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Research Article

A comparative investigation on efficiency of bacteriophage lambda and M13 based vectors for delivering and expression of transgene in eukaryote cells

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Abstract

Gene delivery might be affected by several tribulations based on carrier/vector applied. Bacteriophages lambda and M13 have different genome conformations; linear double-stranded and circular single-stranded respectively. Therefore, it might be expected that these two common classes of gene delivery vehicles will have different capacity for gene delivery and expression in eukaryote cells. To address the possible effects of linear double-stranded and circular single-stranded genome conformations of bacteriophages lambda and M13 on the transgene expression, the transfection efficacy of two vectors based on lambda and M13 were compared in AGS cell line. The GFP encoding sequence was inserted into the Lambda ZAP-CMV XR vector which resulted in λ-ZAP-CMV-GFP construct. The construct was then in vitro packaged using Gigapack® III Gold packaging extract and λ-GFP phage particles were obtained. The λ-GFP phage particles were then used for in vivo excisioning which resulted in M13-CMV-Script-GFP construct. 10^{11} copy of λ-ZAP-CMV-GFP or M13-CMV-Script-GFP constructs were transfected into AGS cells using lipofectamine 2000. Transfection efficiencies were analyzed by FACS. Results showed that linear double-stranded λ-ZAP-CMV-GFP was efficient than single-stranded form of M13-CMV-Script-GFP while its double-stranded form was efficient than the linear double-stranded λ-ZAP-CMV-GFP construct for transgene delivery and expression. Moreover the GFP signals resulted from transfections by single-stranded form of M13-CMV-Script-GFP construct faded more quickly in comparison to others. These findings highlight that genome conformation of gene carriers might be an important factor when seeking for an appropriate gene carrier/vehicle.

***Keywords*:** :Gene delivery; Phage-mediated gene transfection; Vector conformation

Introduction

Bacteriophages are bacterial viruses that infects specifically bacterial cells, however, they have been considered as a class of gene delivery vehicles for eukaryotes by ever-increasing number of researchers (Larocca et al., 1999; Larocca et al., 2001; Piersanti et al., 2004; Lankes et al., 2007; Khalaj-kondori et al., 2010). Among bacteriophages, M13 and lambda are the most studied and exploited ones in molecular biology. These bacterial viruses have several striking features including no tropism for eukaryotic cells, safety, physical stability at various harsh conditions, inexpensive mass production, ease of genome manipulation, nanostructured size and availability of diverse approaches for their specific targeting which have made them attractive for researches of different fields (Poul and Marks, 1999; Larocca and Baird, 2001; Olofsson et al., 2001; Larocca et al., 2002; Catherine et al., 2004). Lambda is a head and tailed bacteriophage with a double-stranded genome of ~48 kb. It has an icosahedral capsid of ~55 nm in diameter surrounding its genome and a fibrous tail of ~145 nm in length (Kaiser, 1966; Zanghi et al., 2005).While, M13 is a filamentous bacteriophage with a ~6.4 kb circular single-stranded genome surrounded by a proteinaceous coat (Petrenko et al., 1996; Calendar and Abedon, 2005).

There are several steps that may affect the efficacy of gene delivery procedure when using bacteriophages as gene delivery vectors/carriers.First of all, as for any other gene delivery vehicles, phage particles should be internalized into target cells, and then they must bypass the cytoplasmic barriers e.g. degradative enzymes of the cytoplasm as well as trafficking-associated problems of transgene toward the cell nucleus (Larocca and

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Baird, 2001). Surface modifications of phage particles through phage display and chemical coupling technologies have been partly overcome these problems. It has been shown that bacteriophages M13 and lambda targeted to mammalian cells by displaying or coupling of host derived ligands on their surfaces can result in expression of the transgene in the target cells (Larocca et al., 2001; Sapinoro et al., 2008; Khalaj-Kondori et al., 2011; Kim et al., 2012). Moreover, displaying of different peptides such as NLS on the surface of bacteriophages and application of proteasome inhibitors indicated an exciting role on efficient intracellular trafficking of phage based vectors (Akuta et al., 2002; Volcy and Dewhurst, 2009). Nevertheless it seems that entering to the nucleus is not sufficient by itself, since several studies indicated that despite displaying of targeting ligands on the phages surface, relatively a small fraction of transfected cells was expressing the transgene.

The final step in a gene delivery attempt is transcription of the transgene and subsequent translation of mRNA to the functional protein. Recently it was reported that phagemid particles bearing inverted self-complementary sequences with capacity of converting to double-stranded DNA can result in higher level of transgene expression than those containing single-stranded DNA (Prieto and Sánchez, 2007). This finding suggests that the bacteriophage genome structure and conformation might be implicated on the processes such as transcription and translation even after successful entering into the nucleus.

In the present study, effects of single-stranded circular conformation of a vector, as seen in M13-based gene carriers, at transgene delivery and expression efficacy were compared to that of double-stranded linear vector conformation such as λ-based ones. To achieve this purpose, the sequence encoding for GFP was inserted into the Lambda ZAP®-CMV XR vector to obtain λ-ZAP-CMV-GFP construct as a vector with double-stranded linear conformation. Then λ-ZAP-CMV-GFP vector was used for obtaining M13-CMV-Script-GFP construct as a vector with single-stranded circular conformation. Finally, efficacy of the two constructs in delivery and expression of GFP transgene in AGS cell line was studied by florescence activated cell sorting (FACS) analysis.

Materials and Methods

Construction of λ-ZAP-CMV-GFP vector and in vitro packaging

λ-ZAP-CMV-GFP vector was constructed according to a previous report (Khalaj-kondori et al., 2010). In brief, the sequence coding for GFP was obtained by PCR using a primer pairs, forward: 5'-GTAGAATTCCGCCACCATGGTGAGCA -3', reverse; 5'-GACCTCGAGTTACTTGTACAGTTCGTCCATG C-3' and pEGFP-N1 plasmid (BD Biosciences Clontech) as template. The PCR product was purified, digested with EcoRI and XhoI (Fermentas, Lithuania) and ligated to the Lambda ZAP-CMV XR vector (figure 1a) (Stratagene La Jolla, CA) digested with the same enzymes. To obtain λ-GFP particles, the ligation product was in vitro packaged using Gigapack® III Gold packaging extract (Stratagene La Jolla, CA) and used to transfect E.coli XL1-Blue MRF´ strain (Stratagene La Jolla, CA). The melted and cooled to 48°C NZY Top agar was added on transfected E.coli XL1-Blue MRF´ cells and immediately plated on prewarmed NZY agar plates. After 12 hours incubation at 37ºC, the recombinant plaques appeared were confirmed by plaque-PCR and amplified.

Amplification of λ-GFP particles

XL1-blue MRF´ cells were cultured in 10 ml of LB broth supplemented with 10 mM MgSO4 and 0.2% (W/V) maltose. The culture was infected by λ-GFP at a multiplicity of infection (MOI) of 1:20 and incubated at 37°C with vigorous agitation until lysis observed. Upon lysis, chloroform was added to a 5% (v/v) final concentration, mixed well and incubated for 15 minutes at room temperature. Then cell debris was removed by centrifugation for 10 minutes at 500×g. DNase I and RNase A (Fermentase) were added to a final concentration of 1µg/ml to eliminate nucleic acids. To precipitate the phage particles, polyethylene glycol 8000 and NaCl were added to a final concentration of 10% (W/V) and 1M respectively and incubated in ice for 60 minutes. Phage particles were collected by centrifugation at 11000×g, 4°C for 10 minutes. Phage pellet was resuspended in SM buffer and tittered

Construction of M13-CMV-Script-GFP vector and M13-GFP particles

Inserts cloned into the Lambda ZAP-CMV XR vector can be excised out of the phage in the form of the kanamycin-resistant pCMV-Script® EX phagemid (figure 1b) vector. So, M13-CMV-Script-GFPvector wasprepared by in vivo excision. In vivo excision was achieved according to the instruction manual of Lambda ZAP®-CMV XR Library Construction Kit (Stratagene). Briefly, 200 µl of XL1-Blue MRF´cells at an OD_{600} of 1 were

coinfected with 250 µl of λ-GFP particles (10^5particles) and 1 µl of Ex Assist helper phage (10^6pfu/µl) and the mixture was incubated at 37°C for 15 minutes to allow the phage to attach to the cells. Then 3 ml of LB broth was added to the mixture and incubated for 3 hours at 37°C with shaking. The mixture was heated at 70°C for 20 minutes and cell debris was pelleted at 1000×g for 15 minutes. The supernatant contains M13-CMV-Script-GFP phagemid packaged as filamentous phage particles (M13-GFP particles). XLOLR cells (Stratagene) were infected with M13-GFP particles and plated on NZY agar containing 30mg/ml kanamycin. Colonies appeared were confirmed by colony-PCR.

Amplification of M13-GFP particles

A 10 ml of 2xYT containing 50mg/ml kanamycin was inoculated with a confirmed single colony of XLOLR and incubated for one hour at 37°C. R408 helper phage (Sratagene) was added to the culture at a MOI of 10 and incubated for 18 hours. The culture was spinned for 10 minutes at 6000×g and Polyethylene glycol 8000 and NaCl were added into the supernatant in a final concentration of 10% and 1M respectively followed by incubation for 60 minutes on ice. The precipitated M13-GFP particles were collected by centrifugation at 17000×g, 4°C for 20 minutes. The pellete was resuspended in SM buffer and tittered.

DNA extraction

The λ-ZAP-CMV-GFP and M13-CMV-Script-GFP constructs were extracted from λ-GFP and M13-GFP particles respectively. M13-GFP and λ-GFP particles were obtained as mentioned above. Phenol-chloroform (1:1 v/v) was added to the phage particles in SM buffer and filliped to destruct the capsid followed by centrifugation 12000×g, 4°C for 10 minutes. The procedure was repeated for 2 times and followed by another round of chloroform extraction. DNA was precipitated by adding one volume of ice cold isopropanol and collected by centrifugation at 12000×g, 4°C for 10 minutes. Moreover replicative form of the M13-CMV-Script-GFP construct was obtained by plasmid minipreparation method. DNA was quantified by Picodrop and evaluated on 1% agarose gel.

Transfection of AGS cells and FACS analysis

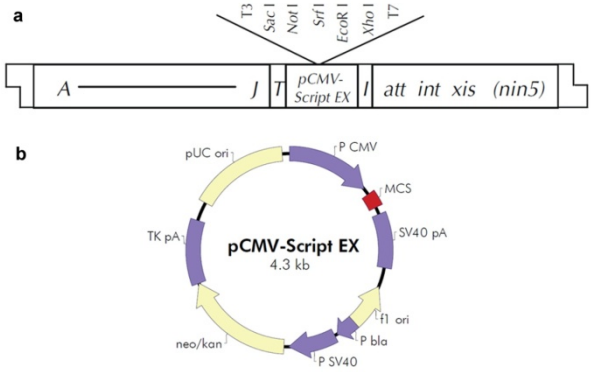
AGS cells were seeded into 24-well plates containing RPMI 1640 with 10% FBS at 4×104 cells/well 24 h prior to transfection. Cells were transfected by 1011 copy of λ-ZAP-CMV-GFP, single-strand or double stranded forms of M13-CMV-Script-GFP constructs using Lipofectamine 2000 (Invitrogene). After 24 and 48 hours incubation, cells were removed from the wells with trypsin–EDTA (Gibco BRL), pelleted and incubated with 5% paraformaldehyde in PBS for 20 min. The cells were washed with PBS and resuspended in PBS containing 0.01% sodium azide and analyzed by FACS (Becton Dickinson Biosciences, San Diego, CA). Transfection efficiency was measured as the percentage of total cells that were GFP positive as detected by FACS analysis. Transfections were done in duplicate and performed three times.

Results and discussion

Preparation and confirmation of constructs

The sequence encoding for GFP was PCR amplified using pEGFP-N1 as template and inserted into the Lambda ZAP-CMV XR vector (figure 1a) to obtain λ-ZAP-CMV-GFP construct. The construct was in vitro packaged and used for transfection of E.coli XL1-Blue MRF´ cells. Plaques appeared were confirmed by plaque-PCR assay. The positive plaques represent λ-GFP particles containing λ-ZAP-CMV-GFP construct.

We obtained M13-CMV-Script-GFP construct from λ-ZAP-CMV-GFP construct by in vivo excision. In vivo excision was resulted in M13-GFP phagemid particles containing the M13-CMV-Script-GFP construct. To plate the excised M13-GFP phagemid, XLOLR cells were infected and colonies appeared were analyzed and confirmed by colony-PCR (figure 2).



Figrure 1. Map and elements of (a) the Lambda ZAP-CMV XR vector and (b) the pCMV-Script® EX phagemid vector

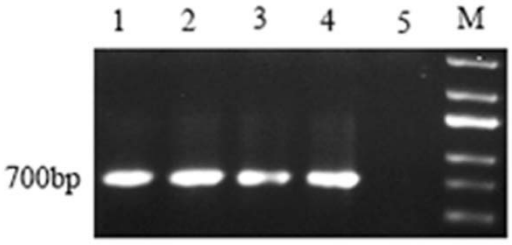


Figure 2. Screening of XLOLR colonies by Colony-PCR.

Lanes 1, 2 and 3; colony-PCR of three well separated colonies, lanes 4 and 5; positive and negative controls respectively, M; size marker.

The Lambda ZAP-CMV XR vector has been designed to allow in vivo excision and recircularization of any cloned insert contained within the lambda vector in the form of the pCMV-Script® EX phagemid vector by the same excision mechanism used with the Lambda ZAP vectors (Stratagene, 2007). This means that the gene inserted would be in the same context of regulatory and vector backbone sequence elements both in lambda and phagemid constructs. This is important because it might eliminate any possible effects of vector backbone sequence on the insert gene expression. In other words, we used this strategy to lower as much as possible any vector sequence-related effects that might affect the transgene expression.

Equaling the copy number of constructs

For comparative study of the efficacy of structurally different constructs in transgene delivery and expression it is needed that different constructs used for transfection have the equal copy numbers. To achieve equal copy numbers, the molecular weight for one molecule of each construct in µg was multiplied by 10^{11} to obtain the µg of each construct needed for each transfection reaction. We chose the copy number 10^{11} for all tests, because it yields 800 ng of λ-ZAP-CMV-GFP construct being in the best DNA concentration range that might be transfected using lipofectamine 2000 for 24-well platform. It should be noted that during M13-GFP phagemid amplification not only the phagemid will amplify but the helper phage R408 amplification will occur as well. Fortunately, the helper phage R408 has been designed to amplify 50 folds less than the phagemid(Stratagene, 2007). Amplification proportion of phagemid to helper phage R408 was tested on gel electrophoresis (figure 3). So, we applied a correction coefficient of one-fifty for calculation of the phagemid copy number. Table 1 summarizes the calculations and represents the micrograms of each construct per one transfection reaction.

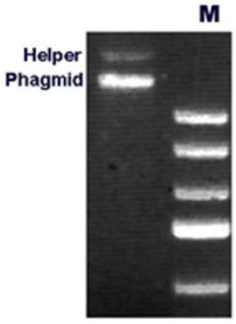


Figure 3. Gel electrophoresis showing the amplification proportion of phagemid to helper phage R408. After phagmid amplification, DNA was purified and electrophoresed on 1% agarose gel. M; size marker.

Table 1. Calculations for equaling the copy number of λ-ZAP-CMV-GFP and M13-CMV-Script-GFP constructs for one transfection reaction.

Construct	Size(kb)	Molecular weight (µg)	Conc. (ng/µl)	Copy No./ µl	Vol. (µl)
λ-ZAP-CMV-GFP	~42.7	4.68005×10^{-11}	830	0.1773×10^{11}	5.640
M13-CMV-Script-GFP	~5	2.74008×10^{-12}	158	0.56×10^{11}	1.76

Transgene expression analysis

To reveal potential effects of vector conformation and structure on transgene delivery and expression, AGS cells were transfected by the same copy numbers of single-stranded circular M13-CMV-Script-GFP and double-stranded linear λ-ZAP-CMV-GFP constructs using lipofectamine 2000. Moreover, double-stranded circular form of the M13-CMV-Script-GFP construct was used as positive control. Transgene delivery and expression efficiencies were evaluated at both individual and total cell population levels. To achieve these, we took advantage of GFP reporter system which is capable to report gene expression accurately even in the level of individual cells (Soboleski et al., 2005). Figure 4 depicts the results obtained from FACS analysis of AGS cells transfected by the above-mentioned constructs. FACS analysis showed that transfections by single-stranded circular M13-CMV-Script-GFP resulted in 36.8% and 25.5% GFP positive cells in 24 and 48 hours post transfection assays respectively. Whereas transfections by double-stranded λ-ZAP-CMV-GFP resulted in 23.3% and 37.3% GFP positive cells in 24 and 48 hours post transfection assays respectively (figure 4a). Mean Fluorescence Intensity (MFI) analysis (figure 4b) also revealed that the MFI of GFP positive cells in 24 and 48 hours post transfection tests of the single-stranded circular M13-CMV-Script-GFP construct was 4.86 and 4.07 respectively. Furthermore, the MFI of GFP positive cells in 24 and 48 hours post transfection tests of double-stranded linear λ-ZAP-CMV-GFP construct was 3.76 and 5.01 respectively. Moreover, the transfections by control construct resulted in 32.6% GFP positive cells with MFI of 7.00 in 48 hours post transfection assays (Figure 4a and b).

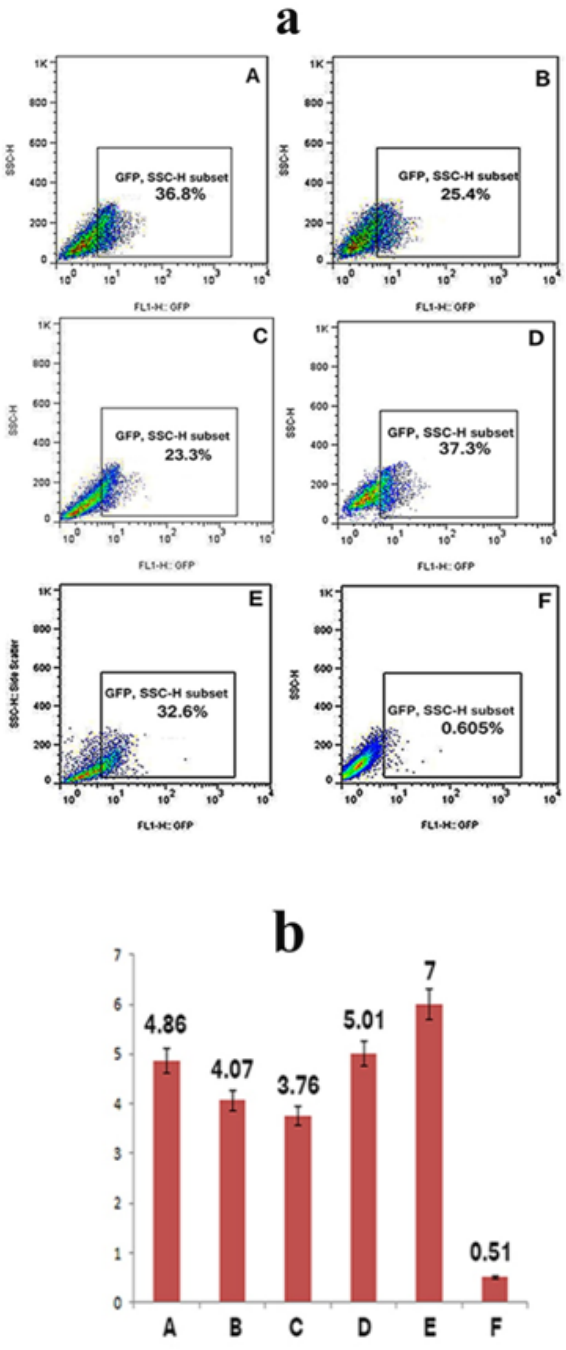


Figure 4. FACS analysis of GFP transgene expression in AGS cell line. AGS cells were seeded in 24 well plates and transfected with 10^{11} copies of λ-ZAP-CMV-GFP and M13-CMV-Script-GFP constructs. 10^{11} copies of double-stranded form ofM13-CMV-Script-GFP construct were usedascontrol to allow comparisons. After 6 hours the medium wasexchanged and cells were incubated until 24 hours or 48 hours. Finally cells were removed from the plate, fixed by paraformaldehyde 2% and resuspended in sodium azide 0.01% and analyzed by FACS. Panel **a** shows GFP positive cells obtained from transfection of AGS cells with 10^{11} copies of A) single-stranded M13-CMV-Script-GFP construct analyzed after 24 hours B) the same construct analyzed after 48 hours, C) double -stranded λ-ZAP-CMV-GFP construct analyzed after 24 hours, D) the same construct after 48 hours and E) double-stranded M13-CMV-Script-GFP construct analyzed after 48 hours. F) no construct. Panel **b** shows the Mean Fluorescence Intensity (MFI) of GFP positive cells of corresponding wells of panel a. All tests have been repeated tree times.

It is worth noting that none of the constructs are able to amplify in the AGS cells, hence the GFP expression level will be depend on merely the copy number and conformation/structure of the construct molecules available for transcription apparatus of the transfected cell. Comparison of the percentage of GFP positive cells achieved at the 24 hours post transfection assays showed that those transfections mediated by single-stranded circular M13-CMV-Script-GFP result in more GFP positive cells (36.8%) than those of the double-stranded λ-ZAP-CMV-GFP (23.3%) construct. This finding implies at first glance that the single-stranded circular conformation might be more efficient than the linear double-stranded conformation for transgene delivery and expression. However, this finding was not true when comparisons were made for the results of 48 hours post transfection assays because those transfections mediated by double-stranded λ-ZAP-CMV-GFP resulted in more GFP positive cells (37.3%) than those of the single-stranded circular M13-CMV-Script-GFP (25.4%) construct. In other words, the percentage of GFP positive cells achieved by transfections mediated by single-stranded circular M13-CMV-Script-GFP construct was decreased over time while that for double-stranded λ-ZAP-CMV-GFP was increased. This finding was true for MFI comparisons too. The MFI of GFP positive cells in 24 hours post transfection assays (3.76) of λ-ZAP-CMV-GFP construct was less than that of 48 hours post transfection assays (5.01). Moreover, the MFI of GFP positive cells achieved in 24 hours post transfection assays (4.76) for M13-CMV-Script-GFP construct was higher than that of 48 hours post transfection assays (4.07). Comparing the results of control to the results of both test constructs revealed that those for the control construct was more close to the results of λ-ZAP-CMV-GFP than those of the M13-CMV-Script-GFP in 48 hours post transfection assays. This might be explained by existence of double-stranded structure in both control and λ-ZAP-CMV-GFP constructs. In other words, it implies that having double-stranded structure might be more critical for a construct to be stable in the transfected cell, because whilst higher percentage and MFI of GFP positive cells in 24 hours post transfection assays mediated by M13-CMV-Script-GFP construct they diminished in 48 hours post transfection assays.

What obvious is from plenty of phage-mediated gene transfer studies to eukaryote cells

(Larocca et al., 1998; Larocca et al., 1999; Di Giovine et al.,2001; Larocca et al., 2001; Urbanelli et al., 2001; Burg et al., 2002; Prieto and Sánchez, 2007) as well as the present study is that constructs having single-stranded circular conformation such as M13-CMV-Script-GFP can mediate transgene expression. However, the mechanism by which this type of constructs is recognized by transcription machinery of eukaryote cells is not clear. This fact raises some questions which need more research to be answered including; are single-stranded circular constructs recognized by eukaryote cell transcription machinery? Can eukaryote cells convert single-stranded constructs to the double-stranded circular conformation? Our 24 hours post transfection assays showed that probably the transcription machinery of eukaryote cells are able to recognize single-stranded circular constructs, hence the GFP positive cells as well as their MFI observed for M13-CMV-Script-GFP were higher than those for 48 hours post transfection assays. Moreover, a research by Michael and coworkers showed that it might be possible converting single-stranded circular constructs to double-stranded form. They transfected many tumor cell lines by EGF-targeted phagemid particles in the presence of genotoxic agents such as camptothecin and obtained expression efficiency up to 30%. Moreover camptothecin treatment resulted in increased stable GFP expression up to 120 hours (Burg et al., 2002). A possible mechanism for this observation might be induction of DNA damage repair system which converts single-stranded DNA to double-stranded form which in its turn may increase the transgene expression efficacy. In addition, other researchers increased transgene expression by designing a construct capable of transforming to double-stranded form. They designed phagemid vectors bearing inverted self-complementary sequences for their expression cassette capable of converting the single strand genome of fF to dsDNA structure in HEK 293T cells (Prieto and Sánchez, 2007).

In conclusion, the results of present study highlights that the double-stranded structure and conformation of vector is critical for proper and stable expression of the transgene. So when one intended to use a carrier such as a bacteriophage for delivering a transgene to eukaryote cells/tissues it might be important to choose a carrier with double-stranded genome such as bacteriophage lambda. However, single-stranded bacteriophages such as M13 also might be of important options if designing a strategy which helps to convert single-stranded structure to double-stranded conformation.

Acknowledgments

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