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Development of a Competitive PCR Method for Physical Titration of Recombinant EBV Vector in a Helper-Dependent Packaging System

Jianlong Wang*,[1] and Jean-Michel H. Vos*,†

*Lineberger Comprehensive Cancer Center and †Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7295

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Epstein–Barr virus (EBV) is a γ-herpesvirus with B lymphotropism and a double-stranded DNA genome of 172 kb that is episomally maintained in permissive cells during latency. EBV-based vectors containing minimal *cis* elements for replication, amplification, and helper-dependent packaging in a producer cell line HH514 have been developed to deliver therapeutic/suicide transgenes as infectious viral particles (miniEBV) to EBV-transformed B lymphoblastoid cells or B lymphoma cells. A quantitative, competitive PCR-based assay was developed to determine the relative packaging efficiencies of miniEBV and helper P3HR1 coproduced in HH514 cells. This provides a rapid and accurate quantitation of the physical titer of the virus preparation, which helps preserve the biological titer of the virus preparation and increase the efficiency of transgene delivery by miniEBV infection. In addition, it provides a sensitive and accurate way to evaluate future development of a helper-free packaging system by detecting any possible helper virus contamination.

Key Words: **Epstein–Barr virus; competitive PCR; viral titers; helper-dependent packaging.**

INTRODUCTION

Epstein–Barr virus (EBV) is a B-lymphotrophic herpesvirus with a double-stranded DNA genome of ~172 kb (1). Basic research on EBV has led to the development of various vectors for gene delivery to B lymphocytes (2). Among the vectors developed are amplicon-based vectors containing minimal *cis* elements (3, 4) for replication and packaging (5, 6) (an example is given in Fig. 1). These amplicon vectors can be expanded and packaged into EBV-like particles (referred to as miniEBV pseudovirions hereafter) in permissive cells containing a replication-competent EBV genome. The gene delivery to targeted cells is then achieved by subsequent miniEBV infection. Therefore, determination of the titer of miniEBV is a prerequisite for subsequent infection/transduction, and in turn, the transduction efficiency is important for successful gene delivery to the target cells.

The biological titer of a virus suspension, expressed in infectious units per unit volume, corresponds to the number of virions (referred to as physical titer, which is expressed in viral nucleic acid molecules per unit volume) that succeed in producing infection under the particular conditions employed in the test. Biological titers of virus stocks can be determined in two different ways: (1) by plaque-forming assay on cell monolayers, the results given in plaque-forming units, or (2) by staining for the expression of a transgene, such as *lac*Z, HSV-tk, or HSV-1 IE-genes, after the infection of permissive cells, the results given in transducing infectious units (TIU). The plaque-forming assay is commonly used for titration of HSV-1 viruses on Vero cells, since Vero cells form confluent monolayers in which plaques are easily detectable upon HSV-1 infection (7). In contrast, EBV does not have an indicator cell line for plaque-forming assay.

The current system for producing infectious miniEBV relies on the permissive human B cell line HH514 coproducing the helper EBV (P3HR1) that is transformation incompetent and replication competent (8–10). Previously, pulsed-field gel electrophoresis and Southern blot hybridization on viral DNA extracted from purified virions have been adapted to quantify the relative packaging efficiencies (and thus physical titers) of the miniEBV and helper P3HR1 genomes in a virus stock. The biological titer of miniEBV was then determined by infection of permissive cells followed by staining for the expression of the *lac*Z transgene (6, 11). However, this process is tedious

[1] To whom correspondence and reprint requests should be addressed at Children's Hospital (Enders 7 Rm 750), Division of Hematology/Oncology, 300 Longwood Avenue, Boston, MA 02115. Fax: (617) 355-7262. E-mail: jianlongnc@genetics.med.harvard.edu.

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FIG. 1. The miniEBV-based amplicon vector pDragon2 used in this study. 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 repeats. "C" indicates the complementary strand in this vector relative to its presence in the native EBV genome. "HygR" and "amp" are hygromycin B resistance and ampicillin resistance genes engineered for selection in mammalian and prokaryotic cells, respectively. Two transgene cassettes for expression of the enhanced version of GFP (EGFP) and suicide genes [HSV-1 thymidine kinase (TK) gene and bacterial cytosine deaminase (CD1) gene] are also shown.

and time consuming, and the process of *lac*Z staining is intrusive at the expense of sample. In addition, the detection of *lac*Z signal is rather insensitive, which frequently results in underestimation of the viral titers. Moreover, the virus stock loses titer with time, even under −80°C storage during the period of titration. The most precise quantitation of viral DNA can, however, be obtained by competitive PCR (12–16).

The quantitative competitive PCR (QC-PCR) assay is based on competitive coamplification of a specific target sequence together with a known concentration of an internal standard (IS) in one reaction tube. The IS shares primer recognition sites with the specific template, both IS and specific template are amplified with the same efficiency during PCR, and their PCR products can be analyzed separately according to either the size or the restriction enzyme digestion. Quantitation is then performed by comparing the PCR signals of the specific target with those of the IS. The concentration (or the copy number) of the specific target initially added into the tube is equal to the concentration (or the copy number) of the IS added (which is known) in the reaction tube that contains the same amounts of PCR signals of both templates. This assay has been applied to measure EBV genome load in peripheral blood samples from both healthy donors and patients with lymphoproliferative disorders (17, 18).

In this study, the QC-PCR assay has been adapted to determine the physical titers of miniEBV and the helper P3HR1. Meanwhile, the biological titer of miniEBV was determined by direct quantitation of GFP-expressing cells using flow cytometry after miniEBV infection of permissive cells. Our results revealed that the amplicon-based miniEBV vector makes up approximately 4% of all the virions packaged in the current helper-dependent packaging system, representing a physical titer of $\sim 4 \times 10^5$/ml of the virus stock. In addition, the biological titer of miniEBV varies depending on the permissive target cell lines used for infection.

MATERIALS AND METHODS

Plasmid Construction

The previously constructed plasmid pH200 containing minimal *cis* elements for EBV latent and lytic replication and packaging (5, 11, 19) was used to construct the new miniEBV-based vector, pDragon2 (Fig. 1). Briefly, two single-cut restriction sites on pH200, *Hind*III and *Bam*HI, were used to clone the EGFP expression cassette and suicide gene expression cassette, respectively. The EGFP expression cassette was constructed by replacing the CMV promoter in pEGFP-N1 plasmid (Stratagene, Inc.) with the 1.2-kb human ubiquitin C promoter (20) (courteously provided by Dr. Peter Angel, Germany). The transgene expression cassette was constructed on backbone pMSCV-IRES-GFP (21) (courteously provided by Dr. Derek Persons, St. Jude Children's Research Hospital, Memphis, TN) by replacing the GFP gene with the bacterial cytosine deaminase gene (courteously provided by Dr. Shannon Kenney in the Center) and inserting the CMV promoter and the 1.4-kb HSV-1 super thymidine kinase gene fragment (22) (courteously provided by Dr. Margaret Black, Washington State University, Pullman, WA) upstream of the IRES element.

Cells and Cell Culture

HH514 is a clone derived from the Jijoye EBV-positive Burkitt's lymphoma (9, 10) that was a gift from G. Miller (Yale University, New Haven, CT). HSC536 cells are lymphoblastoid cells from a patient affected by the inherited autosomal recessive blood disorder Fanconi anemia that were a gift from M. Buchwald (Hospital for Sick Children, Toronto, Canada). Raji is an EBV-positive Burkitt's lymphoma cell line, which was purchased from the American Type Culture Collection (Rockville, MD). All cell lines were grown in RPMI 1640 with 10% fetal bovine serum and supplemented with 1% L-glutamine and 0.1% penicillin/streptomycin.

Preparation of MiniEBV

The plasmid pDragon2 was electroporated into the helper cell line HH514 at 200 V and 960 μF with a Bio-Rad Gene Pulser and the cells were selected in the presence of 200 μg/ml hygromycin B (Boehringer Mannheim) for 2 months. Before induction of virions, the stably transfected HH514 cells were allowed to grow to near confluence ($2–4 \times 10^6$/ml) in a total amount of 500 ml with viability >90%. The cells were cultured in a T-300 with gentle shaking (40 rpm) in a 5% CO_2/37°C incubator. The lytic phase of EBV was induced by adding to the medium 20 ng/ml 12-*O*-tetradecanoyphorbol-13-acetate (Sigma) and 1 mM *n*-butyric acid (NaButyrate; Sigma). Five days later, supernatants were collected by centrifugation at 6000*g* for 10 min at 4°C. The cell pellet was resuspended with 10 ml complete medium and subjected to freeze–thawing three times in a dry ice/ethanol bath and a 37°C water bath. Cellular debris was spun down at 6000*g* for 10 min at 4°C, and the supernatants were combined 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 0.22-μm polyether sulfone filter (Corning). Virions were pelleted from supernatants by centrifugation at 12,500*g* for 2 h in a GSA rotor at 4°C. The medium was discarded and the virion pellet was resuspended in 2.5 ml RPMI 1640 without serum (200-fold concentration). Virions were used for competitive PCR or infection

TABLE 1

Summary of Oligonucleotides Used in This Study

IS	Name	Position in the target template[a]	Sequence
EBNA1	*e*	1161–1180 (+)	5′ GTCATCATCATCCGGGTCTC 3′
	a	1714–1733 (−)	5′ GCATCCTTCAAAACCTCAGC 3′
	na	1647–1664 (−); 1714–1733 (−)	5′ GCATCCTTCAAAACCTCAGCCTTAGCGGGCCAGGTTGT 3′
OriP	*o*	1219–1236 (+)	5′ GCCCGCCCACCTACTTAT 3′
	p	1743–1762 (−)	5′ TAACCCTCACAAACCCCTTG 3′
	ip	1657–1676 (−); 1743–1762 (−)	5′ TAACCCTCACAAACCCCTTGGGGACAAGCCGTAAAGACTG 3′

[a] (+/−) indicates the homology/complementarity of the oligonucleotide sequence relative to the sense strand of the target template.

assay directly. The remaining virus stock was frozen in a dry ice/ethanol bath and stored in a −80°C freezer for further use (although the viral titer decreased with time).

Quantitative Competitive Polymerase Chain Reaction

Generation of IS as competitor template. A rapid and versatile method to synthesize internal standards for competitive PCR has been developed (23, 24) and was employed in this study with modifications. The plasmid pBH140 containing both EBNA1 and OriP (25) was used as template to generate EBNA1 IS and OriP IS by polymerase chain reaction. All primers used in this study were designed with the Primer3 software (Whitehead Institute at MIT). Their sequences and locations relative to the respective gene on the EBV genome are listed in Table 1.

To quantitate the helper P3HR1 virus, the EBNA1-specific IS was first generated using the primer pair *e/na*. The PCR products were then used as template to further amplify a sufficient amount of IS using a second primer pair, *e/a* (Fig. 2, left). Note that the final product from this two-step amplification is 50 nt shorter than that from direct amplification of the EBNA1 gene using the primer pair *e/a* (Fig. 2, left, and Fig. 3A). The IS generated was separated from free dNTPs, primers, and residual pBH140 templates by gel electrophoresis and purified using the QiaQuick gel extraction kit (Qiagen Products). The concentration of the IS was determined by DyNA Quant 200 Fluorometer (Hoefer Pharmacia Biotech, Inc.). The OriP IS was generated in a similar way except that the primer pairs *e/na* and *e/a* were replaced with *o/ip* and *o/p*, respectively, and the difference between the PCR products of the target and IS is 67 nt instead of 50 nt (Fig. 2, right, and Fig. 3A). The PCR conditions for generation of internal standards were the same as that for competitive PCR described below.

Competitive PCR. First, serial dilutions of the IS were made ranging from 1×10^{-6} to 1×10^{-4} ng/μl (1×10^{-6}, 2×10^{-6}, 4×10^{-6}, 8×10^{-6}, 1×10^{-5}, 2×10^{-5}, 4×10^{-5}, 8×10^{-5}, 1×10^{-4} ng/μl). One microliter of the virus stock pretreated with DNase I was mixed with an increasing amount of IS (1 μl each of the serial dilutions above). Reaction conditions were based on the recommendations of the commercial reagent supplier (Gibco BRL), except that each reaction was conducted in a total volume of 50 μl, using 1.25 U/reaction of *Taq* polymerase. PCR was performed using a programmable thermal controller, GeneAmp PCR System 9700 (PE Applied Biosystems), and the following PCR cycle program: preheat at 95°C for 5 min; 30 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s; and finally, 72°C for 5 min.

Sample analysis and quantitation. Approximately 1/5 of each reaction mixture was run on a 3% agarose gel containing 0.5% normal agarose Low EEO (FisherBiotech Chemicals) and 1.25% Synergel (Diversified Biotech) in 1× TAE buffer. Gels were stained with ethidium bromide for visualization under UV light. An image was captured using a CCD camera linked to a frame grabber (UVP Ltd.). The image was exported to the program ImageQuant (version 5.0; Molecular Dynamics) and band intensities were quantified.

Since the comparisons and equivalent point determination in the QC-PCR technique are based on molar amounts, the band intensities associated with the larger PCR products from viral target templates (EBNA1 and OriP) were corrected by multiplication by a factor of 524/573 and 478/544, respectively. This correction enables direct comparison of corrected band intensity of the target products ("CorrV" in Fig. 3C) with measured band intensity of the respective IS (15, 26). A plot of the log of the ratio of the CorrV vs IS against the log copy number of the IS added to each reaction was created with the Microsoft Excel program, and the viral EBNA1 or OriP copy number was deduced from the point where $y = 0$, indicating equal copy number of the viral template and IS template. Note that 1×10^{-6} ng of 524-bp EBNA1 IS corresponds to 1737 copies, and 1×10^{-6} ng of 478-bp OriP IS corresponds to 1904 copies.

The copy number of EBNA1 directly reflects the copy number of the helper P3HR1 in the virus stock. To get the copy number of miniEBV, the copy number of EBNA1 was subtracted from the copy number of total OriP, and the remaining copy number of OriP was divided by 7 or 8 to get the copy number of miniEBV/pDragon2 in the virus stock.

EBV Infection

Raji or HSC536 cells were allowed to reach near confluence ($2–4 \times 10^6$/ml). Five million cells were harvested by centrifugation at 1000 rpm for 5 min (Marathon 10K; Fisher Scientific Product). Cells were resuspended in 1 ml of fresh RPMI 1640 without serum and transferred to small petri dishes. Virus particles were added into cells at an m.o.i. (multiplicity of infection, i.e., virus particles per target cell) of 10 and mixed by swirling. The mixture was incubated at 37°C/5% CO_2 for 1 to 2 h. After incubation, the cells were seeded into 10 ml complete RPMI 1640 and incubated at 37°C/5% CO_2. Three days after infection, cells were subjected to flow cytometry (below) to determine the transduction efficiency.

Flow Cytometry

One million cells (Raji or HSC536) were harvested by centrifugation, washed, and resuspended in 1 ml of PBS for cytometric analysis. Cytometric analysis was performed using a FACScan flow cytometer (Becton–Dickinson Immunocytometry Systems, San Jose, CA) equipped with a water-cooled argon laser emitting at 488 nm. Analysis was performed using Summit Version 3.0 for MoFlo Acquisition and Sort Control (Cytomation, Inc.). Green fluorescence (FL1) was measured using a 530 + 30-nm band pass filter. Gates were set to exclude necrotic cells and cellular debris and the fluorescence intensity of events within the gated regions was quantified. Data were collected from 10,000 to 20,000 events for each sample. Nontransduced cells (Raji or HSC536) were used as negative controls.

RESULTS

Construction of Internal Standards for Competitive PCR

Generation and testing of suitable internal standards and the choice of primer pairs are among the most crucial and time-consuming aspects of setting up a competitive

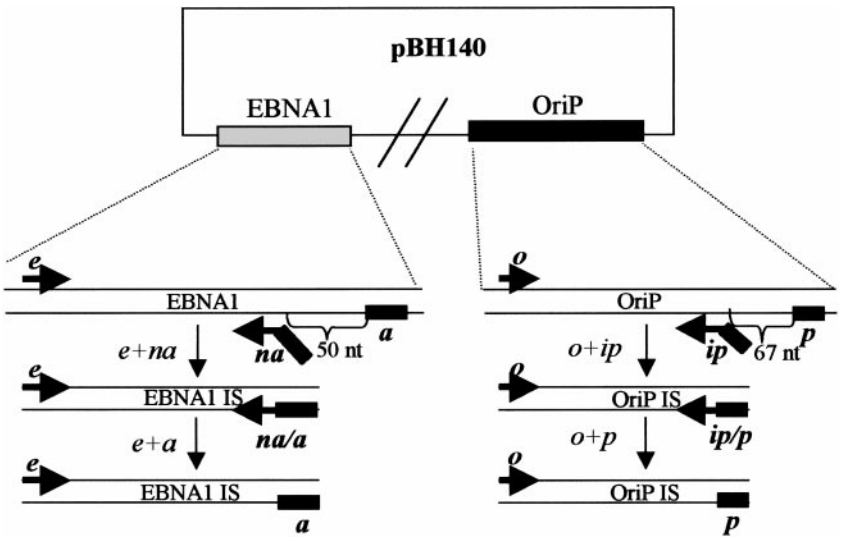

FIG. 2. Generation of the internal standards (IS) for EBNA1 (left) and OriP (right). The primer *na* (Table 1) was designed in such a way that its 5′ half contains the homologous sequence of the primer *a* and its 3′ half complementary to the EBNA1 gene is 50 nt downstream of the primer *a* sequence in the gene. The PCR products (IS) from the template EBNA1 using primer pair *e/na* can be further amplified by a second round PCR with primer pair *e/a.* The generation of OriP IS is performed in a similar way except that OriP-specific primer pairs were used and there is a 67-nt distance between the 3′ half complementary part of the primer *ip* and the sequence of the primer *p* in the OriP.

PCR protocol. Internal standards are DNA fragments sharing the primer recognition sequences with the specific target yet yielding PCR products that are distinguishable from those amplified from the target. The easiest way to distinguish between PCR products of the target template and those of the internal standard is by differences in the size of the two products. This can be achieved by constructing standards having the same sequence as the specific target but containing a deletion (as employed in this study) or insertion.

To quantify the physical titer of miniEBV in a virus stock that also contains the helper P3HR1, the internal standards for both helper P3HR1-specific target EBNA1 and common target OriP were generated (see below for reasons). As shown in Fig. 2 (left), two rounds of PCR using the primer pairs (*e/na* and *e/a*) specific for the EBNA1 gene generated an internal standard for EBNA1 (see Materials and Methods for details). This EBNA1 IS shares the same primer recognition sequences (*e/a*) with the target EBNA1 gene, and its PCR products are 50 bp shorter than the PCR products of the target using the same primer pair, *e/a,* during competitive PCR. The OriP IS was generated in a similar way except that the primer pairs were replaced with the OriP-specific oligonucleotides *o/ip* and *o/p* (Fig. 2, right, and Table 1). The internal standards for both EBNA1 and OriP were purified from agarose gel after electrophoresis and quantified as described under Materials and Methods. One nanogram of the 525-bp EBNA1 IS corresponds to 1.737×10^9 copies (or molecules) of EBNA1 IS, and 1 ng of the 478-bp OriP IS contains 1.904×10^9 copies (or molecules) of OriP IS.

Determination of the Physical Titer of MiniEBV Pseudovirions

The recombinant miniEBV vectors were produced from HH514 cells stably transfected with pDragon2 (Fig. 1). To quantify miniEBV/pDragon2 in the virus stock, the target template and the internal standard generated from the target sequence should be miniEBV specific. However, repeated PCR trials using primers specific for miniEBV targets (either EGFP or hygromycin B resistance gene in pDragon2, see Fig. 1) failed to generate sufficient PCR products for direct quantitation from ethidium bromide-stained signals (data not shown). This suggests that the virus stock produced from the packaging cell line HH514 contains only a small amount of miniEBV pseudovirions. Alternatively, the primers for the EGFP or hygromycin B resistance gene designed in the context of the plasmids carrying the respective target gene may lack specificity for the target gene that is present in the very large EBV genome. Alternative procedures can be employed to detect low-level gene amplification (if it is the case for miniEBV-specific target gene) that is otherwise undetectable by ethidium bromide staining. These include the use of radioactively or fluorescence-labeled dNTP or oligonucleotides during competitive PCR or Southern blot hybridization of the gel blot using gene-specific probes. However, these options were not pursued in this study due to the tedious and time-consuming nature of these procedures and the loss of virus titer over time.

To overcome this template limitation for direct quantitation of miniEBV, a two-step strategy was developed to simultaneously quantify both miniEBV and helper P3HR1 in the virus stock. Since the EBNA1 gene is specific only for the helper P3HR1 and the OriP is present in the genomes of both helper P3HR1 and miniEBV (Fig. 3A), quantitation of the target EBNA1 directly corresponds to the physical titer of helper P3HR1 in the virus stock, whereas quantitation of the target OriP involves both helper P3HR1 and miniEBV. Since EBNA1 and OriP are present at a 1:1 ratio in the helper P3HR1 genome (Fig. 3A), by subtracting the copy number of EBNA1 from the total copy number of OriP the copy number of the OriP amplified from miniEBV can be obtained. Depending on the multimeric properties of the miniEBV genome, the

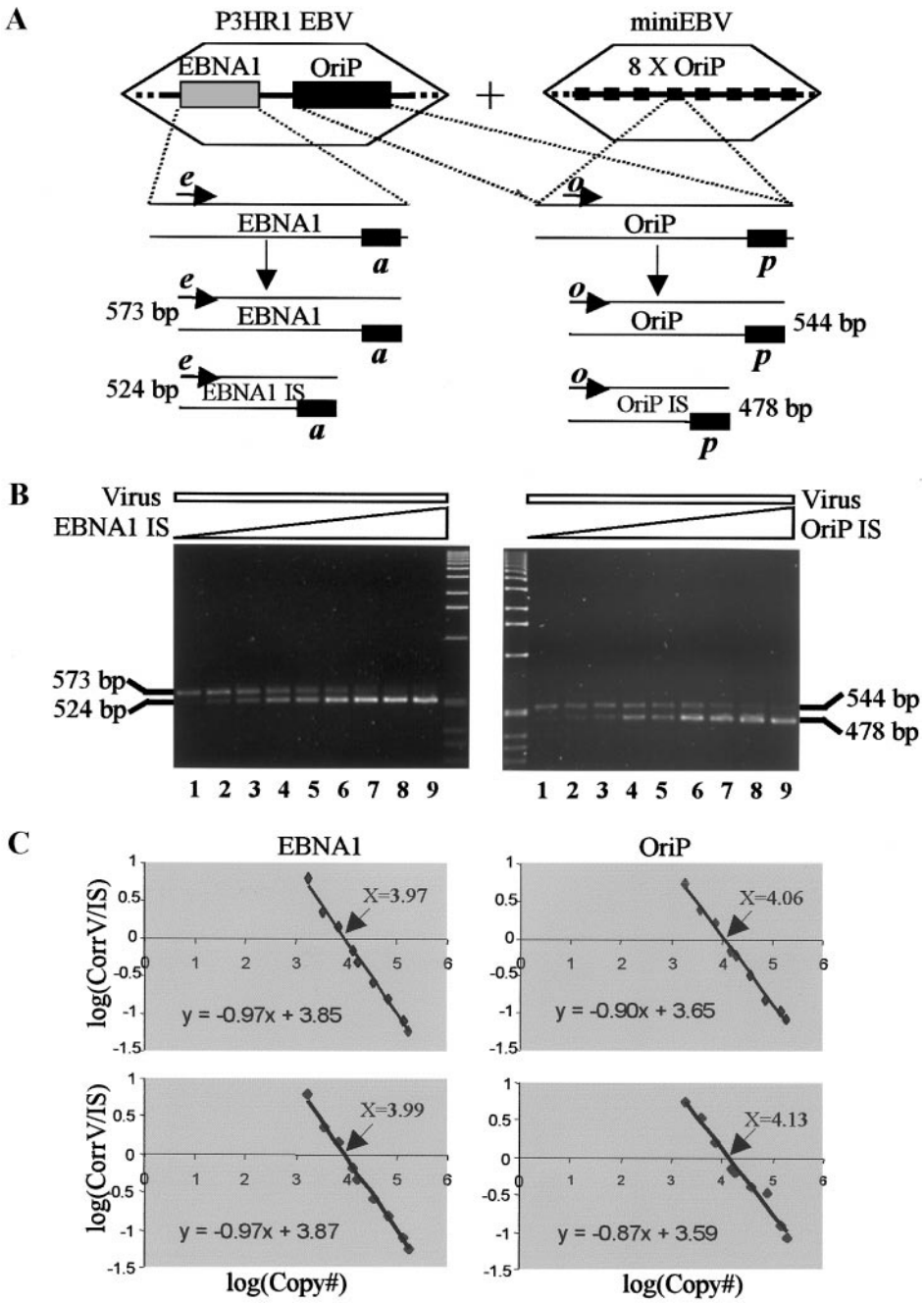

FIG. 3. Quantification of EBNA1 and OriP from a virus stock by competitive PCR. (A) Schematic depiction of the EBNA1 and OriP components in the genomes of helper P3HR1 and miniEBV. An octameric genome of miniEBV is shown. The target template and the IS template during competitive PCR are also indicated below. (B) A representative gel electrophoresis of competitive PCR products. Increasing amounts of IS molecules (from 10^{-6} ng in lane 1 to 10^{-4} ng in lane 9) were added to fixed amounts of virus particles (1 μl) in each reaction tube as described under Materials and Methods. After amplification, products were resolved on 3.0% agarose gels and images captured using a CCD camera linked to a frame grabber (UVP Ltd.). (C) Quantification of ethidium bromide-stained signals of competitive PCR products from (B) (top) and another independent experiment (bottom). Band intensities were quantified using the program ImageQuant (Version 5.0; Molecular Dynamics). A plot of the log of the ratio of the corrected virus amplification signal (CorrV) vs IS signal against the log number of competitor molecules added to each reaction yields a straight line (the equation is shown). The point at which the viral target (either EBNA1 or OriP) copy number equals its corresponding IS copy number is indicated.

copy number (i.e., the physical titer) of the miniEBV can be deduced.

Using a fixed amount (1 μl) of virus stock as target template and an increasing amount of IS (ranging from 1×10^{-6} to 1×10^{-4} ng per reaction) as competitor template, competitive PCR was performed with the primer pairs *e/a* and *o/p* for amplification of EBNA1-specific and OriP-specific sequence, respectively (Fig. 3A). The amplification products were separated by gel electrophoresis (an example is given in Fig. 3B), and band intensities in Fig. 3B were quantified (Fig. 3C, top) using computer software as described under Materials and Methods. The copy number of helper P3HR1 and miniEBV can be determined as follows: the copy number of EBNA1, i.e., the copy number of helper P3HR1, is $10^{3.97}$ (9.33×10^3) in 1 μl of virus suspension. The copy number of OriP amplified from miniEBV (1 μl per reaction) is $10^{4.06}$–$10^{3.97}$ ($\approx 2.15 \times 10^3$). Since the size of pDragon2 is 22.9 kb, and the producer cell line HH514 can package engineered DNA from 150 to 200 kb (11), there should be a 7- to 8-mer of 22.9-kb pDragon2 DNA packaged into virions. Therefore, the copy number of the miniEBV/pDragon2 in 1 μl of virus suspension is $(2.15 \times 10^3)/7$ (307 copies) or $(2.15 \times 10^3)/8$ (269 copies). In a separate experiment, gel

TABLE 2

Helper-Dependent Packaging of MiniEBV by the Producer Cell HH514

Viral stock[a] (HH514/pDragon2)	Viral species	Viral concentration (physical titer)[b]	Percentage (%)
Expt. 1	Helper EBV(P3HR1)	9.33×10^6/ml	97.2
	MiniEBV/pDragon2	2.69×10^5ml	2.8
Expt. 2	Helper EBV(P3HR1)	9.77×10^6/ml	95.5
	MiniEBV/pDragon2	4.65×10^5/ml	4.5
Average ± SD	Helper EBV(P3HR1)	$(9.55 \pm 0.31) \times 10^6$/ml	96 ± 1
	MiniEBV/pDragon2	$(3.67 \pm 1.39) \times 10^5$/ml	4 ± 1

[a] Data from two separate experiments (Expt. 1 and Expt. 2) are presented along with the average and the standard deviation (SD) from the two experiments;
[b] Only the physical titers of miniEBV/pDragon2 with octameric genomes as indicated in Fig. 3A are shown.

patterns similar to those shown in Fig. 3B were obtained (data not shown), and the quantitation data are presented in Fig. 3C (bottom). The physical titers of miniEBV/pDragon2 (and helper P3HR1) from both experiments are summarized in Table 2.

Our results show that approximately 96% ($\sim 9.55 \times 10^6$/ml) of the virus stock is the helper P3HR1, whereas only 4% ($\sim 3.67 \times 10^5$/ml) of the virus stock is miniEBV/pDragon2. This is consistent with our previous data from pulsed-field gel electrophoresis and Southern blot hybridization of viral DNA extracted from purified virions using probes specific for either the helper P3HR1 or the miniEBV, which reveals 95% of the helper P3HR1 and 5% of miniEBV in the virus stock produced from the same helper-dependent packaging system (6, 11). Therefore, the QC-PCR-based assay developed in this study for physical titration of the recombinant miniEBV vector is valid and reliable.

Determination of the Biological Titer of Infectious MiniEBV Pseudovirions

The quick determination of the physical titer of miniEBV/pDragon2 pseudovirions using QC-PCR preserves the virus titer and enables us to perform infection assays immediately on target cells with a certain m.o.i. This will not only help confirm the presence of miniEBV/pDragon2 in the virus stock by detecting miniEBV-specific gene expression (e.g., GFP expression, see Fig. 1), but also determine the biological titer of miniEBV/pDragon2.

EBV-positive B lymphoma Raji cells or EBV-transformed lymphoblastoid HSC536 cells were infected with miniEBV pseudovirions at an m.o.i. of 10, i.e., 10 physical miniEBV particles per cell. Three days after infection, the positively transduced Raji or HSC536 cells were visualized and photographed for GFP expression under fluorescence microscope (Fig. 4). The detection of GFP expression confirms the existence of miniEBV/pDragon2 in the virus stock.

To determine the biological titer of the miniEBV, the number of GFP-positive cells was quantified using flow cytometry as described under Materials and Methods. The transduction efficiencies by miniEBV/pDragon2 pseudovirions on these two target cell lines and the biological titers of miniEBV/pDragon2 (expressed as transducing infectious units per milliliter of virus suspension) are summarized in Table 3. Our results indicate that at an m.o.i. of 10, the miniEBV transduction efficiency in Raji and HSC536 cells is approximately 40 and 15%, respectively. This indicates that approximately 25 and 67

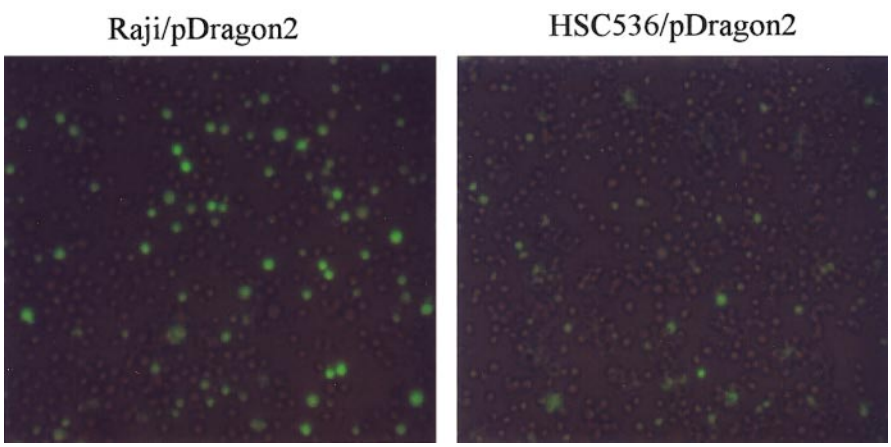

FIG. 4. Infection of EBV-positive B lymphoma cells Raji (left) or lymphoblastoid cells HSC536 (right) with miniEBV/pDragon2. Five million cells were infected by miniEBV/pDragon2 with an m.o.i. of 10, i.e., 10 miniEBV particles per cell. Three days after infection, cells were subjected to fluorescence microscopy and photography. The percentage of GFP-positive Raji or HSC536 cells was determined by flow cytometry as described under Materials and Methods.

TABLE 3

Transduction Efficiencies and Biological Titers of MiniEBV

Target B cells	Viral stock (miniEBV/ml)	m.o.i. (miniEBV/cell)	Transduced cells (GFP^+ cells)	Transducing ratio (miniEBV/GFP^+ cells)	Transducing infectious unit (TIU/ml)
Raji	4×10^5	10	40%	25	1.6×10^4
HSC536	4×10^5	10	15%	67	6.0×10^3

miniEBV physical particles are required for each successful transduction of Raji and HSC536 cells, respectively, which is reflected by the transducing ratio (miniEBV/GFP^+ cells) (Table 3). Since the virus stock contains approximately 4×10^5/ml miniEBV/pDragon2, the biological titer of the miniEBV/pDragon2 pseudovirions is 16,000 TIU/ml for Raji cells and 6000 TIU/ml for HSC536 cells.

DISCUSSION

The determination of viral titers has often relied on tedious and time-consuming procedures such as viral DNA extraction followed by Southern or slot blot hybridization or a plaque-forming assay, which requires an indicator cell line. To fully exploit the application of gene delivery by infectious miniEBV, we have developed an accurate, simple assay for titration of miniEBV vector in a helper-dependent packaging system using QC-PCR. This allows us to obtain the virus physical titer within the same day during virus preparation and perform the infection assay with a certain m.o.i. immediately, thus minimizing the loss of viral titer with time.

The apparent discrepancies between the physical titer and the biological titer of a virus stock suggest that there are inactive virions in virus stock. For some bacteriophages, the number of virions (physical titer) is between 1 and 2.5 times the number of plaque-forming units produced on the most sensitive host bacteria under optimal conditions (27). The differential transduction efficiencies of miniEBV on Raji and HSC536 cells suggest that the biological titer of a virus stock is dependent on the permissiveness of the host cells. Since the virus infection requires specific binding of the viral protein gp350/220 to the receptor (CD21) present in permissive cells, it suggests that fewer CD21 receptors are expressed in HSC536 cells than in Raji cells. Alternatively, there might be a smaller portion of HSC536 cells than of Raji cells expressing sufficient CD21 for successful transduction by miniEBV. In summary, the numerical relation between successful virions (biological titer) and total virions (physical titer) cannot be decided by infection assay alone but requires direct counts of virions. These counts, in turn, cannot distinguish between active and inactive virions or between those that are differentially infectious for different hosts.

The physical titer of miniEBV is not impressive relative to the packaging of the endogenous helper P3HR1. The packaging efficiency of the miniEBV is much lower than that of the helper P3HR1, with the recombinant vector representing only 4% of the total viral stock (Table 2). This suggests that the helper P3HR1 competes with the miniEBV for packaging into infectious virions. In addition, the size of the multimeric miniEBV genome could be suboptimal for packaging compared with that of the helper P3HR1, as the DNA packaging efficacy of EBV is dependent on the length of the DNA being packaged (28). Nonetheless, the therapeutic potential of this system has been established in our lab by *in vitro* correction of the phenotypic defects in lymphoblastoid cell lines established from a Fanconi anemia patient (6) and a Lesch–Nyhan patient (5).

Due to the extremely large size of the EBV genome, the cloning and manipulation of the EBV genome is a challenging task and a helper-virus-free packaging system is currently not available. Although a first-generation packaging cell line for EBV-derived vectors has been developed (29), it still suffers from unwanted recombination between the helper virus genome and the gene vector DNA, resulting in contamination of the vector with the transforming helper EBV (B95.8). Therefore, development of a helper-free miniEBV packaging cell line combined with optimization of miniEBV production will be an important future endeavor. Nevertheless, the method developed in this study provides a rapid and accurate assay for evaluation of the packaging systems by determining the vector titer and detecting possible contamination of the helper virus. This in turn will help improve virus vector production, which is a common issue in the field of viral vector gene therapy.

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