Functional Nucleotide Sequences Capable of Promoting Non-viral Genetic Transfer

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1 Introduction

The major disadvantages of non-viral gene delivery systems are the low efficiency of transfection and transient expression of the transgene. These shortages can be overcome, at least partly, by employing plasmid vectors with particular functional sequences, such as Epstein-Barr virus (EBV)-derived genetic elements (EBNA1 gene and oriP) and the *sleeping beauty* (*SB*) transposable element. Transfection experiments with EBV-based plasmid vectors strongly suggest that the performance of non-viral vectors (gene delivery methods/materials) per se is not as low as widely believed, in terms of the rate of plasmid DNA trafficking from the extracellular space into the cytoplasm. The inability of of plasmid DNA to be transported into the nucleus is the critical hurdle in conventional non-viral transfection, but it may be cleared by using an EBV-based plasmid vector albeit not only by modifying non-viral vectors. This concept may have significant implications for development of novel non-viral gene delivery systems that are applicable to a variety of purposes, including functional genomics and molecular therapeutics.

2 Plasmid Vectors as an Important Component of Non-viral Gene Delivery Systems

Viral vectors consist of recombinant viral particles that are capable of infecting target cells, while non-viral gene delivery systems do not make use of any genetically mod-

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ified infectious viruses. Being free from virus-associated undesired effects, e.g. potential generation of replication-competent retroviruses and a high immunogenicity due to adenoviral proteins, non-viral gene delivery systems may offer promising measures not only in genetically modifying cultured cells and animals but also in treating patients (Niidome and Huang 2002; Mazda 2002). Another advantage of non-viral systems is their large-scale, affordable manufacture. The major problem that needs to be solved is the low efficiency of non-viral systems in transferring and expressing exogenous genes in target cells, compared with the transduction efficiency obtained using their viral counterparts. This is the reason why a technical breakthrough is required in order to develop non-viral gene delivery systems that are feasible for genetic engineering, functional genomics, and molecular therapeutics.

Generally, non-viral gene delivery systems consist of two components, both of which should be taken into account in developing superior non-viral systems:(1) nucleic acids carrying genetic information, most typically plasmid DNA (pDNA), and (2) gene delivery methods/materials (non-viral vector). Therefore, both the pDNA and the non-viral vectors (gene delivery methods/materials) must be improved to obtain ideal non-viral vector systems.

A variety of non-viral vectors have been devised, based on a range of chemical compounds and physical methods. Chemical compounds (carrier molecules) include synthetic and natural macromolecules, such as cationic lipids (Felgner and Ringold 1989; Rocha et al. 2002; Kumar et al. 2003) and cationic polymers (Tang et al. 1996; Kukowska-Latallo et al. 1996; Lemkine and Demeneix 2001) that interact with nucleic acid to form complexes (lipoplex, polyplex, etc.) to be endocytosed into cells. Physical methods, such as electroporation (Herweijer and Wolff 2003), particle bombardment (gene gun) (Cui and Mumper 2003), and other procedures (Plank et al. 2003; Hosseinkhani et al. 2003), promote transfer of nucleic acid into cells in an endosome-pathway-independent manner. "Naked pDNA" methods are another means to transfect exogenous genes into tissue/organs (Wolff et al. 1990).

However, the other component of the non-viral gene delivery systems, pDNA, has not been very extensively studied, in an effort to improve non-viral gene delivery systems. Indeed, nucleotide sequence drastically affects not only intensity but also longevity of transgene expression. More importantly, the use of particular nucleotide sequences drastically improves the efficacy of gene delivery, as will be discussed later. If ideal sequence elements are devised, potentially every non-viral vector may be significantly improved in terms of the transfection efficiency by combining them with plasmid vectors containing such elements.

3 Plasmid Vector Sequence Crucially Affects the Efficacy of Non-viral Gene Transfer

This section discusses the powerful impact of plasmid vector components on the efficiency of non-viral gene delivery by providing evidence from a very simple, but profound and evocative, experiment (Fig. 1).

pS.CD8α and pSES.CD8α are plasmid vectors harboring exactly the same expression cassette for murine CD8α cDNA as a reporter gene (Satoh et al. 1997). The pSES.CD8α possesses EBV nuclear antigen 1 (EBNA1) gene and oriP sequence, I

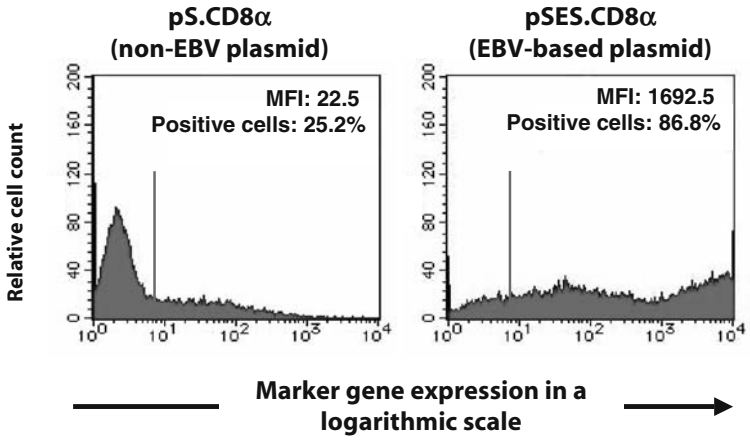

Fig. 1. Plasmid vector composition critically affects the efficacy of non-viral gene transfer. Transfection with an EBV-based plasmid vector resulted in an extremely high proportion of marker-gene-positive cells as well as a tremendously high intensity of marker gene expression on a single-cell basis (*right*). This indicates that a non-viral vector, in this case electroporation, operates at a considerably high efficiency (both plasmids were transfected by the same procedure), while the low efficiency of a conventional plasmid vector (*left*) can be attributed to the inability of the plasmid sequence to appropriately function inside the cells. See text for details

discussed later, while pS.CD8α is a conventional plasmid vector without the EBV sequences. These plasmid vectors were transfected by electroporation into KE cells, which are an EBNA1-expressing transformant derived from the K562 human leukemic cell line (Mazda et al. 1997). Two days after cultivation without any drug selection, cells were stained with a fluorochrome-conjugated anti-mouse CD8α antibody followed by flow-cytometric analysis.

Figure 1 shows the histograms. The KE cells that had been transfected with pS.CD8α expressed murine CD8α at a low level on the surface. This is not surprising because electroporation-mediated transfection into a cell line does not achieve very high efficiency without drug selection. In striking contrast, transfection with pSES.CD8α resulted in tremendously strong expression of the marker gene on the cell surfaces.

Comparing the two histograms in more detail, the results clearly reveal that the pSES.CD8α transfection succeeded in both (1) a higher transfection efficiency, in terms of the increase in percentage of CD8α-positive cells (87% (pSES.CD8α) vs. 25% (pS.CD8α)), and (2) a much stronger expression in each single CD8α-positive cell, as demonstrated by a dramatic elevation in the fluorescent intensities (1690 (pSES.CD8α) vs. 22.5 (pS.CD8α)).

This simple experiment provides insights into non-viral transfection. The two cell populations shown in Fig. 1 were transfected and cultured in exactly the same way, while the plasmids shared a common expression cassette for the marker gene. The only difference was the presence or absence of the EBV genomic elements (EBNA1

gene and oriP). It is quite unlikely that the electric pulse increased the permeability of plasma membrane in such a manner that only pSES.CD8α was effectively penetrated into cells while pS.CD8α selectively remained impermeable. Instead, the experimental result that nearly 90% of the pSES.CD8α-transfected cells expressed the transgene at a high level indicates that the electroporation procedure worked at an extremely high efficiency in this experimental setting, and that pS.CD8α also should have been transmitted from extracellular space into the cytoplasm at a comparable efficiency. The failure of pS.CD8α to result in high CD8α expression can thus ascribed to the different behaviors of the two plasmids after being introduced into the cells, namely intracellular distribution.

Therefore, the experimental data strongly suggest that the major obstacle to non-viral gene transfection, i.e., the failure of cytoplasmic pDNA to be transported into the nucleus (see below), can be overcome by employing an EBV-based plasmid vector, but not by improving the electroporation procedure, which already has reached maximum efficacy. Similar results can be obtained by electro-transfecting various types of cells with plasmid vectors with or without EBNA1 gene/oriP (Mazda et al. 1997; Hirai et al. 1997; Satoh et al. 1998; Tomiyasu et al. 1998).

Interestingly, non-viral vectors other than electroporation also give similar outcomes. Indeed, it has frequently been shown that differences of several-fold to several-thousand-fold between the transfection efficiencies of EBV-based and conventional (non-EBV-based) plasmid vectors are obtained when they were transfected into various cells in culture by means of cationic lipids (Satoh et al. 1997; Harada et al. 2000; Asada et al. 2002) or cationic polymers (Tomiyasu et al. 1998; Harada et al. 2000; Maruyama-Tabata et al. 2000; Tanaka et al. 2000; Ohashi et al. 2001; Iwai et al. 2002; Nakanishi et al. 2003). Significant difference in transfection rates were also obtained by transfection in vivo into various tissues or tumors by means of cationic polymers (Maruyama-Tabata et al. 2000; Iwai et al. 2002; Nakanishi et al. 2003), electroporation (Kishida et al. 2001; Ohashi et al. 2002), and naked DNA methods (Tomiyasu et al. 1998; Cui et al. 2001).

The experimental evidence indicates that the efficacies of non-viral vectors are not as poor as widely believed. Successful transfection with EBV-based plasmid vectors strongly suggest that non-viral delivery systems enables transfer of pDNA into cytoplasm at a considerable rate, although the efficiencies vary according to the types of delivery systems, target cells, etc.

This notion raises a fundamental question as to whether or not "transfection efficiency" can accurately be assessed using the widely accepted experimental systems, which are typically as follows: (1) appropriate target cells are transfected with pDNA carrying a marker gene by means of a delivery system to be examined, (2) cultivation of the cells for an appropriate period, (3) the cells, cell extracts, or culture supernatants are collected and the expression level of the marker gene is measured. The procedures are modified depending on the purpose of the study, e.g. to examine transfection efficiency in vivo, appropriate organs/tissues are substituted for cells in step (1), and instead of (3), the organs/tissues are collected to evaluate marker gene product. Apart from such variations, the efficiencies of non-viral gene transfer systems are assessed by performing experiments that are based on the same concept as described above.

However, interpreting the data obtained from such experiments requires consideration of the following: Several independent steps are critically involved in marker

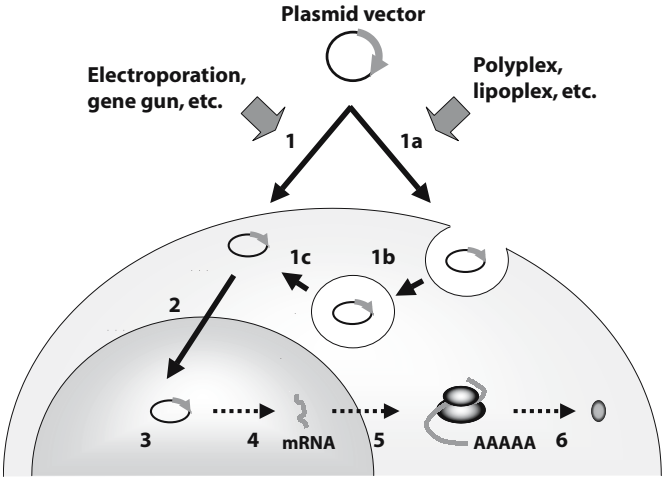

Fig. 2. Multiple steps are involved in successful non-viral gene transfection.
The performance of a non-viral vector cannot be simply evaluated by the magnitude of marker gene expression, which depends on efficiencies of multiple steps. Indeed, intra-nuclear delivery of pDNA (*step 2*) is the most critical step that determines the rate of non-viral transfection. Use of the EBV-based plasmid vector may elevate the transfection rate by accelerating this step. See text for details

gene expression after transfection (Fig. 2). These include: (1) transfer of pDNA from the extracellular space to the cytoplasm through the plasma membrane, (2) transfer of pDNA from the cytoplasm to the nucleus through the nuclear pore complex (NPC), (3) maintenance of pDNA in the nucleus, (4) transcription, (5) maturation of mRNA and its transfer from the nucleus to the cytoplasm, and (6) translation and post-translational modification of the protein. When polyplexes or lipoplexes are used as the non-viral vectors, additional steps are usually required instead of (1), i.e., (1a) transfer of pDNA from the extracellular space to the endosome/lysosome compartment by endocytosis, (1b) maintenance of pDNA in the compartment, (1c) release of pDNA from the endosome/lysosome compartment to the cytoplasm.

Although the efficiency of step (1) may reflect the capability of the non-viral vector of interest, the efficiencies of the other steps depend on different factors. For example, the rate of step (4) has been attributed mainly to the transcriptional activity of the promoter/enhancer element contained in the plasmid. Therefore, the expression level of a marker gene is proportional to the product of the efficiencies of all these steps, but not simply to the performance of the non-viral vector tested.

Actually, step (2) is the most critical process, in which efficiency crucially determines marker gene expression in many non-viral gene delivery systems (Lechardeur and Lukacs 2002; Hebert 2003)(see below). Without ameliorating this step, non-viral gene delivery cannot be improved, because transfer of pDNA from the extracellular space to the cytoplasm has succeeded at a considerably high rate as demonstrated in Fig. 1 and by other, similar experimental evidence for a variety of non-viral vectors,

as discussed above. Basically, non-viral vectors are not capable of promoting step (2), which is the reason why plasmid vector sequences are extremely important.

From another point of view, in order to devise or modify non-viral vectors, use of EBV-based plasmid vectors instead of conventional plasmid vectors may greatly help in assessing the capability of novel non-viral vectors, because the efficiency of step (2) should be sufficiently high so that the efficiency of step (1) is measured at high sensitivity and accuracy.

4 Epstein-Barr Virus-Based Plasmid Vectors

The rationale for EBV-based plasmid vectors, as well as concrete examples of their use in non-viral gene delivery systems, has been reviewed elsewhere (Mazda 2000, 2002). Here, the EBV system is briefly explained in order to support the concepts presented above. The EBV-based plasmid vector contains the EBV nuclear antigen 1 (EBNA1) gene and oriP sequence, which were originally described as elements to support replication of the EBV genome (double-stranded circular DNA of approximately 172 kb) in latently infected human cells (Adams 1987; Haase and Calos 1991; Yates and Guan 1991). EBNA1 is a nuclear phosphoprotein that binds specific motifs in the oriP sequence, conducting DNA replication in concert with cellular cofactors. Therefore, EBV-based plasmid vectors are maintained in transfected cells as an extrachromosomal circular DNA (episome). Some groups have developed autonomously replicative artificial chromosomes, so that transgene expression persists for a sufficiently long period of time (Sun et al. 1994; Westphal et al. 1998; Kelleher et al. 1998; Black and Vos 2002; Stoll et al. 2001; Stoll and Calos 2002; Sclimenti et al. 2003). The episomal maintenance is particularly important when target cells proliferate after transfection.

EBNA1 also facilitates trafficking of oriP-bearing plasmid DNA from the cytosol to the nucleus (Ambinder et al. 1991; Fischer et al. 1997) and its maintenance in the nucleus (Jankelevich et al. 1992). Another function of EBNA1 is the activation of transcription through binding to oriP (Gahn and Sugden 1995; Puglielli et al. 1996; Reisman and Sugden 1986; Sugden and Warren 1989; Wysokenski and Yates 1989). We hypothesized that these characteristics explain why plasmid vectors with EBNA1 gene and oriP enable high-level gene transfer and expression.

We then estimated the contribution of each activity of the multifunctional viral elements to the high efficiency of transfection. We determined that transfer of pDNA from the cytoplasm into the nucleus is actually the most critical step, and that the efficacies are quite different between conventional and EBV-based plasmid vectors (Kishida et al., in preparation). This is consistent with previous reports indicating that most pDNA molecules introduced into a cell are entrapped and degraded in the cytoplasmic and endo-lysosomal compartments without successful transport into the nucleus, and this barrier is the critical obstacle of conventional non-viral gene delivery strategies (reviewed in Lechardeur and Lukacs 2002). We also found that replication of pDNA does not contribute to the high rate of transfection of EBV-plasmids, although this function plays key roles in prolonged transgene expression in cultured human cells.

Taking advantage of the high transfection efficiency, preclinical gene therapy studies were carried out with EBV-based plasmid vectors in a variety of animal model

systems. Briefly, subcutaneous tumor transplants such as melanoma (Asada et al. 2002; Kishida et al. 2001), hepatocellular carcinoma (Iwai et al. 2002), prostate cancer (Nakanishi et al. 2003), and Ewing's sarcoma (Maruyama-Tabata et al. 2000) were treated with cytokine-gene-transfected tumor vaccine (Asada et al. 2002) or intratumoral delivery of cytokine genes (Kishida et al. 2001), a suicide gene (Maruyama-Tabata et al. 2000; Iwai et al. 2002), or an apoptosis-inducing gene (Nakanishi et al. 2003). Metastatic malignancies were also attempted, including hepatic metastasis of melanoma (Asada et al. 2002; Kishida et al. 2003a), lung metastasis of lymphoma (Kishida et al. 2003b), and hepatic metastasis and peritoneal carcinomatosis of reticulum cell sarcoma (Itokawa et al. 2004). Therapeutic/prophylactic experiments were also performed against nonmalignant disorders including cardiomyopathy (genetic transfer of the β2-adrenergic receptor into failing cardiomyocytes) (Tomiyasu et al. 2000), diabetes mellitus (intrahepatic delivery of the insulin gene under the control of a glucose-responsive promoter) (Yasutomi et al. 2003), and acute herpes simplex virus type 1 (HSV1) infection (DNA vaccination using a viral antigen gene with/without cytokine genes as adjuvants) (Cui, et al. 2003, 2005). Significant therapeutic outcomes were obtained from all these trials due to the high efficacy of the non-viral delivery systems employing the EBV-based plasmid vectors.

5 The Sleeping Beauty Transposable Element

In the folllowing, DNA transposons, and especially the *sleeping beauty* (*SB*) element, are discussed as another example of using a plasmid vector with particular nucleotide sequences as a component of non-viral gene delivery systems. Although EBV-based plasmid vectors are maintained as episomes in target cells, resulting in prolonged expression of the transgene, employment of a transposable element enables chromosomal integration of the transgene, providing an alternative strategy to prolonging the expression period obtained after non-viral gene delivery.

Unlike retrotransposons, which replicate and jump in a copy-and-paste manner, DNA transposons are excised from their original genomic location and subsequently reinserted elsewhere in the genome (a cut-and-paste process). DNA transposons have been utilized for insertional mutagenesis as well as germline transgenesis in invertebrate organisms including *Drosophila* and *C. elegans*, but similar systems were not available in vertebrates until recently. While some DNA transposons in invertebrates, such as the Tc3 element in nematode (Raz et al. 1998) and *Minos* in fly (Zagoraiou et al. 2001), were shown to function in cells of vertebrate species, a more efficient DNA transposon for vertebrates was developed by "awakening" a salmonid fish transposon belonging to the Tc1/mariner superfamily, which are molecular fossils inactivated millions of years ago by the accumulation of mutations (Ivics et al. 1997). Ivics et al. constructed a synthetic transposon based on phylogenetic data so that inactivating mutations were eliminated. The reconstructed element, the *SB* transposon, precisely worked in fish as well as in mouse and human cells (Ivics et al. 1997).

The structure of *SB* and the mechanisms of transposition have been described elsewhere in detail (Izsvak and Ivics 2004; Ivics and Izsvak 2004). Briefly, natural *SB* consists of a single open reading frame encoding an enzymatic polypeptide (transposase),

which is flanked by two terminal inverted repeats (IRs) at both ends. The transposase binds the recognition sequences in the IRs in a highly sequence-specific fashion, leading to mobilization of the transposon. *SB* was engineered to transpose a gene of interest, by inserting the transgene segment between the IRs while supplying the transposase either in *trans* (Izsvak et al. 2000; Dupuy et al. 2002) or in *cis* (Mikkelsen et al. 2003). In the original *SB*, the gene to be mobilized should be less than several kilo base pairs in size, since the efficiency of transposition declines with increasing the length (Izsvak et al. 2000).

More recently, the *SB* transposon was further improved through a mutagenesis approach so that the modified version is capable of transposing DNA segments more than 10 kilo base pairs in size (Zayed et al. 2004). The transposable activity was also augmented, particularly when combined with the DNA-bending, high-mobility-group protein, HMGB1, which is a host-encoded cofactor of *SB* transposition (Zayed et al. 2003).

Plasmid vectors harboring the *SB* transposon can be combined with variety of non-viral vectors, including cationic lipid (Liu, L. et al. 2004b), cationic polymer (Belur et al. 2003) and other carrier molecules (Harris et al. 2002; Zayed et al. 2003, 2004), while microinjection (Fischer et al. 2001; Dupuy et al. 2002; Horie et al. 2003), polybrane shock (Ortiz-Urda et al. 2003) and naked DNA (Yant et al. 2000; Montini et al. 2002; Belur et al. 2003) procedures have also been used. *SB* transposes not only in vertebrate cells in culture, but also in somatic (Yant et al. 2000; Montini et al. 2002; Belur et al. 2003; Liu et al. 2004a, Liu, 2004b) and germline (Fischer, S. E. et al. 2001; Dupuy et al. 2002; Horie et al. 2003) tissues of mice in vivo. The major advantage of using *SB* is the prolonged expression of the transgene in transfected cells and tissues due to chromosomal integration of the recombinant transposon, while the integration sites in host chromosomes are almost random (Vigdal et al. 2002; Roberg-Perez et al. 2003).

Some studies have suggested the feasibility of the *SB* system for gene therapy, based on therapeutic experiments in which *SB*-bearing plasmid vectors were administered to model animals (reviewed in Izsvak and Ivics 2004; Ivics and Izsvak 2004). Yant et al. treated hemophilic mice with a rapid intravenous administration of a *SB* construct carrying the α1-antitrypsin gene together with a transposase construct, partially ameliorating the bleeding phenotype for more than 5 months (Yant et al. 2000). Montini et al. transfected fumarylacetoacetate hydrolase (FAH) gene knockout mice with a mixture of a FAH gene-containing transposon construct and a transposase expression plasmid, as a therapeutic model against hereditary tyrosinemia type I, resulting in long-lasting expression of the therapeutic gene in the liver (Montini et al. 2002). Ortiz-Urda et al. performed ex vivo transfection experiments in which a *SB* vector encoding both laminin 5 and a selectable antibiotic marker was delivered into keratinocytes obtained from patients with junctional epidermolysis bullosa (JEB), a lethal hereditary skin disorder (Ortiz-Urda et al. 2003). After drug selection, the genetically modified cells were transplanted into immune-deficient mice, so that the skin tissue regenerated. Long-term expression of laminin 5 at a therapeutic level and phenotypic correction of the skin (hemidesmosome formation and blistering) were obtained, strongly suggesting that *SB*-mediated genetic engineering of self-renewing cells results in stable maintenance of transgene expression in the progeny cells (Ortiz-Urda et al. 2003).

6 Conclusions

Currently, significant advances in the development of non-viral vectors are being made by means of recent technological innovations, including nanotechnology (Vijayanathan et al. 2002). Employment of functional genetic elements, such as EBNA1/oriP and the *SB* transposon, may further improve their efficacy, compensating for the shortcomings of non-viral systems. EBV-based plasmid vectors are transported into and maintained in the nucleus as well as replicated as episomes, overcoming the problems of the low transfection rate and the transience of transgene expression. The *SB* transposon enables chromosomal integration of a transgene in transfected cells, potentially enabling its permanent expression, although random integration into the host genome may cause aberrant expression and/or silencing of cellular genes that locate close to the integration site. Both the EBV-based plasmid vector and *SB* have been shown to work not only in vitro but also in vivo. Plasmid vector sequences should be focused on as an important component of non-viral gene delivery systems, while their combination with other devises, such as polypeptides containing a nuclear localization signal (reviewed in Hebert 2003), may also be useful in developing promising strategies.

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