

Combination of Epstein-Barr Virus-Based Plasmid and Nonviral Polymeric Vectors for Enhanced and Prolonged Gene Expression

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An Epstein-Barr virus (EBV)-based plasmid contains the EBV nuclear antigen 1 (EBNA1) gene and EBV replication origin (oriP) sequence. Since EBNA1 (the only EBV-encoded protein) is combined with oriP, it is replicated simultaneously with chromosomal DNA in human, primate, and canine cells and is faithfully segregated at a stable copy number upon cell division. Consequently, it can be used to stably express gene inserts over a prolonged time in target cells. We have previously shown that the polyamidoamine (PAMAM) dendrimer can be surface-modified with L-arginine. Arginine is present at a high frequency in the trans-activator of transcription (Tat) sequences of human immunodeficiency virus (HIV). It presents high membrane permeability and permits effective transfer of DNA inside the cells. In this study, we constructed two kinds of recombinant DNA by inserting the luciferase gene and enhanced green fluorescence protein (eGFP) gene as reporter genes into the pCEP4 plasmid vector. We measured dynamic light scattering (DLS) and zeta potential after preparing PAMAM-based cationic polymer/EBV-based plasmid complexes. We performed transfection of HEK 293 cell lines with the polyplexes, and monitored luciferase activity and green fluorescence protein (GFP) expression. Our results show that PAMAM-based cationic polymer/EBV plasmid complexes provide enhanced and sustained gene expression.

Key Words : Epstein-Barr virus-based plasmid, Dendrimer, Polyplex, EBNA1, Transfection

Introduction

For optimal human gene therapy, various viral and non-viral vectors (plasmid DNA using gene gun or electroporation methods, liposomes, and polymers) have been studied.^{1,2} Non-viral gene delivery systems may be favored over viral vectors because of their safety and high reproducibility, but their transfection efficiency is not as good as that of viral vector systems. To overcome this disadvantage, we used an Epstein-Barr virus (EBV)-based plasmid DNA. The EBV-based plasmid contains the EBV nuclear antigen 1 (EBNA1) gene and the Epstein-Barr virus replication origin (oriP) sequence. Since EBNA1 (the only EBV-encoded protein) is combined with oriP, it is replicated simultaneously with chromosomal DNA in human, primate, and canine cells and is consequently maintained and expressed for a long time in the cell.³⁻⁶ EBNA1 also enables the binding of plasmid to the nuclear matrix, nuclear localization of the plasmid, and transcriptional upregulation.⁶ Studies on EBV itself and EBV-related plasmids encompass a broad range of life science fields, ranging from molecular biology and immunology to pharmacy.^{1,7-11}

We conducted experiments involving a polyplex of PAMAM-Arg as a non-viral vector and the EBV plasmid in order to provide prolonged and enhanced transfection efficiency. Polyamidoamine (PAMAM) dendrimers that are

modified with L-arginine (amino acid present at high frequency in the Tat sequence of HIV) are already reported to have lower cytotoxicity and higher transfection efficiency than other non-viral vectors.¹²⁻¹⁴ The Tomalia group reported a starburst PAMAM dendrimer for the first time in 1984, and this dendrimer has been studied in various fields including biotechnological applications.¹⁵ The PAMAM dendrimer has a unique repeating structure and can readily form complexes with plasmid DNA.^{16,17}

In this study, we created two kinds of recombinant DNAs by inserting the luciferase gene and enhanced green fluorescence protein (eGFP) gene as reporter genes into the pCEP4 plasmid vector. The recombinant DNA and PAMAM-Arg polymers were allowed to form polyplexes, and the transfection efficiency was determined in the HEK293 cell line. Using this system, we found that PAMAM-Arg/EBV plasmid complexes provide much enhanced and sustained gene expression for longer period of time.

Experimental Sections

Materials and Reagents. pCEP4 was purchased from Invitrogen (San Diego, CA, USA). Dulbecco's modified eagle medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), trypsin (0.25% trypsin with ethylenediaminetetraacetic acid [EDTA]), and antibiotic-antimycotic solution

(100 unit/mL penicillin, and 100 μ g/mL streptomycin) were purchased from Gibco (Gaithersburg, MD, USA). The luciferase assay kit was purchased from Promega (Madison, WI, USA). The micro BCA protein assay kit was purchased from Pierce (Rockford, IL, USA). Starburst polyamidoamine (PAMAM G4, NH_2 core), *N,N*-dimethylformamide, anhydrous 99.8% (DMF), *N,N*-diisopropylethylamine (DIPEA) and piperidine were purchased from Sigma-Aldrich (Seoul, South Korea). Fmoc-Arg(Pbf)-OH was purchased from Novabiochem (San Diego, CA, USA). *N*-hydroxybenzotriazole (HOBt) and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Anaspec Inc. (San Jose, CA, USA).

Plasmid Constructions. An EBV-based plasmid vector expressing firefly luciferase or enhanced green fluorescent protein (eGFP) as a reporter gene for monitoring gene expression efficiency was constructed by subcloning either the luciferase gene of pGL3-basic or the eGFP gene of pEGFP-N3 into pCEP4. pGL3-basic was cut with *Bam*HI and *Hind*III, and the resulting ~2 kb fragment contained the luciferase sequence. This fragment was ligated using T4 DNA ligase (Invitrogen, San Diego, CA, USA) with pCEP4 digested with *Bam*HI and *Hind*III for 1 h at 25 °C. Recombinant DNA carrying the eGFP gene was constructed using a similar approach, but by using the restriction enzymes *Hind*III and *Not*I. The sequences of the recombinant DNA were confirmed by DNA sequencing.

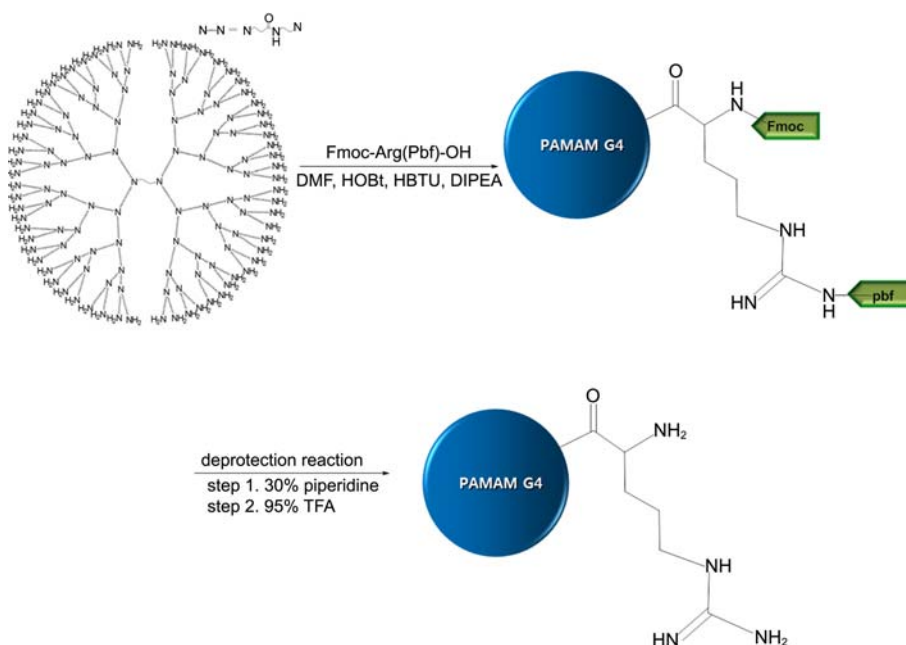
Synthesis of PAMAM-Arg. PAMAM-Arg was synthesized using the procedure reported in a previously published article¹³ (Scheme 1). Arginine was coupled to PAMAM in the presence of the coupling reagent HOBt (4 eq.), HBTU (4 eq.), Fmoc-Arg(pbf)-OH, and DIPEA (8 eq.) in anhydrous DMF. To remove the Fmoc group of the Fmoc-Arg(pbf)-coupled PAMAM dendrimer, 30% piperidine in DMF (v/v) was added. After a 1 h reaction, the mixture was precipitated

in cold ethyl ether and washed with excess ether twice. A TFA solution was added in the presence of TIS and distilled water (DW; TFA:TIS:DW = 95:2.5:2.5, v/v/v, 25 °C, 6 h) for deprotection of the pbf groups of arginine, and the mixture was precipitated in cold ethyl ether and washed with excess ether twice. The product was solubilized in distilled water and dialyzed overnight. The final product was collected by freeze drying, yielding a white powder (conjugation yield is over 90%).

Preparation of Cationic Polymer/DNA Complex and Particle Size and Zeta Potential Measurement. Complexes of cationic polymers and plasmid DNA (polyplexes) were prepared at a weight ratio of 4:1 in 150 μ L of DW. The mixture was incubated for 30 min at room temperature in order to allow optimal polyplex formation. The size distribution and zeta potential of polyplexes were determined using a Photol ELS-Z2 (Otsuka Electronics Co. Ltd. Japan) instrument.

Cell Culture and Transfection *in vitro*. Human embryonic kidney 293 (HEK293) cells were maintained in continuous growth in cell culture media (DMEM supplemented with 10% FBS and 1% antibiotic solution) at 37 °C in a humidified atmosphere containing 5% CO_2 , in tissue culture T25 polystyrene flasks. The polymer/pDNA complexes (polyplex) were added to the growth medium when the cells reached 60-70% confluence, following which the culture was still incubated in the CO_2 incubator. The transfected cells were subcultured every 2 days.

Transfection Efficiency. After the growth medium was removed, the cells were washed with DPBS and lysed for 15 min at RT by using 50 μ L of 1X Reporter lysis buffer (Promega, Madison, WI). Luciferase activity was measured using a LB 9507 luminometer (Berthold, Germany), and the protein content was determined using a micro BCA protein assay kit using a VERSAmax microplate reader (VERSAmax;



Scheme 1. Synthesis scheme of PAMAM G4-arginine.

Molecular Devices, Sunnyvale, CA).

GFP Expression. Transfection efficiency was determined by fluorescence microscopic evaluation of cells treated with polyplexes of PAMAM-Arg polymers and pCEP4-eGFP. The TE2000 microscope (Nikon, Japan) was used for these experiments.

Results and Discussion

Constructions of EBV-Based Plasmid DNA. Two kinds of recombinant DNA were constructed by inserting the luciferase gene and the eGFP gene into the pCEP4 plasmid vector (Figure 1). pCEP4-luciferase was a 12,326 bp plasmid carrying ampicillin resistance. We performed a midi-prep for mass production of recombinant DNA and obtained 280 μ g of pCEP4-luciferase, the purity of which was measured ($A_{260}/A_{280} = 1.65$). In order to confirm that the intended fragment size was obtained, the size of the bands was observed on an agarose gel after restriction enzyme cutting. In addition, we verified that the correct insert was present by using sequencing (data not shown). Three restriction enzymes,

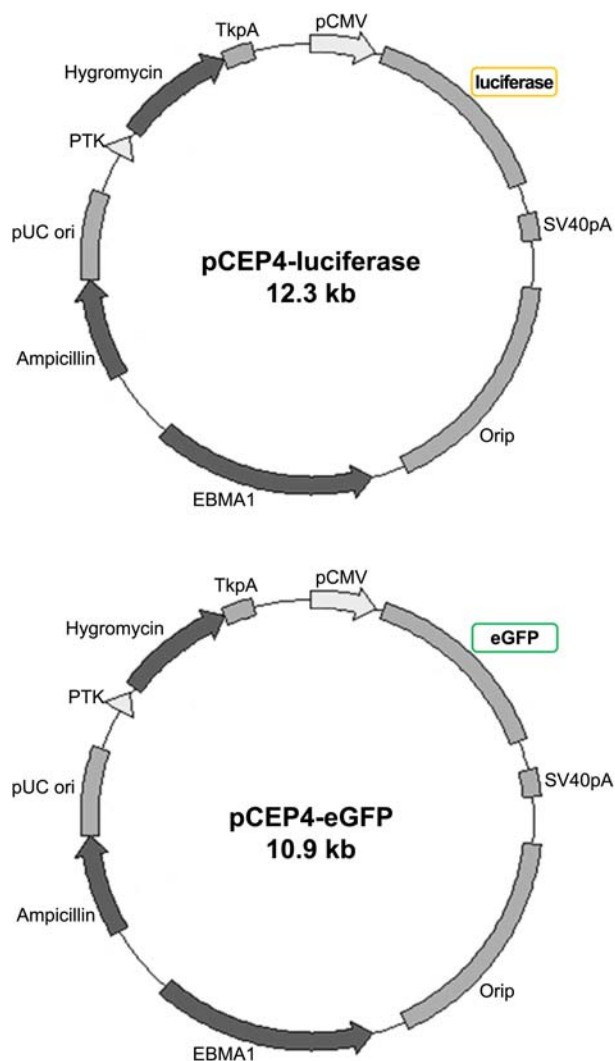


Figure 1. Maps of recombinant plasmids containing the luciferase or eGFP gene.

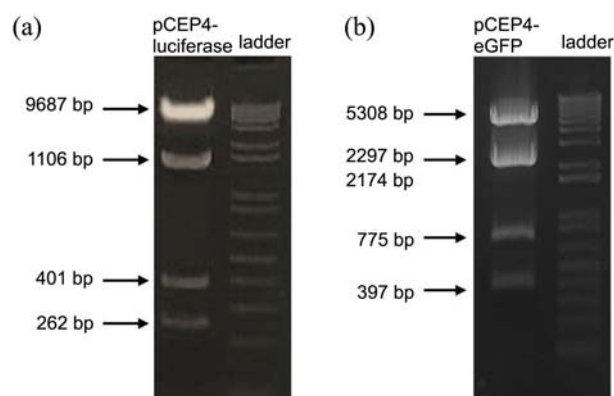


Figure 2. Products of restriction enzyme digestion of the recombinant plasmid DNAs (A, pCEP4-luciferase; B, pCEP4-eGFP).

HindIII, *BamHI*, and *XbaI*, were incubated with pCEP4-luciferase, and 4 fragments of the predicted band sizes (9687, 1106, 401, and 262 bp), were confirmed (Figure 2(a)). pCEP4-eGFP is a 10,950 bp plasmid. After digestion with *EcoRI* and *XhoI*, the predicted band sizes, that is, 5308, 775, and 397 bp, were evident on an agarose gel (Figure 2(b)). An additional 2 predicted bands of 2297 and 2174 bp appeared as a single band on the agarose gel, probably because their similar sizes could not be resolved under these electrophoretic conditions.

Formation of Polymer/pDNA Complexes (Polyplexes). An agarose gel electrophoresis retardation assay was per-

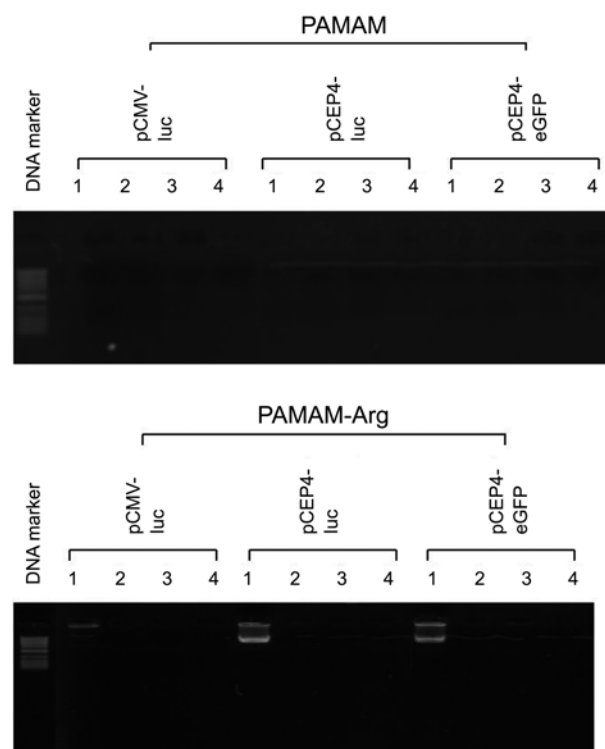


Figure 3. Agarose gel retardation assay for comparison of various plasmid DNAs. Ratios of polymer/DNA (w/w) = 1:1, 2:1, 4:1, and 8:1 (lanes 1, 2, 3, and 4, respectively), (upper: PAMAM; lower: PAMAM-arginine).

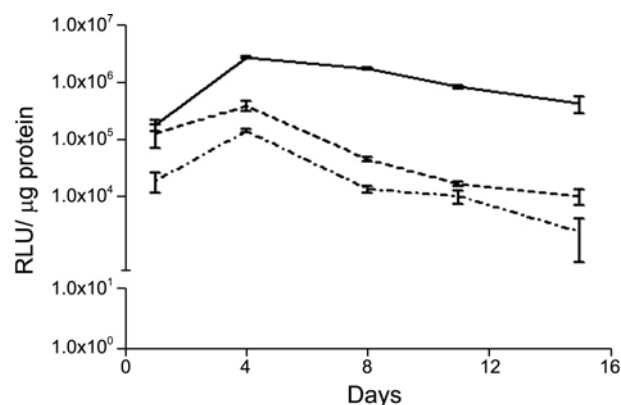
Table 1. The mean diameter and zeta potential values of various polyplexes

	Size (nm)	Zeta potential (mV)
pCEP4-luciferase	1116.0	-34.65
pCEP4-eGFP	2582.3	-21.51
pEGFP-N1	3156.3	-37.58
pCMV-luciferase/PAMAM-Arg	232.5	+37.74
pEGFP-N1/PAMAM-Arg	91.5	+21.74
pCEP4-luciferase/PAMAM	761.7	+30.36
pCEP4-luciferase/PAMAM-Arg	88.9	+22.56
pCEP4-eGFP/PAMAM	168.6	+31.42
pCEP4-eGFP/PAMAM-Arg	153.1	+26.27

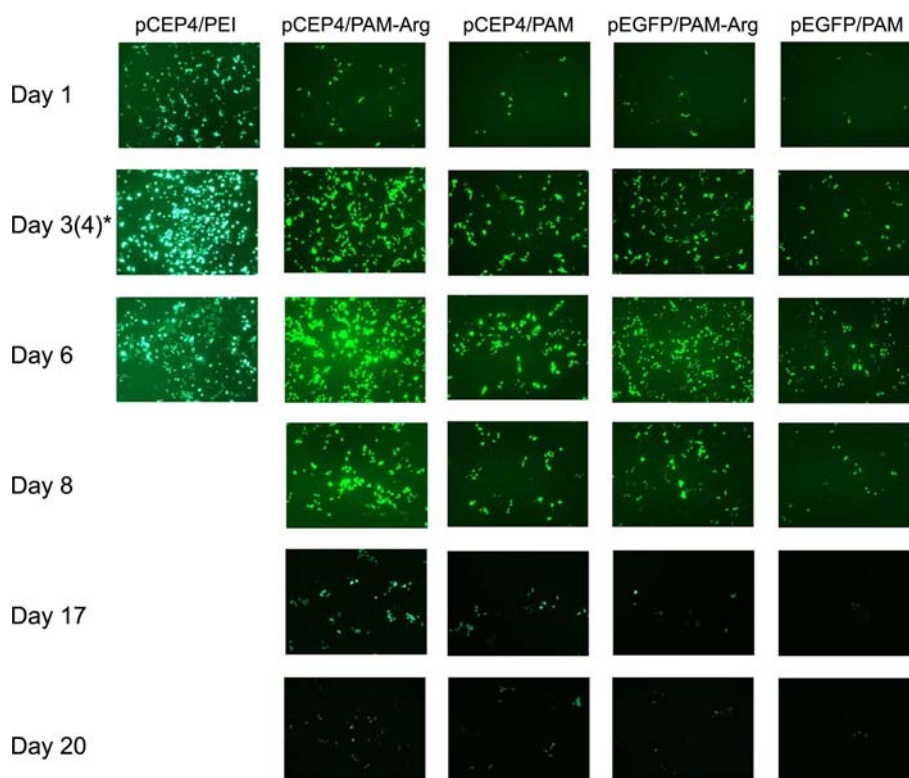
formed to assess the optimal complex ratio between recombinant DNA and PAMAM derivatives. The DNA (0.5 μ g) of the pCEP4-luciferase and pCEP4-eGFP, as well as pCMV-luciferase as a control, formed an electrostatic interaction with the polymer at ratios of 1:1, 2:1, and 4:1 (w/w). As shown in Figure 3, the migration of each plasmid was completely delayed at the 1:1 ratio with PAMAM G4, but 2:1 with PAMAM-Arg.

Size Measurements and Zeta Potential of the Polyplexes.

On the basis of the results of the agarose gel retardation assay, the particle size and zeta potential values of the polyplexes were measured at a fixed 4:1 (polymer:DNA) weight ratio (Table 1). The size of plasmid DNA before complex formation was around 1-3 μ m. This size decreased to the

**Figure 4.** Comparison of the transfection efficiency of various plasmid DNAs with PAMAM-arginine polymer (solid line, pCEP4-luciferase; dashed line, pCN-luciferase; dash-dot, pCMV-luciferase).

nanometer scale after complexes were formed. For example, pCEP4-luciferase/PAMAM-Arg and pCEP4-eGFP/PAMAM-Arg complex sizes were in the range of 80-150 nm. We presume that the arginine-grafted PAMAM dendrimer is more effective at promoting DNA condensation than native PAMAM dendrimer, because the arginine residues of PAMAM-Arg increased the positive charge density of the polymer itself. As a result, the arginine-grafted PAMAM dendrimer displayed much improved capability of complex formation with plasmid DNA compared to native PAMAM G4. This enhanced ability of PAMAM-Arg to form complexed DNA

**Figure 5.** Enhanced and prolonged GFP expression by using an EBV-based plasmid DNA and PAMAM-arginine polymer in HEK293 cell lines (* pCEP4-luciferase/PEI was observed at 4 days after transfection).

particles at a nanometer scale is thought to be particularly desirable for gene delivery approaches.

Based on the results of surface charge measurement of DNA and the polyplexes, the negative values of naked DNA are likely to be due to the presence of phosphate groups in the nucleotide backbone. However, upon complex formation, the values became positive (+20 ~ 40 mV). The positive charge of the polyplexes could facilitate endocytosis by promoting interaction with the negatively charged cell surface.

Luciferase and GFP Expression with Polymer/pDNA Complexes (Polyplexes). Transfection efficiency (measured by luciferase activity) was compared using three different kinds of plasmid DNA containing a CMV promoter, which were all complexed with PAMAM-Arg (Figure 4). The transfection efficiency of luciferase-encoding plasmids lacking EBNA-1 decreased by approximately 10-fold after 7 d, whereas pCEP4-luciferase showed sustained high-level gene expression. While the overall efficiency of pCEP4-luciferase/PAMAM-Arg was approximately 10-fold higher than that of plasmids lacking EBNA-1, there was a significant difference of about 100-fold at 8 d after transfection.

The time-dependent GFP expression was observed using a fluorescence microscope. Polyethylenimine (PEI), which is known to promote high transfection efficiency, and native PAMAM were used as control polymers, and pEGFP-N1 containing no EBNA1 gene was used as a control plasmid. As shown in Figure 5, PEI/pCEP4 and PAMAM-Arg/pCEP4 complexes yielded the most robust fluorescence when they were measured on days 3-6 after transfection, whereas all the other combinations showed much weaker fluorescence. However, cells transfected with PEI showed no fluorescence at 7 d due to the known severe cytotoxicity of this polymer. The transfection efficiency of pCEP4-eGFP at 7 d after transfection was similar in the presence of either PAMAM-Arg or PEI. Moreover, although the pEGFP-N1/PAMAM-Arg polyplex did not contain the EBNA1 gene or oriP sequence, the fluorescence expression was considerably stronger than that of PAMAM polyplexes lacking the arginine modification. We observed that the cells transfected with EBV-based DNA maintained GFP fluorescence, whereas little fluorescence was evident at 3 weeks after transfection.

The above results show that transfection efficiency increases gradually, and it is effectively sustained and enhanced when a competent transfection vector like PAMAM-Arg could be combined together with the plasmid DNA containing the EBNA1 gene and the oriP sequence. On the basis of these results, we expect that the sustained gene transfection could

be established by combination of the EBV-based plasmid gene expression and an effective gene carrier.

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