene therapy of inherited diseases requires prolonged expression of a therapeutic gene product, and thus it is important that a packaging cell line free of infectious helper virus be created. Finally, of paramount importance for the success of gene therapy is the availability of disease-specific delivery systems that direct the activity of therapeutic/suicide genes specifically to the sites of disease/malignancy. The transductional targeting by the infectious miniEBV vectors restricts the transgene expression to B lymphocytes and probably some other cell types, including those of epithelial origin. More restricted transgene expression could be achieved by transcriptional targeting using tumor- or tissue-specific promoters to drive the transgene expression. Therefore, development of a helper-free packaging system and enhancement of the vector targeting specificity would deserve our future endeavor in perfecting the infectious miniEBV vectors for use in gene therapy.

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This chapter is dedicated to the memory of Dr. Jean-Michel H. Vos, who succumbed to cancer on November 29, 2000, at the early age of 44. For more than 10 years, Dr. Vos had been a pioneer in the fields of chromosome engineering and gene therapy, and he had made important contributions to the development of mammalian artificial chromosomes and mini-herpesvirus based episomal vectors for animal transgenesis and gene therapy.