

Nuclear localization signals in phage terminal proteins provide a novel gene delivery tool in mammalian cells

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Terminal proteins (TPs) of bacteriophages prime DNA replication and become covalently linked to the genome ends. Unexpectedly, we have found functional eukaryotic nuclear localization signals (NLSs) within the TP sequences of bacteriophages from diverse families and hosts. Given the role of bacteriophages as vehicles for horizontal gene transfer (HGT), we postulated that viral genomes that have covalently linked NLS-containing terminal proteins might behave as vectors for HGT between bacteria and the eukaryotic nucleus. To validate this hypothesis, we profited from the *in vitro* Φ 29 amplification system that allows the amplification of heterologous DNAs producing linear molecules of DNA with TP covalently attached to both 5' ends. Interestingly, these *in vitro*-generated TP-DNA molecules showed enhanced gene delivery in mammalian cells, supporting a possible role in HGT by transferring genes between prokaryotes and eukaryotes. Moreover, these TP-DNA molecules are a useful tool to amplify and subsequently deliver genes efficiently into the eukaryotic nucleus. Here, we suggest various possible applications and further developments of the technique with biotechnological and therapeutic purposes.

understanding of gene transfer mechanisms in prokaryotes.³ One of the more interesting theories on prokaryotes-to-eukaryotes gene transfer is the so-called “you are what you eat” hypothesis, by which prokaryotic genes from symbiotic or food bacteria could have replaced ancient eukaryotic genes over evolutionary time.^{4,5} In agreement with this theory, the occurrence of HGT in eukaryotes is higher in unicellular organisms that do not require specific germ lines to be reproduced and to spread the newly acquired genes.⁶ However, once inside the eukaryotic cell, the nuclear envelope constitutes a barrier that prevents access to the cell genome. Only small molecules can overcome this blockade and most nuclear proteins are gifted with a specific amino acid portion, called “nuclear localization signal” (NLS), that label them as nuclear and as such they are detected by a specific nuclear transport pathway. This is a fine-tuned process that requires one or more carrier proteins that mediate the transport through the nuclear pore complex in an energy dependent manner.⁷ In the same way, DNA molecules over 300 bp cannot penetrate efficiently through the nuclear envelope, which constitutes an obvious obstacle for foreign DNA uptake, either from natural origin or from human-developed technologies, such as cell transfection.⁸

We have found a widespread occurrence of predicted NLSs in phage terminal proteins (TPs) from different families of bacteriophages and with diverse hosts and show that the TPs of five different bacteriophages, Φ 29, Nf, PRD1, Bam35 and Cp-1, localize to the mammalian cell

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As more complete genome sequences become available, the role of horizontal gene transfer (HGT) in shaping them becomes clearer.^{1,2} However, its importance in the evolution of eukaryotic genomes is often overshadowed by the greater prevalence and the more advanced

nucleus.⁹ Moreover, after the latter work was accepted we noticed that the TP of the recently discovered phage YS61,¹⁰ which has been proposed to constitute a new independent subfamily within the Podoviridae virus family, also contains a putative NLS in its sequence (residues 126–155, according to NLStradamus¹¹ prediction), which further confirms the widespread presence of NLSs in TPs.

The use of TP-mediated protein-primed DNA replication has been reported or suggested in diverse replicons from all domains of life, including prokaryotic and eukaryotic viruses, linear plasmids and transposons. In this mechanism, the OH group of a specific serine, threonine or tyrosine of the TP is used by the replicative DNA polymerase to start DNA synthesis from both ends of the linear double-stranded genome and thus the TP remains covalently linked to such 5' ends, giving rise to TP-DNA genome molecules.¹² Moreover, since bacteriophages, and viruses in general, are known active vehicles for HGT, we hypothesized that viral genomes that have covalently linked NLS-containing terminal proteins may behave as vectors for HGT between bacteria and the eukaryotic nucleus, thus providing the next step to the “you are what you eat” theory. In order to demonstrate that the linked TP can mediate the nuclear translocation step we took advantage of the described TP-mediated Φ 29 minimal amplification system¹³ that requires only four proteins, DNA polymerase, TP, single-stranded DNA binding protein and double-stranded DNA binding protein. This system generates in vitro DNA molecules containing the TP covalently linked to the 5' DNA ends that confer resistance to exonuclease degradation. We used a circular plasmid DNA, which contains the *yfp* gene expression cassette for mammalian cells and the phage Φ 29 origins of replication. This plasmid, once linearized, can be amplified in vitro by the phage replication system, thus incorporating the TP covalently linked at both 5' DNA ends. We found that the attached Φ 29 TP increased *yfp* expression with respect to that of a control linear DNA and, accordingly, this positive effect was reversed by removing the TP with proteinase K treatment.⁹ These results show that TP-linked DNA

enhances gene delivery into the eukaryotic nucleus, supporting a possible role in HGT by transferring genes between prokaryotes and eukaryotes. Furthermore, the in vitro-generated TP-DNA molecules are a useful tool to deliver genes into eukaryotic nucleus. Although the use of TPs as gene delivery vectors had been already suggested,^{14,15} we have now an amplification system that allows generating the in vitro TP-DNA molecules with increased stability and transfection yield (Fig. 1). This TP-DNA might be generated using either a DNA that already contains the phage origins of replication (Fig. 1A, left side) or any linearized DNA after ligation of linkers containing the required origin sequences¹³ (Fig. 1A, right side). The possible applications of in vitro-generated TP-DNAs are numerous. On the one hand, we have shown that they can be complexed with standard transfection agents and used for routine cell transfection with an increased yield. On the other hand, the use of fusion proteins with several functional motifs, such as DNA binding domains, NLS, cell penetrating domains (TAT), endosome disruption enhancers (diphtheria toxin) or even cell-specific antibodies have been shown to improve gene delivery in cultured cells.^{16–18} To test whether the Φ 29 amplification system is able to use a TP with some fusion domains, we performed in vitro amplification assays with an YFP-TP fusion protein, which has the yellow fluorescent protein (YFP) fused to the N-terminal end of Φ 29 TP and found that the YFP-TP protein can be incorporated to the Φ 29 genome origins with similar yield than the wild type TP (not shown). Thus, different motifs or proteins could be fused to Φ 29 TP in order to obtain TP-DNA molecules with specific features, like the ability to penetrate into an specific cell type and providing expression of the desired gene. It is also interesting to consider further refinements of the system, like the addition of site-specific nuclease domains that may target selected human genes and excise or correct regions of genes implicated in monogenic diseases.¹⁹ Altogether, in vitro generation of linear DNA molecules with 5'-linked TPs constitutes a potential novel strategy for the assembly of synthetic therapeutic gene delivery vehicles.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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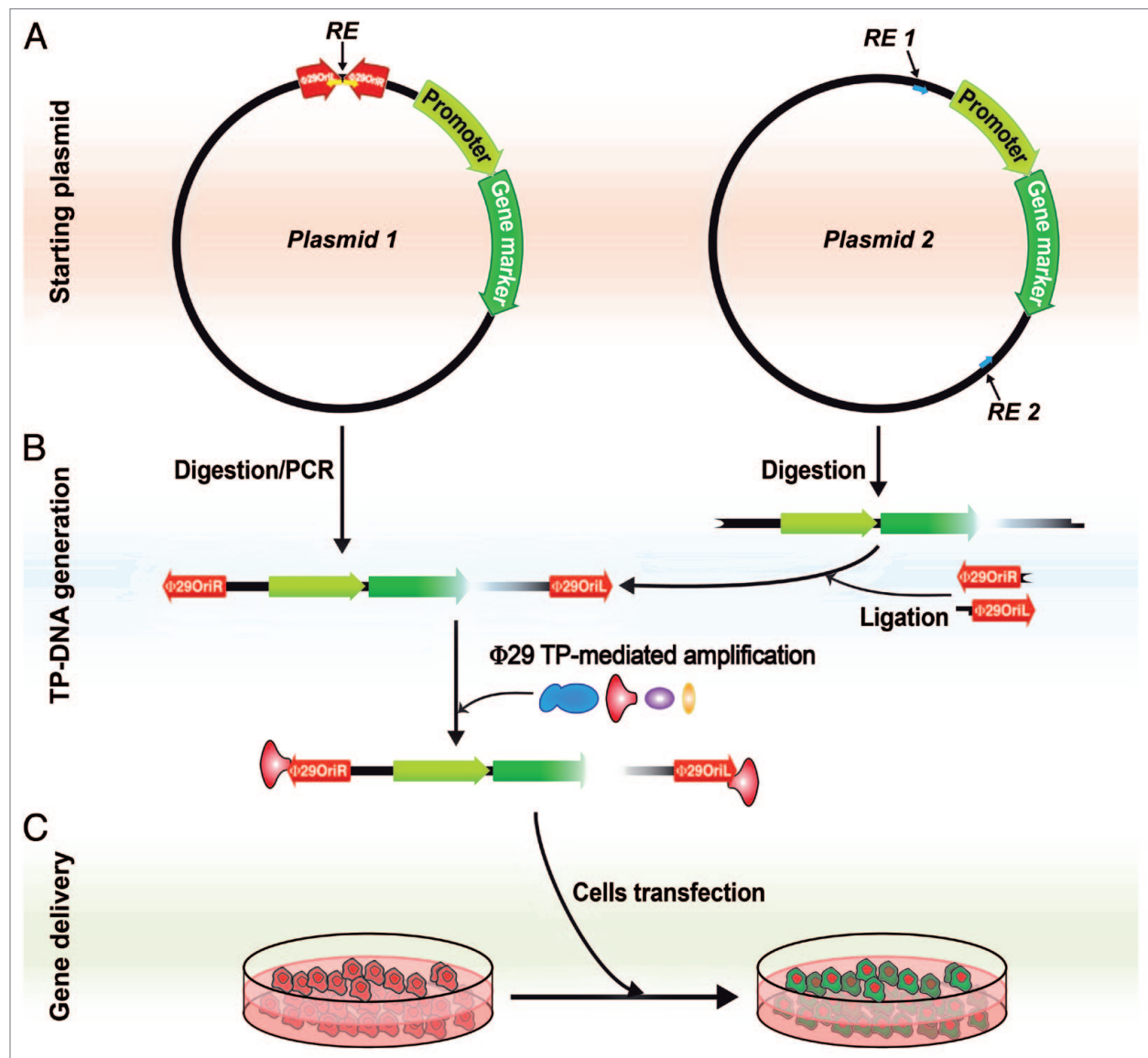


Figure 1. Generation of $\Phi 29$ TP-linked DNAs for enhanced gene delivery. **(A)** Starting plasmid. The transfected DNA should contain a gene marker under a suitable promoter for expression in mammalian cells. In *Plasmid 1* (on the left), the $\Phi 29$ genome replication origins have been cloned into the plasmid, which allows the generation of linear DNA by cleavage with the appropriate restriction endonuclease (RE) or by PCR amplification with specific primers. Alternatively (on the right), $\Phi 29$ replication origins may be linked as specific adapters using incompatible restriction enzyme sites (RE 1 and RE 2). In the latter case, the digested linear DNA fragment may be originated either from an expression plasmid¹³ or a PCR-generated fragment containing the same restriction sites. Yellow and blue arrows stand for the specific PCR primers in each case. **(B)** TP-DNA generation. Linear DNAs containing $\Phi 29$ replication origins at both ends can be amplified with the addition of four $\Phi 29$ DNA replication proteins, DNA polymerase (blue), TP (red), double-stranded DNA binding protein (purple) and single-stranded DNA binding protein (yellow). **(C)** Gene delivery. The amplification mixture can be directly used for gene delivery experiments, like cell transfection. Evaluation of the gene marker expression should be checked by western blot or suitable activity assays.

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