Construction and Characterization of a Robust *in vivo* Technology for the Production of Superior DNA Vectors with Applications in Gene Therapy and Vaccine Developement

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Plasmid DNA (pDNA) vectors are the current conventional technology driving therapeutic gene transfer, whether for use toward mal/nonfunctional gene replacement, DNA vaccination, or production of therapeutic proteins in mammalian cells. However, the conventional pDNA vector suffers from several safety and efficiency limitations: 1) it imparts adverse immune responses to bacterial sequences required for maintenance and amplification in prokaryotes; 2) its bioavailability can be compromised due to size; and 3) it may be genotoxic due to its potential to integrate into the host chromosome and yield an oncogenic event. In this study we have constructed a robust *in vivo* bacterial platform for the production of bacterial sequence-free linear covalently closed (LCC) DNA vectors, termed DNA Ministrings, through the manipulation and application of bacteriophage-encoded recombination systems. Phage N15 and PY54 lysogenize their bacterial hosts as a linear plasmid with covalently closed ends (LCC plasmid). LCC morphology is conferred by the phage-encoded telomerase *via* a single cleaving-joining reaction of the perfect palindrome target site. This system was exploited to generate DNA Ministring vectors, encoding only the gene(s) of interest and necessary complementary eukaryotic expression/enhancement genetic elements that are devoid of unwanted bacterial sequences and are linearized through a single *in vivo* enzymatic reaction.

The *tel* and *telN* prokaryotic telomerase (protelomerase) genes were amplified from PY54 and N15 lysates, respectively, and cloned into a bacterial vector that expresses the gene under control of the temperature sensitive bacteriophage λ CI857 repressor that confers conditional expression from λ *pL/pR* promoters. This regulatory circuit was integrated into a *RecA*$^{+}$ *lacZ*$^{+}$ *E. coli* K-12 strain *via* homologous recombination, where successful recombinants were disrupted for the *lacZ* gene. Recombinant cells are capable of conditional expression of the phage-derived telomerase enzymes by shifting the temperature to >37 °C. Phage P1-derived Cre recombinase was applied as a positive control, since its functionality in generating DNA minicircle vectors has been previously shown. A multi-purpose 342 bp target site termed Super Sequence (SS) that possesses the Cre, Flp, Tel, and TelN target sites in addition to two flanking SV40 enhancer sequences was cloned into two different sites of a GFP expression eukaryotic pDNA vector. The amplification of this DNA vector through *telN / tel* or *cre* expressing Recombinant *E. coli* cells (R-cells) generated bacterial sequence-depleted (LCC) DNA Ministring and (CCC) Minicircle vectors, respectively, as evidenced by digestion patterns of the purified vector. Transfection efficiency of these vectors was assessed in rapidly dividing human ovarian cancer and in relatively slowly dividing human embryonic kidney cell lines. *In vitro* experiments with DNA Ministrings in human cells lines resulted in significantly higher transfection efficiency, bioavailability, and cytoplasmic diffusion levels compared to the parental plasmid precursor and isogenic DNA Minicircle counterparts. The safety of the LCC DNA vector conformation, with respect to insertional genotoxicity, was assessed by insertion of LCC pDNA vectors into bacterial and human genomic DNA. The integration of LCC DNA into bacterial and human host genomic DNA resulted in chromosomal DNA disruptions at site of integration, loss of genome stability, and subsequent cell death. LCC integration-induced apoptotic cell death and natural elimination of the integrant from human cell population improves the safety profile of DNA Ministrings by eliminating integrants following the potential genotoxic side effects of undesired vector integration into the host genome.

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DEDICATION

To my beloved hausband

Mohammad

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List of Abbreviations

a.a.	amino acid
[]	Denotes plasmid-carrier state
AGE	agarose gel electrophoresis
bp	base pair(s)
CAT	chloramphenicol acetyl transferase
CCC	circular covalently closed
CMV	cyto megalo virus
CpG	CpG dinucleotide
DMEM	glucose dulbecco's modified eagles medium
ds	Double-stranded
DTS	DNA nuclear targeting sequence
EBV	Epstein-Barr virus
EGF	epidermal growth factor
EGFP	enhanced green fluorescence protein
ELISA	enzyme-linked immunosorbent assay
EtBr	ethidium bromide
FIV	feline immunodeficiency virus
GFP	green fluorescence protein
GOI	gene of interest
GT	gene therapy
GTA	gene transfer agent
HBsAg	hepatitis B surface antigen
hMSC	human mesenchymal stromal cells
IF	integration frequency
IPTG	isopropyl β-D-1-thiogalactopyranoside
Kb	kilo-base(s)
kDa	kilo Dalton(s)
LB	Luria-Bertani medium

LCC	linear covalently closed
MCS	multiple cloning site(s)
MIDGE	minimalistic immunogenic defined gene expression
NLS	nuclear localization sequence/signal (both)
NPC	nuclear membrane pore complex
p	promoter
PAGE	polyacrylamide gel electrophoresis
pDNA	plasmid DNA
PEG	polyethylene glycol
PEI	polyethylenimine
PI	propidium iodide
PP	parent plasmid
Protelomerase	prokaryotic telomerase
R	recombinant cells
RISC	RNA-induced silencing complex
SDS	sodium dodecyl sulfate
ss	single-stranded
SS	super sequence
SV40	simian virus 40
SV40E	SV40 enhancer
TE	transfection efficiency
TF	transcription factors
Tf-PEG-PEI	transferrin-PEG-PEI
TLR	Toll-like receptor
X-Gal	5-bromo-4-chloro-3-indolyl β-d-galactoside

Chapter 1

General introduction

1.1 Bacteriophage recombination systems and biotechnical applications

Bacteriophage recombination systems have been widely used in biotechnology for modifying prokaryotic species, for creating transgenic animals and plants, and more recently for human cell gene manipulation. In mammalian cells, in contrast to homologous recombination, which benefits from the endogenous recombination machinery of the cell, site-specific recombination requires an exogenous source of recombinase. The mechanism of bacteriophage evolution and their co-existence with bacterial cells has become a point of interest to study ever since the bacterial viruses' life cycle has been explored. Phage recombinases have been applied as valuable genetic tools and today characterization of new phage enzymes and their potential applications in genetic engineering and genome manipulation, vectorology and generation of new transgene delivery vectors, and cell therapy are attractive areas of research. The significance and role of phage recombination systems in biotechnology is reviewed in this chapter, with specific focus on homologous and site-specific recombination conferred by the coli-phages, λ and N15, the integrase from the *Streptomyces* phage ΦC31**,** the recombination system of phage P1, and the recently characterized recombination functions of *Yersinia* phage PY54. Key steps of the molecular mechanisms involving phage recombination functions and their application in molecular engineering, our novel findings on the PY54-derived recombination system and its application to the development of new DNA vectors are discussed.

1.1.1 Bacteriophage

Bacteriophages (phage) are viruses that infect bacterial cells and use the host cell machinery to produce more progeny. Like any other virus, bacteriophages are divided into two major families: “lytic” and “temperate”. After infection of a sensitive host cell, lytic phages immediately propagate themselves by using the host cell molecular machinery releasing thousands of new phage particles into the environment upon lysis of the infected cells. In contrast, temperate bacteriophages must ‘make a decision’ between lytic growth and lysogenic propagation. Lysogeny is a latent state in which phage lytic genes are repressed and the phage genome (prophage) is often integrated into the infected host chromosome or is harboured as an episome, where it will be vertically replicated until the prophage is induced (*1*). To facilitate integration, temperate bacteriophages encode an integrase enzyme that mediates unidirectional site-specific

recombination between two DNA recognition sequences, the phage attachment site, *attP*, and the bacterial attachment site, *attB*. The lysogen in this case is flanked by two hybrid sites, *attL* and *attR*, which consists of half *attP* sequence and half *attB* sequence and becomes the substrates for excessive recombination by excisionase. Several unfavorable environmental factors affect the exclusive reaction which is followed by lytic phage release and initiate a new infection cycle. The ability of phage integrases to unidirectionally recombine two short DNA sequences makes them useful tools for many genetic engineering purposes (*1*). These properties give phage integrases growing importance for the genetic manipulation of living eukaryotic cells, especially mammals and most plants that possess large genomes, for which there are few tools for precise genetic manipulation. Integrases of different phages have been shown to work efficiently in mammalian cells, carry out efficient integration at introduced native *att* sites or pseudo sites available on host genome with partial identity to *att* sites. This reaction has applications in areas such as gene therapy, construction of transgenic organisms, and manipulation of cell lines. Each phage-encoded integrase recognizes specific individual sequences. Some integrases act autonomously, while others act with the help of other phage proteins and/or bacterially encoded host factors (*2*).

1.1.2 Homologous recombination

The process of homologous recombination involves the alignment of similar DNA sequences to form a mobile junction between four strands of DNA, termed “Holiday junctions” that are highly conserved in both prokaryotic and eukaryotic cells. Homologous recombination needs energy and cofactors from host cells (*3*). The frequency of integration by homologous recombination appears to be generally about 10^{-6} for most mammalian cells, although it was reported that this frequency can be increased up to 20 fold by using completely isogenic DNA, or up to 100 fold by making a double-strand break at the target site but providing the resources to generate such a break in endogenous sequences is another challenge (*4*).

A highly efficient genetic engineering system was developed based on homologous recombination, termed "recombineering", which was defined as genetic engineering technique with phage-encoded recombination system that utilizes short sequence similarity. Recombineering and homologous recombination were applied as a method of choice for knock-in and knock-out strategies to either manipulate bacterial and mammalian genome or as alternative strategy to traditional plasmid vectors cloning with PCR products (*5-7*). Homologous recombination is carried by two major pathways, the RecA-dependent and RecA-independent pathways.

1.1.2.1 *E. coli* RecA-dependent recombination pathway

Homologous recombination is a major DNA repair process in bacteria. It is also important for producing genetic diversity in bacterial populations. One of the most well-studied pathways is the RecA-dependent or

the RecBCD pathway that is useful in the repair of chromosomal DNA double-strand breaks (*8*). The RecBCD recombination complex recognizes specific sequences within the bacterial chromosome called "*chi* sites" that prevent DNA degradation. Foreign DNA segments that lack *chi* sites are thus degraded to protect the *E. coli* cell from invading nucleic acids. RecBCD also known as Exonuclease V (Exo V) is an enzyme that recognizes a double-stranded (ds) DNA break and initiates recombination and repair. The enzyme complex is composed of three different subunits called RecB, RecC, and RecD, hence the complex name RecBCD. Both the *alpha* (RecD) and *beta* (RecB) subunits are energy dependent helicases that unwind and separate the strands of DNA, where the *beta* subunit is also a nuclease that produces single-stranded nicks in the DNA. The *gamma* subunit (RecC) recognizes the 5'-GCTGGTGG-3' (*chi*) sequence to initiate recombination (*9*).

RecA is another significant protein in *E. coli* recombinational repair of damaged DNA and has a functional homolog in every species. The RecA activity is energy dependent, where the protein strongly binds to and coats the single-stranded (ss) DNA generated by the exonuclease activity of ExoV to form a nucleoprotein filament. Since it has more than one DNA binding site, RecA can hold a ssDNA and dsDNA together. This feature makes it possible to catalyze a DNA synapse reaction between a DNA double helix and a homologous region of ssDNA. The reaction initiates the exchange of strands between two recombining DNA double helices, then progresses down the strand by DNA branch migration to complete recombination (*8*).

In this study, $RecA^+$ *E. coli* K-12 (W3110) cells were applied to process homologous recombination in order to insert the bacteriophage-derived recombinase genes into the *lac* operon of *E. coli* using the pBRINT-*cat* integrating plasmid (*10*). The pBRINT family of integrating vectors facilitates the homologous recombination-mediated chromosomal integration of cloned sequence of interest into the *lacZ* gene of *E. coli* in *a* RecA-dependent manner (*11*). In contrast, for cloning, and plasmid maintenance and in an *E. coli* host, *recA* is often mutated to ensure the stability of extrachromosomal DNA.

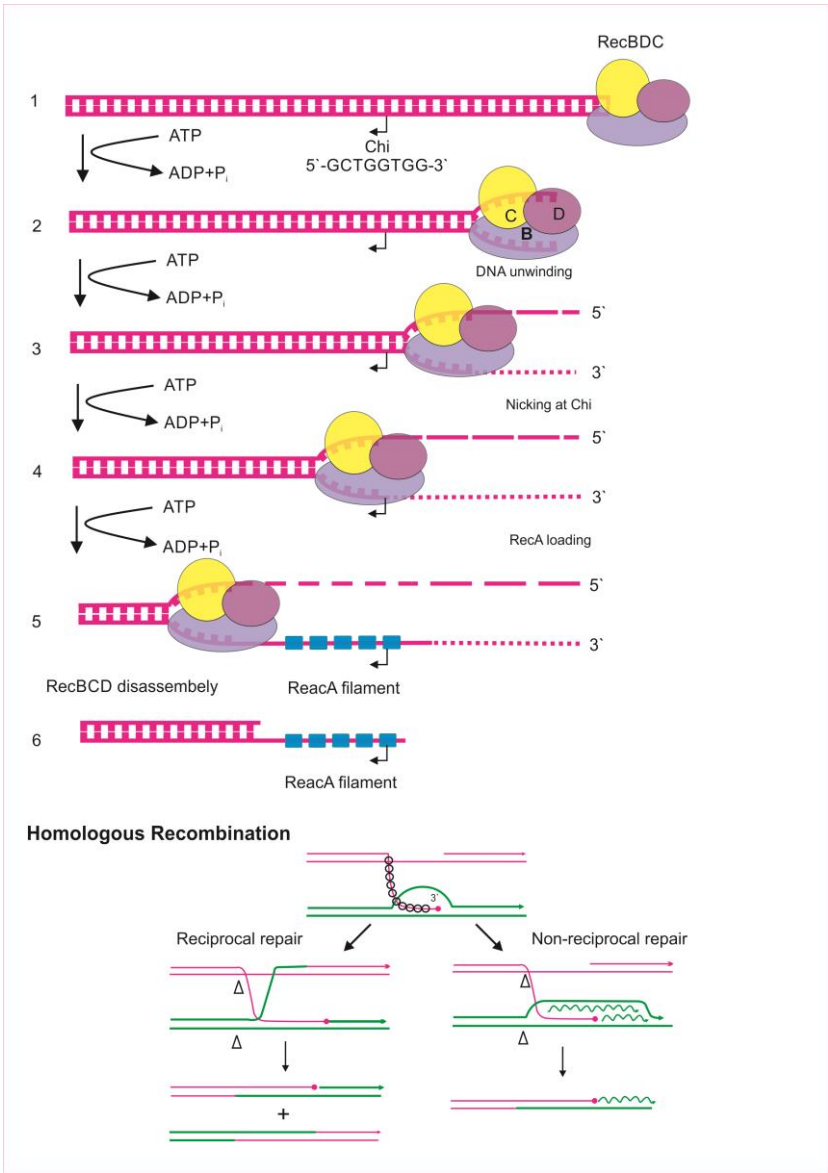


Figure 1- Molecular model of RecA-dependent homologous recombination in *E. coli*.

1) RecBCD binds to a DNA double strand break, **2)** RecB and RecD initiates unwinding of the DNA duplex through ATP-dependent helicase activity, **3)** RecB and RecD continues its unwinding and moves down the DNA duplex, cleaving the 3' strand much more faster than the 5' strand, **4)** RecBCD loads RecA protein onto the *chi* tail. At some undetermined point, the RecBCD subunits disassemble, **5)** The RecA-ssDNA complex invades an intact homologous duplex DNA to produce a D-loop, which can be resolved into intact, recombinant DNA in two ways. [Modified from (*12*)]

1.1.2.2 The bacteriophage λ Red recombination system

The recombination genes of the bacteriphage λ Red system were among the earliest genes to be described. Following the isolation of *recA, recB, recC,* or *recD* recombination-deficient mutants, it was found that phage λ could effectively initiate homologous recombination independent of any of the bacterial recombination pathways due to its own recombination enzymes. λ Red recombination was thus considered to be a separate pathway (*13*).

The "Red system", is comprised of *exo* (α), *bet* (β) and *gam* (γ) genes, all clustered in the *pL*-governed operon of the λ genome, and their expression is regulated by the *cI* repressor (*14*) .The *exo* gene encodes a 24 kilo Daltons (kDa) 5′-3′ exonuclease (Exo) that targets dsDNA nicks; *bet* encodes a 28 kDa ssDNA-binding protein (Bet) capable of annealing complementary ssDNA strands; and *gam* encodes a 16 kDa polypeptide (Gam) that inhibits Exo V activity and confers protection against nuclease attack and digestion of the λ linear dsDNA genome. During the λ lytic cycle, the λ Red recombination is stimulated by endonuclease activity of the λ terminase at *cos* sites, which provide the dsDNA break following the 5′-3′ exonuclease activity of Exo, which provides the 3′ overhang to initiate recombination. In this case, if an intact dsDNA is the only available partner, recombination proceeds through the *E. coli* RecA-dependent mechanism by strand invasion. In contrast, if the available partner is a damaged/nicked dsDNA or a replicating chromosome with a free double-strand end, then recombination can proceed in a RecA independent manner through Bet-mediated annealing. The Red recombination system is facilitated by the λ Gam function. Gam inhibits *E. coli* RecBCD exonuclease activity to prevent further digestion of λ phage DNA by host cell, thus promoting Exo and Bet access to DNA ends to promote Red-mediated recombination (*15*).

The Red recombination system was applied for the first time in 1998 for efficient and simplified genetic engineering of bacterial cells (*16*). The system was exploited by expressing the λ *red* genes, either *via* plasmid or chromosomally inserted, to promote recombination between the bacterial chromosome and a non-replicative linear dsDNA molecules introduced into the cell. Most of the recombination systems that had been developed previous to this study required long (500^{+} bp) homology arms, whereas the plasmid-encoded *red* system facilitated insertion and recombination events between PCR products with short homology arms (50^{+} bp) into the bacterial chromosome with high efficiency. This technology has since been applied for genome editing in different prokaryotic cells, inserting genes for long term and stable production of recombinant proteins and enzymes (*17*).

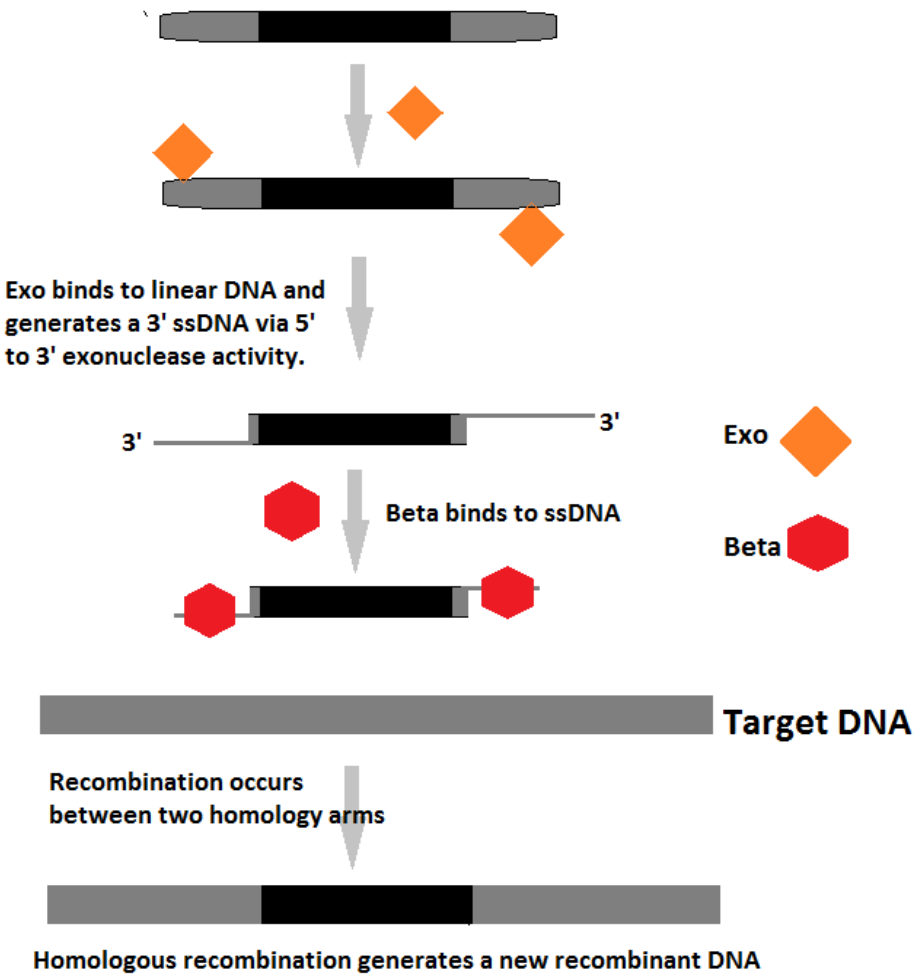


Figure 2- Molecular mechanism of Red-mediated recombination.

1.1.3 Site-specific recombination

Permanent modification of the target genome is of great utility in genetic engineering and is generally carried out by three different approaches: 1) random integration (illegitimate recombination); 2) homologous recombination; 3) site-specific recombination. Although random integration can be used to place the introduced gene into the genome, the lack of control over the position of introduced DNA can result in undesirable side effects, including unpredictable expression of the introduced gene and potential mutagenesis of neighbouring genes. As such, it's a method best suited for insertional mutagenesis. A method that can exclusively yield efficient site-specific integration into safe locations in the target genome would be representing an ideal approach. While homologous recombination provides excellent specificity

in integration sites, it occurs at too low frequency to be optimal for genetic engineering in multicellular organisms (*18*).

Site-specific DNA recombination systems are derived from prokaryotes and unicellular yeasts and among them bacterial viruses provide a repertoire of recombinational systems, a number of which have been exploited to facilitate efficient DNA exchange in human cells. As it was mentioned earlier, temperate bacteriophages are often integrated into the host chromosome at a specific site by recombination, a process that requires specialized 'attachment sites', *attP* and *attB* in the phage and the host chromosomes, respectively. In the prophage, the enzymes generate two junction sites, *attL* and *attR*, consisting of hybrid sequences of *attP* and *attB*, and the integration reaction strictly conserves the original sequences in the recombined product sequences. Lysogeny is reversible, and a minority of cells within a population of lysogens will spontaneously lyse, releasing progeny phage. The integrated prophage is flanked by *attL* and *attR*, which are the substrates for excisionase recombination, a process that reforms *attP* and *attB* and releases the prophage. During the initial stage of the conversion to the lytic phase, a phage-encoded excisionase catalyzes the excision of the prophage genome by cooperating with the phage integrase, and the original *attP* and *attB* sequences are regenerated *via* site-specific recombination between *attL* and *attR* (*19*).

Site-specific recombination facilitates integration, excision, and inversion of defined DNA segments that usually requires no DNA synthesis or high-energy cofactor (*1*). Site-specific recombinases are classified into two major families of tyrosine or serine, which were named based on the amino acid residue that forms a covalent protein-DNA linkage in the intermediate reaction. Although enzymes of the site-specific recombinase families share high specificity and efficiency, their recombination mechanisms are distinctly different. In fact, all recombinases typically mediate efficient "cut-and-paste" type DNA exchange between recognition sites in the range of 30–40 bp or longer (*1*). Recombination is conservative and is characterized by the following events: 1) recombination occurs at a specific site on the interacting DNA molecules; 2) expression and synthesis of the recombinase enzyme; 3) strand exchange occurs at small regions of DNA homology within the recombining sites; 4) pairing of the interacting recombination sites followed by strand exchange resulted in structural intermediates; and 5) resolution of intermediates followed by strand migration (*1*). However, tyrosine recombinases form "Holliday intermediates" by cleaving one strand of each duplex at a time; each crossover site is nicked and must be joined to its partner before the second strand can be cut. In contrast, serine recombinases cut all strands in advance at both crossover sites before any exchange, following 180° rotation and rejoining of cleaved substrate (*20*). The tyrosine-type recombinases are subdivided into two families based on the directionality of site-specific recombination: 1) unidirectional tyrosine-type phage integrases; and 2) bidirectional tyrosine-type simple

recombinases. Generally, the tyrosine family of integrases tends to recognize longer *attP* sequences, and require other proteins encoded by the phage or the host bacteria for their activity. In contrast, serine recombinases recognize short *attP* sequences, but these *attP* sequences are still long enough to be specific on a genomic scale, and do not require host cofactors. Serine-type recombinases are subdivided into two families based on protein size: 1) small serine-type resolvase/invertases; and 2) large serine-type phage integrases (*21*).

Some integrases function with no requisite for cofactors, permitting their activity in foreign cellular environments. From this group, bacteriophage P1 derived Cre (cyclization recombinase), bacteriophage lambda derived Int (integrase), and *Saccharomyces cerevisiae* derived Flp have been applied widely in gene manipulation of higher eukaryotic organisms and in the production of transgenic species (*2*). Recombinases such as Cre, Flp, and Int perform both integration and excision at the same target sites, and thus, their net integration frequency in mammalian cells is generally low. An ideal application would see that the transgene remain inexcisable following integration (*1*); a property shown by some phage derived recombinases such as the *Streptomyces* phage ΦC31 integrase (*22*), *Escherichia* phage N15 TelN protelomerase (*23*), and *Yersinia* phage PY54 Tel protelomerase (*24*).

Here we examine the application of phage-derived recombination systems toward: 1) human genome engineering strategies and regenerative medicine, and 2) vectorology and in engineering of superior DNA transgene delivery vectors.

Table 1- Characteristics of site-specific recombinases active in mammalian cells.

	Catalytic residue	Recombination mechanism	Recombinase family	Protein size	Recombination directionality	Minimal recognition site
Phage λ Int	Tyrosine	Formation and resolution of Holliday junction	Tyrosine-type phage integrases	356 a.a.	Unidirectional	Non-identical 250 bp λ *attP* and 25 bp *attB*
Phage P1 Cre	Tyrosine	Formation and resolution of Holliday junction	Simple recombinase	343 a.a.	Bidirectional	Identical 34 bp *loxP*
Saccharomyces cerevisiae FLP	Tyrosine	Formation and resolution of Holliday junction	Simple recombinase	423 a.a.	Bidirectional	Identical 48 bp *FRT*
Phage ΦC31 Integrase	Serine	180° Rotation and rejoining of cleaved DNAs	Phage integrase (large recombinase)	613 a.a.	Unidirectional	Non-identical ~40 bp *attP* and *attB*
Phage N15 TelN	Tyrosine	Cleaving joining activity at perfect palindrome target sequence to generate DNA intermediate with hairpin ends	Protelomerase	630 a.a.	Unidirectional	Perfect palindrome *telRL* sequence 56 bp
Phage PY54 Tel	TelN-like	Cleaving joining activity at perfect palindrome target sequence to generate DNA intermediate with hairpin ends	Protelomerase-like	617 a.a.	Unidirectional	Perfect palindrome 42 bp *pal* sequence

1.1.3.1 Phage λ Integrase

Phage λ-derived Integrase (Int) is a conservative site-specific recombinase that belongs to the tyrosine-type integrase family of recombinase. The natural function of Int is to carry out integration of the λ genome into and out of the *E. coli* genome. In combination with the Xis (excisionase) protein, Int is also involved excision upon prophage induction. Although the Int-*att* system was considered as a potential candidate for human cell genome manipulation, this was not selected due to certain obstacles encountered in clinical studies. Int requires the host-encoded proteins (Integration Host Factor; IHF) for efficient integration, which limits its integrative recombination activity in heterologous cells. However, several attempts to overcome the problems associated with using these integrases in heterologous cells have been made. As an example, IHF-independent mutant λ integrases have been generated that catalyze intramolecular integration and excision reactions at low levels in the absence of accessory proteins in mammalian cells (*25*). In the current study, the "CRIM (conditional-replication, integration, and modular)" integrating plasmids (*26*) possessing the λ *attP*, were used to insert an operon into a Rec^+ *E. coli* K-12 (W3110) genome. These modified "CRIM" plasmids were integrated into the host bacterial attachment (*attB*) site by supplying phage λ integrase (Int) from a helper plasmid. This system proved very efficient for site specific integration of the gene of interest (GOI) into the host genome (*10*). However, λ integrase usually catalyzes bidirectional site-specific recombination in cells other than natural *E. coli* host, which limits its application in clinical studies where the strict unidirectional integration of foreign DNA into a heterologous genome is desirable (*21, 25*). As such, λ Int is not an ideal recombinase for stable and long term human genome modifications.

1.1.3.2 Phage P1 Cre recombinase and *Saccharomyces cerevisiae* Flp recombinase

Phage P1-derived Cre ("causes recombination") recombinase and *Saccharomyces cerevisiae* derived Flp ("flipase") recombinase were discovered by Austin *et al.* (1981) and Broach *et al.* (1982), respectively. These recombinases catalyze reversible site-specific recombination events between two short, identical sequences, the 34 bp *loxP* target site for Cre, and the 48 bp *frt* site for Flp. Both Cre and Flp belong to the simple/bidirectional tyrosine-type recombinase family, and as such, do not require host-encoded accessory proteins, or specific DNA structures (*21*). The simplicity of the Cre/*loxP* and Flp/*frt* systems has led to their extensive use as tools for *in vivo* genomic engineering such as DNA rearrangements and gene knock-in and knock-outs in a wide variety of heterologous environments, including human cells. Indeed, they have been used to activate or switch gene expression in clinical applications. However, integration of foreign DNA seqeunces into chromosomally integrated *loxP* or *frt* sites is reversible since

wild type Cre and Flp recombinases predominantly catalyze re-excision of the integrated DNA molecule *via* the reverse reaction. This feature has been widely applied in knock-out studies when excision of a particular gene is required.

Cre recombinase differs from typical tyrosine-type temperate phage integrases, which is necessary as P1 as a prophage does not integrate into the host chromosome, but rather replicates as an episomal replicon in host *E. coli* cells. The P1 lysogenic cycle begins with circularization of the injected linear P1 DNA into infected cells by site-specific recombination at *loxP* sites in the phage genome. P1 plasmids replicate independently from the host chromosome and Cre activity resolves dimeric P1 plasmids into monomeric P1 genomes thereby facilitating stable partitioning of P1 into daughter cells during cell division (*27*). Cre's activity in cointegrate resolution limits the utility of the Cre/*loxP* system for applications involving stable integration into the genome.

The Flp recombinase from yeast 2-μm circle, similar to Cre, also differs from typical temperate phage integrases in its physiological function. Initially, the Flp recombinase leads to amplification of the 2-μm circle by inverting the DNA segment flanked by the two *frt* sites; this rearrangement initiates rolling-circle replication by changing the direction of the replication fork. Next, the enzyme resolves the multimeric array generated by rolling-circle replication into 2-μm circle monomers (*28*). Thus, the physiological function of Flp recombinase is the inversion and excision of a DNA segment flanked by two *frt* sites. As such, Flp/*frt*, like Cre/*loxP* is also not ideal for applications involving stable integrative recombination.

Several strategies have been employed to overcome the issues associated with application of these recombination systems for unidirectional integrative recombination. For instance, the recombination-mediated cassette exchange (RMCE)" and the "LE/RE mutant system" have led to the development of two types of mutant *loxP* sequences to facilitate unidirectional site-specific recombination by the Cre recombinase. The Cre recombinase recognizes 34 bp *loxP* sites, each of which consists of two 13 bp inverted repeats that bind to Cre recombinase, and a central 8 bp spacer region where strand exchange occurs. If one nucleotide in the central 8 bp spacer region is modified, Cre recombinase is still able to recognize the modified target site and carry out the integration event, but cannot execute its excision function. This was shown in heterologous genomes, where a pre-inserted DNA sequence flanked by two mutant *loxP* sites containing modified spacer sequences resulted in loss of Cre endonuclease capacity (*29*). Modified *loxP* sites have been used to integrate foreign DNAs into a wide variety of heterologous host genomes including those of plants (*30*), mammals (*31*), and bacteria (*32*).

RMCE-type recombination based on the Flp/*frt* system has also been utilized to facilitate unidirectional integrative recombination, as *frt* target sequences are longer and more complex than *loxP* sites (*19*). Each

48 bp *frt* site consists of three 13 bp repeats and an 8 bp spacer region in which strand exchange occurs. Two nearly identical 13 bp inverted repeats bind the Flp recombinase and flank the 8 bp spacer, while the additional 13 bp repeat follows a 1 bp gap forming a perfect direct repeat with the repeat at one side of the spacer. While the inversion and excision reactions occur with a 34 bp core site consisting of the two 13 bp inverted repeats and 8 bp spacer region, the Flp-mediated recombination is more efficient with the intact *frt* site that contains the third repeat that serves as an extra Flp-binding sequence (*21*).

Both λ and P1 phages derived site-specific recombination systems have been exploited toward production of modern plasmid-derived DNA transgene delivery vectors (Table 2). The first system described in 1997 for bacterial sequence-free DNA minicircle production, employed the λ Int/*attP* site-specific recombination system. In this study, recombinant Int was expressed endogenously from *E. coli* and two *attP* target sequences were inserted into a plasmid DNA vector flanking the eukaryotic expression cistron. Gene *int* expression resulted in site specific excision of *att* sites and generation of two mini circular constructs, one of which contained the bacterial sequence to form the miniplasmid and the other encoding the gene of interest (GOI) cistron to form the desired DNA minicircle (Figure 3). Despite the obvious advantage, the system however, demonstrated several disadvantages such as the toxicity of phage λ Int recombinase in bacterial cells and hence, the necessity for tight control over the recombinase induction process in order to produce the DNA minicircle in culture, and inefficient recombination (40–70%) (*33, 34*). As such, the project instead turned to an alternative strategy using the Cre-*loxP* recombination system. Similar to the first attempt, the Cre recombinase was expressed either endogenously or episomally (*35*). The Cre recombinase would then catalyze site-specific recombination between *loxP* sites available on standard plasmid DNA vector (pDNA) leaving the bacterial sequence-free DNA minicircle for further application (*35*).

Until recently, the Cre-*loxP* recombination system was the method of choice to generate DNA minicircle vectors, despite its limitation in generating pure minicircle with no conventional plasmid residual contamination. The *in vivo* efficiency of this system was <100% and the undigested parent pDNA was the one of the most challenging steps in purification. As such, other groups applied different recombination systems and tactics to improve DNA minicircle production leading to the achievement of highly pure DNA minicircles with no parent plasmid or miniplasmids residual contamination described in the next section (*36*).

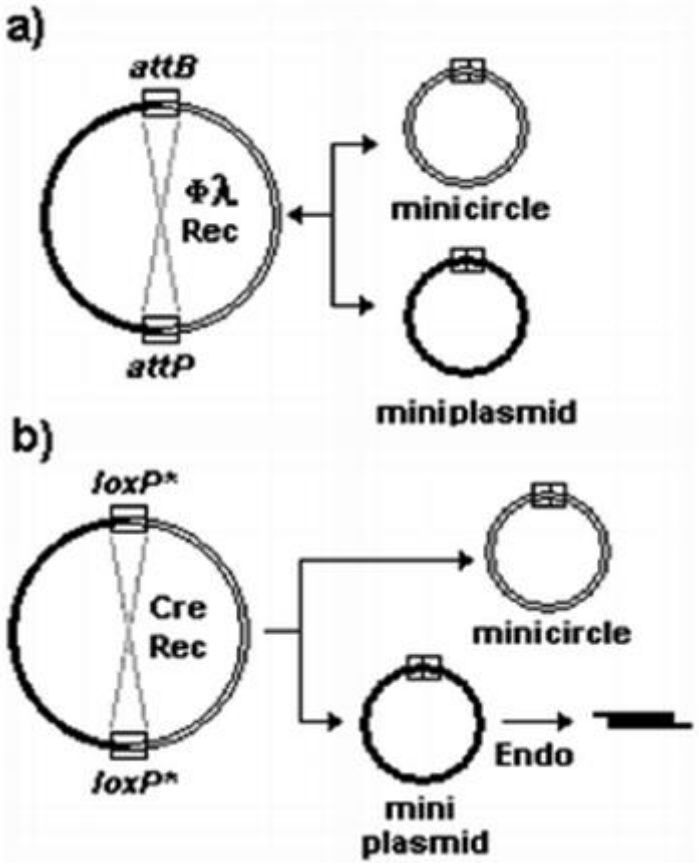


Figure 3- *In vivo* production of minicircle DNA vectors.

Vectors are generated by site-specific recombination *via* two excision sequences (*white, gray boxes*), which flank the mammalian expression cassette and the bacterial amplification unit in a recombinant plasmid vector. *Arrows* indicate the direction of the recombination reaction. **A)** System employing the phage λ *Int* recombinase supplied by the bacterial host strain and the corresponding target sequences *attB* and *attP* (*boxes*). **B)** System employing the Cre recombinase of P1 phage and mutated *loxP* sites for unidirectional recombination [Reproduced with permission from (*37*)].

1.1.3.3 Phage ΦC31 integrase

In the 1990s, actinophage ΦC31 integrase was discovered and found to be homologous to resolvase/invertases (*38*). This enzyme belongs to the large integrase serine-type recombinase family and catalyzes unidirectional site-specific recombination between *attP* and *attB* sites, both of which are normally ~40 bp in size and do not require any supplementary host factors or specific DNA structures. In general, both *att* sites have an identical 2 bp core sequence flanked by 20–25 bp imperfect, inverted repeats (P and P′ in *attP*; B and B′ in *attB*), where there is little sequence similarity between *attP* and *attB* sequences. This integrase requires the slightly longer *attP* sequence for efficient integration and *attP* encodes more perfect inverted repeats than *attB*. The integrase possesses highly homologous N-terminal

catalytic domains (140 a.a.) and diverse C-terminal DNA-binding domains (300–500 a.a.) and the DNA binding domains confer sequence-specific DNA-binding to distinct *att*-integrase pairs (*38*). These integrases function on a recombination mechanism similar to that of the resolvase/invertases serine type recombinases, involving the 180° rotation and rejoining of cleaved *att* DNAs, except that they recognize two different sites and catalyze a tightly regulated unidirectional recombination. The unidirectional recombination is mediated by the larger C-terminal DNA-binding domains of the integrase that possess a motif controlling recombinational direction. The excision reaction requires the phage-encoded excisionase, Xis, which binds tightly to the *attP*- and *attB*-bound dimers, inhibiting the integration reaction, where it binds less strongly to the *attL*- and *attR*-bound dimers to facilitate the excision reaction. It is this characteristic in particular that makes integrases the most ideal recombination enzyme of choice in clinical applications where there is a need for insertion of specific gene into heterologous genome and for provision of a long term / stable expression of the protein product (*39*).

The relative simplicity of serine-type phage integrase-mediated recombination has spurred the development of heterologous gene integration systems based on these enzymes. Genomic integration systems based on ΦC31 integrase have been developed for human genomes editing (*40*). This integrase efficiently catalyzes the site-specific integration of an *attP*-containing DNA vector into pre-existing (engineered) *attB* or *attP* sites in the mammalian genome. The second strategy (*attP*) imparted with better outcome owing to the complexity and lower accessibility of the chromosomal DNA compared to that of the pDNA structure (*41*). In addition, "pseudo *attP*" sites with only 40% identical sequence to wild-type *attP* have been identified that can bind to ΦC31 integrase and mediate ΦC31 integrase-dependent integration at 10-fold higher rates than random sites. Interestingly, these "pseudo *attP"* sites are often present in transcriptionally active regions of genome and euchoromatin chromosomal DNA, and thus, the insertion of a vector into pseudo *attP* sites often provides higher expression levels than those inserted randomly into the human genome. These findings indicate that multiple distinct site-specific recombination systems, based on various serine-type phage integrases, may be employed to simultaneously or sequentially introduce multiple foreign DNAs into *att* sites pre-inserted into heterologous genomes.

The integration frequency mediated by the ΦC31 integrase in mammalian cells is approximately 10- to 100- fold higher than Cre-mediated integration at an inserted wild-type *loxP* site, higher than Flp-mediated integration at an inserted *frt* site, and more efficient than phage integrases of the tyrosine-catalyzed site-specific recombinase family, such as λ Int (*42*).

Table 2- Systems developed for production of bacterial sequence depleted DNA vectors.

Type of DNA vector	General features	Generation Systems	Improvements	Limitations
Minicircle	Non-replicating, CCC, *in vivo* production available, provide sustained and high expression of GOI, no immunostimulatory effect, site specific recombination dependent, high yield production is available	Phage λ *attB-attP* recombination	–	Size exclusion/sequence-specific purification. Low recovery due to bidirectional recombination reaction
		Cre/mutated loxP recombination	Unidirectional recombination reaction, tight control of recombinase gene expression	Size exclusion/sequence-specific purification
		ΦC31 Integrase	Unidirectional recombination reaction, tight control of recombinase gene expression, high yield production of minicircle	
Ministring	Non-integrating, non-replicating, *in vitro* and *in vivo* production available, LCC topology, provide sustained and high expression of transgene, no immunostimulatory effect, TelN/*telRL* or Tel/*pal* protelomerase dependent for *in vivo* production, no limit on high yield production, no isoform generation	"MIDGE"	Enhanced targeting	Enzymatic production process, high cost, limited high yield production
		Purified N15 phage protelomerase TelN	No need to ligase to generate covalently closed ends	Size exclusion/sequence-specific purification, limited high yield production, need purified TelN
		PCR amplified	Enhanced targeting	Synthetic production process, high cost, limited high yield production
		TelN/*telRL* and Tel/*pal* recombination	Unidirectional recombination reaction, tight control of recombines expression, high yield production of ministrings	

1.1.3.4 Phage N15 TelN protelomerase

Bacteriophage N15 (NC_001901) is unique among bacteriophages with respect to its genetic composition and organization. In 1964, Victor Ravin discovered *E. coli* phage N15 in Moscow. *E. coli* phage N15 not only represents the first example of a linear prophage genome with covalently closed ends but it remains to be the only known naturally occurring example of a linear plasmid in *E. coli* (*43*). While N15 virions are very similar to phage lambda in respect to morphology, burst size, plaque morphology, and length of genome, N15 prophage is very unusual in that it is the only colliphage that exists and autonomously replicates as a LCC DNA molecule instead of integrating into *E. coli* genome in the lysogenic state. Like λ, the linear N15 mature phage DNA possesses single-stranded cohesive ends termed as *cos*L and *cos*R. The linear plasmid prophage termini, *telR* and *telL (telRL),* are located near the centre at position 24.8 kb on the mature N15 DNA map. The *telRL* site is located in a similar site that is occupied by the attachment site, *attP*, in λ and defines the boundary between the left and right arms (*44*). In the mature N15 genome, the genes responsible for formation and maintenance of the linear plasmid in host cell, such as the protelomerase, the anti-repressor, as well as replication and partitioning genes are located on the left (0-10 kb) arm. This cluster has some overlap with genes involved in the lytic growth of phage although most lytic genes are located on the left arm. Ravin showed that if the sequences between the left arm and the right arm are deleted , the 5.2 kb mini plasmid product, which is about 30% of the genome, will still be able to replicate individually (*45*).

Figure 4 shows the path of events that occurs in a lysogeny cycle, following injection of the linear N15 genome into the host. First, the genome circularizes by annealing and ligating its terminal cohesive *cos* ends. Next, the telomere-forming *telRL* site on the circular plasmid prophage becomes the target for N15 encoded protelomerase cleaving-joining enzyme (*46*). The *telRL* sequence contains a 56 bp palindrome with two different base pairs at the 12th and 14th positions on both sides of the palindrome centre (*telO*), which forms a 14 bp perfect palindrome recognized and cleaved by TelN protelomerase to create single-stranded termini (Figure 4). After cleavage, the self-complementary single-stranded ends reanneal and create ideal hairpin structures at each ends (*43*).

TelN is a slightly acidic 630 a.a. protein that is about 72.2 kDa in size. It is a key enzyme not only in the generation and replication of a linear plasmid prophage, but it also plays significant role in the lytic growth of N15 phage. It was shown that TelN and λ Int play a similar role in the creation of lysogeny (*46*). As it was explained earlier, the C-terminal segments of integrases are highly conserved, particularly in catalytically active domains. In addition, analytical studies on TelN structure revealed a region (residues 390 – 427) that is analogous to the C-termini of integrases that belong to the tyrosine recombinase family. TelN possesses a tyrosine at the same position as integrases, which suggests that protelomerases and tyrosine recombinases possess similar catalytic mechanisms for DNA cleavage and ligation and may have evolved from a common ancestor. Both

generate a 3′-phosphotyrosine DNA intermediate that enables the covalent rejoining of cleaved DNA strands without the use of a high-energy cofactors (*43*).

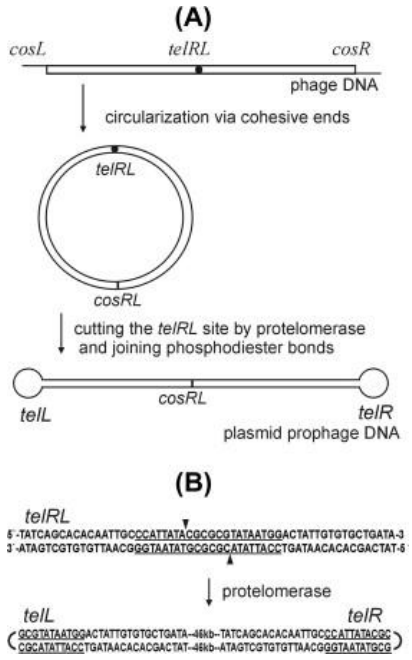


Figure 4- Mechanism of conversion of phage DNA into linear plasmid.

A) *cosL***,** *cosR*, single stranded cohesive ends; *cosRL***,** *cos* site after annealing and ligation of cohesive ends; *telRL*, uncut target site of protelomerase; *telL* and *telR*, left and right hairpin ends of the prophage created by protelomerase. **B**) Sequences of *telRL* site and hairpin ends of the prophage. The positions of the cleavage sites are marked by a filled triangle. [Reproduced with permission from (*45*)].

TelN cleaving-joining activity is functional in the absence of any other N15-encoded factors and purified TelN is the only protein required to convert circular DNA substrates to the linear molecule with covalently closed ends(*47*). I*n vitro* studies on the functionality of purified TelN demonstrate that in the presence of the 56 bp *telRL* target site, and in particular the 22 bp *telO* target site, purified TelN processes circular and linear pDNA *in vitro* with the same covalently closed end DNA topology as is observed *in vivo* (*23*). In addition, it was shown that the central telomerase occupancy site (*tos*), within the *telO* perfect palindrome, is occupied by two TelN molecules to create a stable TelN-*telRL* complex. The *telO* palindrome alone is not sufficient for specific stable complex formation and requires the additional sequences of *telRL* to stabilize the TelN-target complexes that process the binding reaction of TelN homodimers to the 3′-phosphoryl of the cleaved strands and generation of the LCC DNA (*46*).

TelN protelomerase represents a significant addition to the repertoire of exploitable enzymes in biotechnology with respect to its unique property in linearizing the plasmids carrying its *telRL* target site. The "pJAZZ" series of transcription-free, linear cloning vectors and linear miniplasmids are N15-based linear cloning systems that facilitate cloning of AT-rich inserts of up to 30 kb and DNA sequences with inverted repeats, respectively, which are very difficult or impossible to clone in standard plasmid vectors (*44*). TelN/*telRL* cleaving joining activity has also been applied to enhance *in vitro* production of DNA vectors, whereby linear closed mini DNA vectors (Figure 5) showed higher and more sustained expression of the GOI both *in vitro* and *in vivo* (*47*).

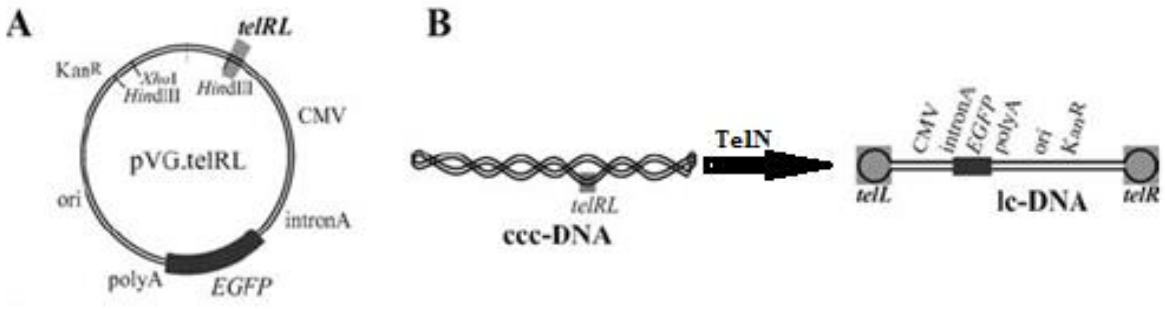


Figure 5- *In vitro* application of TelN/*telRL* protelomerase system.

- **A)** Schematic presentation of eGFP expressing plasmid DNA carrying the *telRL* target site.
- **B)** Schematic representation of the processing of the *telRL* substrate by recombinant TelN protein and XhoI restriction enzyme. TelN cuts the *telRL* site and produces a linear closed (LC) plasmid DNA; XhoI cuts its restriction site and produces linear open (LO) plasmid [Modified from (*47*)].

1.1.3.5 Phage PY54 Tel protelomerase

Bacteriophage PY54 (NC_005069) was isolated in 2003 and classified as a temperate phage specific for *Yersinia enterocolitica.* PY54 also exhibits a λ-like morphology and similarly belongs to the *Siphoviridae* family with a comparable 46 kb dsDNA genome (*48*).

Like N15, PY54 also lysogenize and co-excist with its infected bacterial hosts as a linear, circularly permuted, and covalently closed plasmid with similar telomere-like hairpin ends. In addition to the similarities between phages N15 and PY54 with respect to genome size and morphology, both phages also replicate their prophages as LCC plasmid. However, despite these similarities, these two phages are evolutionary quite distant, where N15 is far more closely related to λ than to PY54 (*49*).

The homologue of the N15 *telN* gene, encoding a protelomerase called Tel, encodes a 77 kDa TelN-like enzyme with observably identical function, able to process recombinant plasmids containing the 42 bp *telRL*-like palindrome (*pal*) (*49*). The *pal* site (Figure 6) is a 42 bp perfect palindrome, that unlike N15 possess only partial function *in vivo* in the absence of adjacent sequences (*49*). Sequencing and nucleotide BLAST of *telRL* and *pal* showed that the ten nucleotides "(5'-TACGCGCGTA-3')" in the centre of the paralogous palindromesare 100% identical. Furthermore, the amino acid BLAST alignment of TelN and Tel enzymes show 60% similarity (Figure 7).

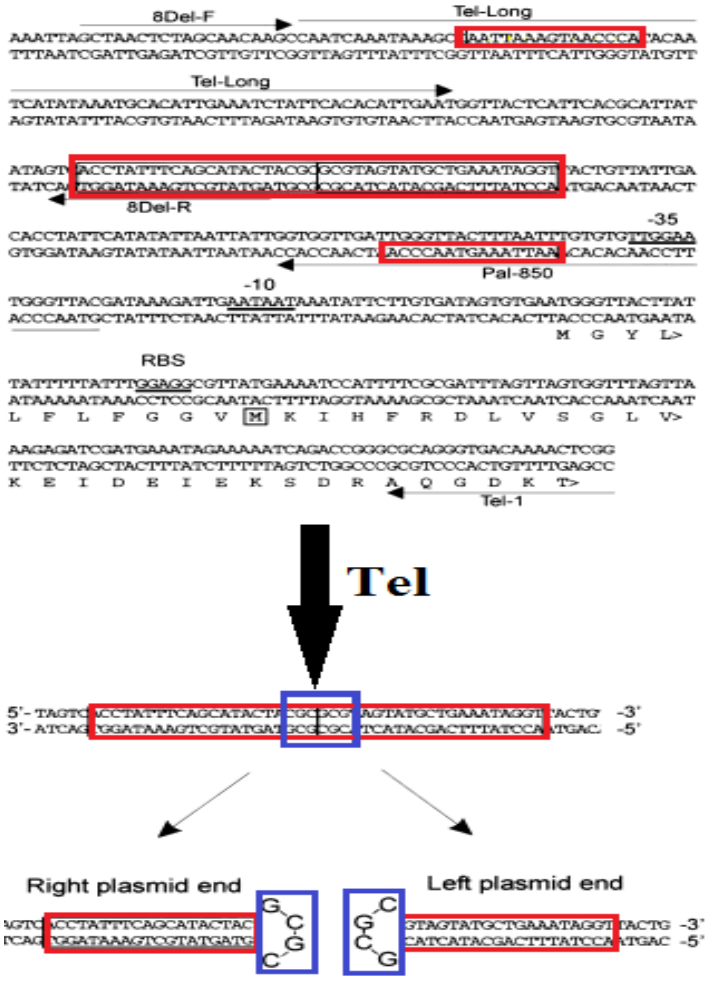


Figure 6- *Pal* sequence and mechanism of inversion into closed ends.

The 42 bp palindrome is flanking inverted repeat and the putative start codon are boxed. [Modified from (*48*)].

To date, there are no reports showing the *in vivo* applications of the Tel-*pal* system of PY54 such as the construction of Tel-expressing cells in genome engineering, or in the production of new pDNA vectors that exploit this system. While the generated LCC and mini LCC DNA vectors have been employing the TelN-*telRL*

system, enzyme production was limited and exploited *in vitro*. This study focuses on applying the N15 and particularly the PY54 protelomerase enzymes to develop a novel *in vivo* technology for production of superior bacterial sequence-free mini DNA vectors for transgene delivery (*10*).

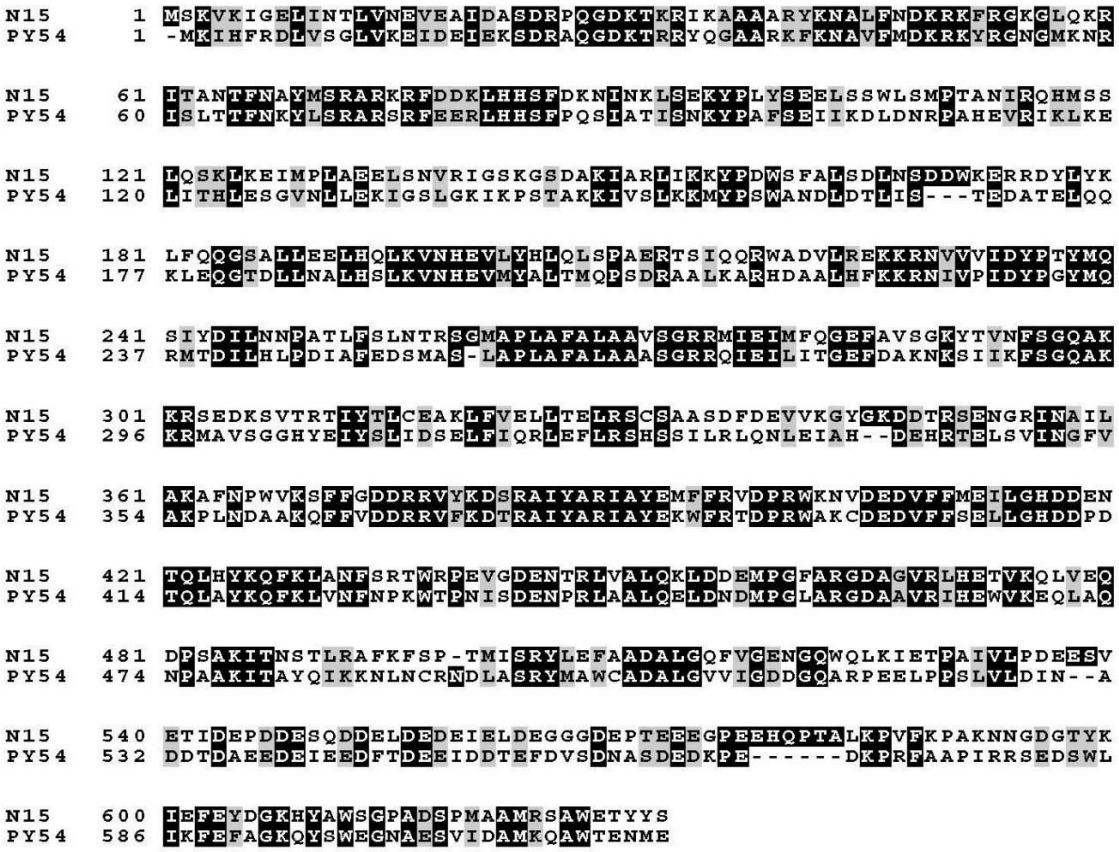


Figure 7- Alignment of the PY54 and N15 protelomerases.

The amino acid alignment of N15 TelN and PY54 Tel proteins showing ~60% identity (black box) and similarity in gray box.

1.1.4 Conclusion and perspective

In recent decades, a number of site-specific recombinases have been discovered from various microorganisms and applied to *in vivo* genome engineering for a wide variety of heterologous cells. These enzymes are classified into tyrosine-type and serine-type recombinases according to their catalytic mechanisms, where both families are further subdivided into two subgroups identified in Table 1. Tyrosine-type phage integrases, such as λ integrase, catalyze tightly regulated unidirectional recombination. Their applications to gene integration into heterologous

genomes, however, remain difficult due to their characteristic bidirectional recombination in heterologous cells. In contrast, the tyrosine-type simple recombinases, such as Cre and Flp, catalyze bidirectional recombination and have been widely used as tools for gene integration into heterologous genomes by development of RMCE and LE/RE mutant site systems. Serine-type phage integrases, such as ΦC31integrase catalyze unidirectional recombination between short attachment sites with no need to host-encoded proteins, cofactors, or specific DNA structures, which, due to simplicity, favours their use in the development of human gene integration systems. Homologous recombination is generally favored for site-specific introduction of foreign DNAs into a wide variety of heterologous genomes, but the efficiency of homologous recombination is dependent on the host cell features and DNA structure of targeting region.

As it is explained in the following chapters, in this study, homologous recombination was used to insert Cre, TelN, and Tel encoding genes derived from the phage P1, N15, and PY54, respectively into the *E. coli* chromosome. The productions of recombinant enzymes were governed by a temperature-controllable expression system toward construction of a one-step *in vivo* production system of prokaryotic sequence-free mini DNA vectors. We showed that Cre/*loxp*, TelN/*telRL*, and Tel/*pal* cleaving-joining systems are active in a new heterologous prokaryotic environment. Expression of the three enzymes from *E. coli* genomic DNA conveyed digestion of the plasmids carrying their target sites. We also applied site specific recombination to insert the PY54 derived *tel* and its target site *pal* into human embryonic kidney (HEK 293) chromosomal DNA to assess the functionality of Tel/*pal* protelomerase-mediated cleaving-joining activity in human cells. In contrast to prokaryotic cells, the wild type Tel did not show protelomerase activity in human cells during the first trial. Therefore, additional consideration and modification to the *tel* sequence seems necessary for testing Tel/*pal* activity in human cells due to complexity of these cells compared to bacterial cells.

1.2 Gene Therapy

Gene therapy (GT) is defined as the introduction of a transgene into a cell using a vector to replace a mal- or non-functional gene, or for transient or sustained expression of a protein to arrest, prevent, or reverse a pathological process. The concept of GT has persisted for many years but by the development of new techniques for *in vivo* expression of a therapeutic transgene in human cells, and recently with the ability to alter genes *ex vivo* from autologous cells and transplant them back into patient, gene-based therapeutics have recently received significant attention.

GT was initiated using viral vectors that exploit the natural benefit of efficient viral infection systems to target human cells. Despite the outstanding preclinical success, the clinical application of early recombinant viral vectors remained limited. One of the greatest challenges was to only transduce the targeted cells without effecting or harming the surrounding tissue. Another challenge was the short-lived and inefficient transgene expression conferred by these vectors. The 1999 GT trial of an adenoviral vector targeting correction of the X-linked disease, ornithine transcarbamylase deficiency, resulted in Jesse Gelsinger's death (*50*). The patient's death can be directly attributed to the severe immune reaction to the vector and massive organ failure (*50*). This tragedy directed attention to viral vector immunocompatibility and immunostimulatory considerations in the use of viral vectors (*51*). In 2000, following the first GT success with a lentiviral insertional vector, a rare but serious immunodeficiency disorder in children was reported to be cured. Some children who were treated showed a leukaemia-like disease due to insertional mutagenesis of the viral vector into the target genome that activated proto-oncogenes adjacent to the insertional site (*52*). These severe outcomes called into question the safety of viral GT strategies and subsequently fueled interest in the development of nonviral gene-transfer technologies in the past decade. Arising from these outcomes and limitations, it was quickly realized that any future success in GT required profound understanding of vector biology and synthesis mechanisms, molecular basis of bio- and immune- interactions between vector and host, and cellular barriers to transgene delivery to develop vectors with improved efficiency and safety.

The focus of this chapter is on nonviral GT systems, which serves as a brief review on the improvements on the design and application of nonviral DNA vectors, key challenges, and current direction and application of these vectors in molecular medicine. Although the strategies summarized here are also directly applicable to generating transgenic animals in biotechnological settings, the issue of safe and efficient transgene expression in the human context is the primary intent.

1.2.1 Viral vectors

Different viruses have been applied as vectors for gene delivery within the past two decades and have shown strong potential to treat various diseases, such as cystic fibrosis, hemophilia, Alzheimer's disease, and some cancers since viruses have evolved in nature to efficiently deliver their own genetic material to specific cells, and standard molecular biology methods can be employed to replace therapeutic transgenes in place of some or all of the viral genes. More than half of all clinical trials to date have employed adenoviral (Ad), retroviral/lentiviral, or adeno-associated viral (AAV) vectors for gene delivery (*53*).

1.2.1.1 Adenovirus

Adenoviruse is a non-enveloped virus with a 36-kilobase (kb) dsDNA genome and a potential candidate as a gene delivery vehicle since the recombinant virus is able to grow as high-titer with large transgene capacity, and efficient transduction of both dividing and non-dividing cells. Some serotypes of this virus have been found to mediate gene delivery to a wide range of tissues, such as the respiratory tract, eye, liver, and urinary tract. Considering all the positive points about this virus there are still some issued that should be considered before applying adenoviruses as vectors such as lengthy production, difficult production for helper-dependent virus in case of engineered viruses lacking the replication ability, preexisting immunity and most importantly vector immunogenicity (*54*).

1.2.1.2 Adeno-Associated Virus

AAV is a non-pathogenic, non-enveloped virus with a 4.7 kb ssDNA genome. This virus requires the presence of certain proteins from a helper virus to complete its lifecycle. AAV vectors have been shown long-term gene expression in a variety of tissues including dividing and non-dividing cells *in vivo,* which promotes their potential as gene delivery vectors. However, there are several limitations that have to be overcome before application of this virus in gene delivery including its genome packaging size, preexisting immunity, poor transduction of some cells, and infection of off-target cells (*53*).

1.2.1.3 Lentevirus

Lenteviruses are retroviruses with a diploid, 7–12 kb single-stranded, positive sense RNA genome that express different proteins essential for their lifecycle. One of the most important encoded proteins is the integrase that helps the virus to integrate its genome into the infected cell chromosome, which permits stable and prolonged expression of delivered therapeutic genes and makes retroviruses an attractive platform for gene delivery. Moreover, retroviral vectors show low immunogenicity and relatively large packaging capacity. However, disadvantages such as low production yields, instability of envelope proteins, and most importantly safety issues based on the random integration patterns must be considered (*55*). Thus, prior to application of lentiviral based vectors as therapeutic agents some important issues

should be considered, most importantly, the safety concerns of possible generation of replication competent lentiviruses during vector production, integration of the viral genome into the target cell chromosome which in case produce other problems such as insertional mutagenesis leading to cancer, germ line genome alteration resulting in long lasting effect inherited to future generations, and spreading of new viruses from gene therapy patients (*56*).

1.2.2 Bacterial vectors

The ability of bacteria to mediate gene transfer has recently been investigated and the results have led to the application of different bacteria such as attenuated strains of *Salmonella, Shigella, Listeria*, *Yersinia*, and non-pathogenic *E. coli* in gene therapy. Gene transfer from bacteria to a very broad range of recipients including yeast, plants, and mammalian cells has been described before; however, in some cases, the exact mechanism of DNA transfer from bacteria to the mammalian cell is not fully understood. Therapeutic benefits have been observed in vaccination against infectious diseases, immunotherapy against cancer, and topical delivery of immunomodulatory cytokines in inflammatory bowel disease. In regards to cancer immunotherapy, *Salmonella* strains have been used to deliver tumor antigens such as *β-galactosidase* carrying on a eukaryotic expression vector. The mouse models suffering from fibrosarcoma, renal carcinoma, and melanoma were protected against the relevant antigens by oral administration of *Salmonella* vectors. Application of this bacterial strain in delivery of IL-18 and INF-*γ* was also reported. In addition, the anaerobic bacterial strains such as *Clostridia* and *Bifidobacteria* were used for depletion of tumor cells based on the replication of these bacteria in the hypoxic environment of the tumors (*57*).

1.2.3 Nucleic Acid vectors

1.2.3.1 RNA vectors

RNA interference, or RNAi, is a process that sequence-specifically destroys mRNA, causing null phenotypes. RNAi technology is an excellent way to fully or partially suppress the expression of a specific gene, allowing targeted gene knock-out in many different models including mammalian cells. Small interfering RNAs, or siRNAs, are short RNA molecules of 19 to 22 nucleotides in length. The siRNAs are generated *via* cleavage of dsRNA templates by RNAse III ribonuclease. The siRNAs are then incorporated into an RNA-induced silencing complex (RISC) and unwound into single-stranded siRNAs. Next, the single-stranded siRNAs guide the RISC complex to the target mRNAs for destruction, causing RNA interference. Depending on the amount of siRNA expressed and its inhibitory efficiency, expression of the target gene can be either completely blocked or dramatically suppressed. By this means scientists are able to determine the function of different genes (*58*).

1.2.3.2 DNA vectors

DNA vectors are double-stranded generally circular plasmid DNA that combines sequences required for replication and selection in bacterial cells with sequences necessary to express the encoded transgene in eukaryotic cells after delivery to an organism and transfection of target tissue.

1.2.4 Synthetic vectors

Based on the fact that viral and bacterial vectors showed some limitations for gene therapy, and considering the relatively better biocompatibility and easier large scale production of synthetic vectors, scientists started to think about complexing the DNA vectors with synthetic vectors to make gene delivery experiments more efficient. Since 1970s variety of modifications have been done to formulate a better and safer synthetic vector in order to overcome several barriers such as DNA complexation, cellular uptake, endosomal escape, cytoplasmic mobility, and nuclear entry (*59*). Here, some of the most important synthetic vectors are listed.

1.2.4.1 Lipid-based vectors

Liposome-mediated gene transfer was one of the earliest strategies applied to introduce exogenous genetic material into host cells and several publications recorded the delivery of exogenous globin mRNA, chromosomes, and DNA into host cells using lipid bases vectors. The term "lipofection" was introduced to explain lipid based gene transfection. Now it is clear that the lipofection occurs through endocytosis. Then the cationic lipoplex destabilizes the endosomal membrane, leading to reorganization of the phospholipids following neutralization of the lipoplex and causing the DNA to dissociate into the cytoplasm. Also, the two key points that affect the liposome-mediated gene delivery was explained as the density and nature of the cationic head groups and the hydrocarbon moiety (*59*).

1.2.4.2 Polymer-based vectors

The cationic polymers showed the ability to complex with DNA and form 'polyplexes' regardless of its size and kind. Although these vectors are promising candidates for synthetic vectors, the inefficient gene transfection *in vivo* remains as the primary obstacle toward a successful gene therapy using the cationic polymers. To achieve an improved gene transfection using polyplexes variety of studies have been performed by different approaches such as the chemical synthetic engineering approach which aims to modify the kind and composition of the polymers, the biochemical approach to target ligands such as galactose, mannose, transferring, or antibodies into the polymers, the functional molecular engineering approach in order to stimulus-response polymers with light and thermal reactivity and design high performance vectors, and physical engineering approach in which different physical stimulation are applied to increase the transfection efficiency like electroporation, gene gun, ultrasound and

hydrodynamic pressure. Also, several groups have been focused to modify and standardize the molecular structure of cationic polymers like changes in the polymer chain length and composition of polymers (*60*).

1.2.4.3 Dendrimer-based vectors

Dendrimers are novel, three-dimensional polymers that have the ability to interact with different forms of nucleic acids such as plasmid DNA, antisense oligonucleotides, and RNA and form complexes to protect the nucleic acid from degradation through purely electrostatic interactions. In fact, cationic dendrimer condenses the anionic nucleic acids and produce a positively charged complex with efficient transfection ability *via* the negatively charged cell membranes. However, it is worthy to mention that highly cationic systems are cytotoxic. The nature of the dendrimer nucleic acid complex depends on numerous factors such as stoichiometry, concentration of dendrimer-amines and nucleic acid-phosphates, as well as solvent properties such as pH, salt concentration, buffer strength, and dynamics of mixing (*61*). Despite these limitations and due to ease of synthesis and chemistry of dendrimers/dendrons, they have become the most utilized synthetic vectors for gene transfer and well recognized commercially available product, "nano-diagnostics (Stratus®, Siemens, Germany), nano-scale gene vectors-(Superfect®, Qiagen, Germany) and siRNA vectors (PrioFect® Starpharma/Dendritic Nanotechnologies,Inc., Australia)" are some examples (*62*).

1.2.4.4 Polypeptide based vectors

Electrostatic conjugation of peptides and oligonucleotides is a potential gene delivery strategy into mammalian cells with high efficiencies and cell-specificity by the means of short sequences of basic amino acid residues which are called "protein transduction domains or cell-penetrating peptides" (*59*).

1.2.5 Main barriers to overcome in a successful gene therapy

While nonviral DNA delivery systems provide safety advantages over their viral counterparts in terms of immunocompatibility and genotoxicity, these outcomes generally come at the cost of inferior targeting and cellular uptake. In addition to a plethora of safety concerns regarding viral manipulations, the production and storage of viral vectors in large scale calls into question their feasibility. In addition, the size of the transgene into a viral vector is limited by the size of the viral capsid, which is generally not a concern with nonviral vectors. Nonviral gene delivery systems offer the potential to provide nucleic-acid-based therapeutics that closely resembles traditional biologic therapeutics. However, in order to provide effective nucleic-acid-based therapeutics, vectors must meet basic criteria. These criteria include, but are not limited to: 1) repeated administration with minimal immune response generated; 2) production in large quantities with high reproducibility and purity; 3) low scale up costs; 4) stable storage profile; and 5) operationalization into a final product offering for patient administration.

Currently, the simplest nonviral gene delivery system uses physical methods to transfer 'naked' transgene DNA vectors into target cells by electroporation, ultrasound, gene gun, or hydrodynamic injection. When "naked" DNA is injected directly into certain tissues, particularly muscle, it produces significant and sustained levels of gene expression. The popularity of naked DNA as a nonviral GT strategy has continued to grow in human clinical applications (18.3% of trials in 2012 compared to 18% in 2007 and 14% in 2004). In fact, naked DNA vectors represent the most popular nonviral system used in clinical trials (18.3 %), followed by 5.9 % "lipoplexed", which involves the interaction of cationic lipids with DNA. A small number of trials have used a range of modified bacterial vectors (20 trials) or yeast vectors (seven trials) [Gene therapy clinical trials worldwide to 2012 – an update]. Four pDNA products have already been licensed as DNA vaccines for viral infections and for treatment of cancer in veterinary applications (*63*).

There are several major challenges that need to be address in order to develop a highly safe and effective nonviral DNA vector therapeutic. These barriers fall into three classes: 1) manufacturing and formulation; 2) extracellular barriers; and 3) intracellular barriers. Successful transgene delivery first requires that the DNA vector enter the target cell. The vector must then pass through the highly dense cytoplasmic network in order to reach the nucleus after which it must translocate across the nuclear envelope. Finally, upon entering the nucleus, the transgene is expressed, mRNA is exported to the cytoplasm for translation, if necessary, and produced into functional intended product. While simple in theory, there are challenges that diminish the efficiency of transgene delivery and acceptable expression levels.

1.2.5.1 Bio- and immunocompatibility of vectors

The cytotoxic or hemolytic effect of vector particles and /or activation of the host immune reaction against the transgene product and/or vector particles can dramatically compromise the safety and efficiency of GT. The transgene delivery system must be resistant to degradation by serum nucleases and its immunogenicity minimized, unless desirable, while in the extracellular surroundings. Plasma nucleases digest unprotected or nicked DNA within just a few minutes, and as such, DNA vectors must rapidly reach target cells and cross the cytoplasmic membrane of target cells. This requirement is complicated by the fact that the cytoplasmic membrane is composed of dense phospholipoprotein barriers that intrinsically inhibit efficient DNA translocation. Interaction of DNA with conventional nonviral vectors such as diverse lipid, proteins, and polymer formulations assist transgene delivery unit survival in exteracellular environment and cell entry by binding or enveloping DNA through a charge interaction (*64*). Although nonviral vectors are generally less immunogenic than their viral counterparts, the combination of carriers with methylated CpG dinucleotide motifs that are extensively distributed into the

bacterial backbone of plasmid DNA vectors, stimulate cytokine-mediated immune reactions that can cause severe toxicity *in vivo* (*65, 66*). Therefore, a better understanding of the vector and transgene expression cargo and their interactions with host immune system is essential to design and develop safe delivery systems (*67, 68*). Some different strategies have been designed to overcome these problems in a way that is similar to viral vectors. For instance, mixing pDNA by DNA condensing agents like linear polyethylenimine, PEG-PEI (poly(ethylene glycol)-conjugated PEI) for surface shielding, and Tf-PEG-PEI (transferrin-PEG-PEI) to provide a ligand for receptor-mediated cell uptake provides a specific tumor-targeting DNA complexes with subsequent application as medical products (*69*). In a different study, it was shown that in systemic delivery of polyplexed or lipoplexed DNA, by masking the surface charge of DNA complexes, they could avoid interactions between DNA cargo and plasma components like erythrocytes and the reticuloendothelial cells. Among many different masking reagents, the polyethylenimine (PEI)-based DNA complexes shielded with polyethylene glycol (PEG) and linked to the receptor binding ligands transferrin (Tf) or epidermal growth factor (EGF) have been shown strong affinity and specificity to the target tissue and mediated efficient gene transfer into the target tumor cells in a receptor-dependent and cell-cycle-dependent manner (Tumor-targeted gene transfer with DNA polyplexes). These new formulations for nonviral vectors possess an improved *in vivo* stability profile, reduced affinity for intracellular proteins and cell surfaces, and are capable of reaching to target cells in their active form with minimal recognition and rejection by immune systems (*69*).

1.2.5.2 Vector specificity, cell uptake, and lysosomal escape

Once a nonviral vector successfully passes the extracellular barrier and has localized to the target site, it must be taken up by the target tissue in order to express the transgene. Many different parameters influence the distribution of the vector to improve GT. These include, but are not limited to the blood supply and endothelial barriers of the target tissue, in the case of both systemic and localized gene delivery, and the vector size and its interaction and absorption by the target cells. Despite many efforts to increase specificity of the vectors, empirically, it has proven considerably challenging to design either viral capsids with modified specific cell-type receptor-targeting capabilities or nonviral vectors with cell specific ligands and associated DNA vectors with cell- or tissue-specific promoters. Depending on the vector, target cell, or protein product, the degree of toxicity caused by vector uptake by non-target cells would be very variable (*64*). Furthermore, even upon successful cellular endocytotic entry of the nonviral vector, escape from the endosome, cellular trafficking and nuclear penetration still remain to be accomplished. Several strategies have been attempted, often in combination, to increase nonviral vector efficiencies. These include: 1) complexing DNA vectors with synthetic nano-particles to form a structure similar to the target cell membrane; 2) adding peptide sequences to nonviral vectors to assist DNA

compaction to minimize its size and improve receptor-mediated endocytosis (*68, 70, 71*) ; 3) targeting liposomes with receptor ligands (*72*); 4) adding endosomal disruption sequences such as adding the pH-sensitive fusogenic peptide, "GALA (WEAALAEALAEALAEHLAEALAEALEALAA)" to the transferrin-modified liposomes (*73*).

While nonviral gene delivery techniques facilitate the persistence of the transgene expression unit in the extracellular environment, and improve target cell membrane uptake, they generally perform poorly in the intracellular environment, in particular, in cytoplasmic diffusion and nuclear uptake. However, many techniques are currently being tested to enhance levels of nonviral gene transfer by targeting vectors to the nucleus, including: 1) modification of pDNA with DNA nuclear targeting sequence (DTS); 2) covalent linkage of nuclear localization signals (NLS) to the constructs (*74*, *75*); and 3) attachment of karyopherins (or other import receptors) to vectors that promote uptake through the nuclear pore(*70*).

The SV40 enhancer is a DTS that encodes binding sites for a variety of transcription factors. As transcription factors are proteins that are translated in cytoplasm but function in the nucleus, they require NLSs within their primary amino acid sequence that serves to interact with importin proteins and direct nuclear import (Figure 8).

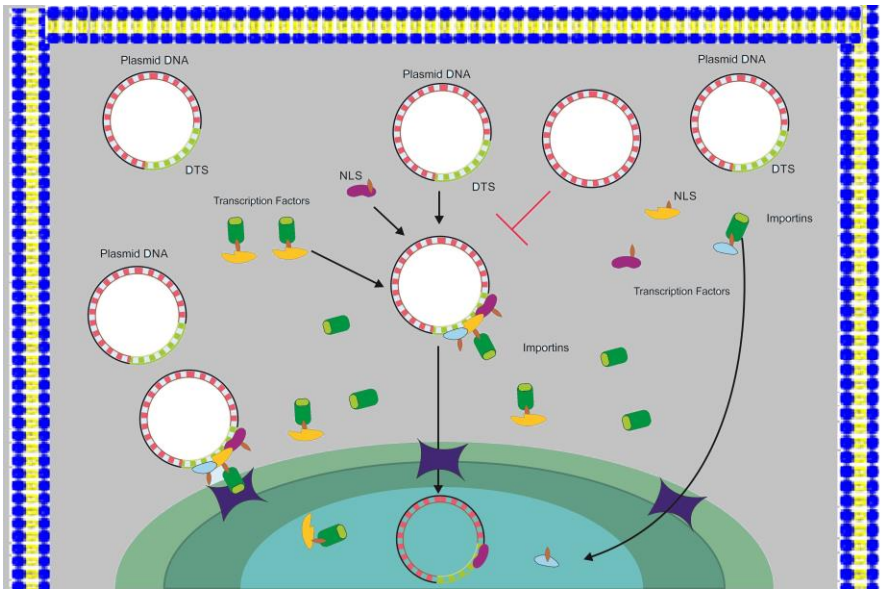


Figure 8- Nuclear entry mechanism of pDNA vectors.

It was shown that SV40 enhancers facilitate sequence-specific nuclear entry of DNA vectors through the nuclear pore complexes. The SV40 enhancer binds to transcription factors in cytoplasm and creates a DNA-protein complex passing through nuclear pore complex by importins (*64*, *75*, *76*).

1.2.5.3 Vector genome persistence

A thorough understanding of the pathology and the diseased target tissue is essential in order to appropriately and successfully design strategies in GT. As an example, if the intended therapy is to fix acquired disorders such as infection or cancer, or if the targets are slow or non-dividing organs like the liver, the brain, the heart or the muscles, then more limited periods of expression *via* non-replicative DNA vectors that exist episomally will be more desirable. However, non-dividing episomal vectors are lost during cell division and as such would not often be used in rapidly dividing cells such as white blood cells. Furthermore, in the case of genetic disorders that require lifelong and stable expression, gene replacement or compensation would be best achieved using integrating vectors. Integrating vectors do however, carry inherent risk of insertional mutagenesis if integration is not controlled *via* homologous recombination (*77, 78*), or integration of a transgene into the regions of the genome that are not associated with cell proliferation or tumor suppression through exonucleases (*17*). Furthermore, transgene expression can be extinguished by epigenetic modifications to the vector sequence (*79*), whether from integrated DNA (*80*) or from a persisting episomal vector (*81*). The structure of the DNA vector after it reaches the nucleus and how accessible the transgene is to transcription factors plays an important role in sustained transgene expression. Histones, as the basic units of chromatin structures, associate with chromosomal DNA to form heterochromatins and euchromatins, which are transcriptionally silent and active, respectively, the former of which can negatively impact transgene expression. Different approaches have been tested *in vitro* to influence transgene methylation levels and inhibit transgene silencing and to improve durability of translation. Some strategies include applying human "housekeeping" promoters (*82*), or adding transcription factor binding sites to tissue specific promoters to increase expression levels (*83*). Immediately following transfection, any bacterial sequences within vector DNA are recognized by human cells as heterochromatic structures and are silenced or down-regulated for transgene expression (*84*).

The plight to generate more efficient and safer vectors for GT has spurred several different proposals and gene transfer strategies, described herein. As previously mentioned, the majority of DNA vectors that have been used in GT are standard expression plasmids. Typically, pDNA vectors possess bacterial sequences such as the prokaryotic *ori*, origin of replication, and antibiotic selection marker(s) that are necessary for maintenance and replication of theses vectors in bacteria cells. They of course must also possess eukaryotic sequences such as the transgene, promoter, polyadenylation sequence and other transcription and translation regulatory elements that are similar to the cassette in a viral vector. Prokaryotic sequences are undesirable in clinical applications and can cause cytotoxic reactions of host immune system, horizontal transfer of antibiotic resistant genes to normal microbial flora, and even

silence of the intended transgene (*85*). In contrast, bacterial sequence-depleted DNA vectors offer higher and more persistent expression, generally at levels 100 to 1,000 times greater than their standard plasmid precursor (*66*). Two different approaches have been developed for generation of bacterial sequence-free DNA vectors including mini circular and mini linear closed DNA vectors. Due to clinically improved results achieved by these vectors, they very likely will replace standard pDNA vectors in future transgene delivery trials.

1.2.6 New generation of DNA vectors

1.2.6.1 Mini circular DNA vectors

Circular bacterial sequence-depleted transgene expression cassettes, commonly known as DNA minicircles, have been constructed employing phage site-specific recombinases, such as λ integrase (Int) (*34, 86*), P1-derived, Cre (*35*), or phiC31 Int (*36*). Recombinase-mediated site-specific recombination results in two well-characterized circular molecules that are generated from the single parent plasmid: 1) the minicircle comprising the GOI expression cassette, and 2) the mini-plasmid containing the bacterial backbone elements. Further purification of the minicircle showed that it could be therapeutically applicable (*37*). For the first time, Darquet and coworkers (1997) and Scherman's group (1999) showed that DNA minicircle confers much higher transgene expression levels *in vitro* and *in vivo*, respectively, compared to the parental plasmid or other conventional control plasmids encoding the same transgene (*33*). This result was further confirmed, and showed an even more significant increase in the expression of the encoded transgene, when the same amount (weight-to-weight basis) of DNA minicircle and parental plasmids were transfected or by better delivery techniques (*33, 34, 85, 87-90*).

1.2.6.2 Mini linear DNA vectors

LCC DNA vectors are small-sized and "dumbell-shaped" DNA molecules that possess the transgene expression unit, including promoter, GOI, and mRNA-stabilizing sequences, flanked by two short hairpin oligonucleotide sequences to close the free ends and provide protection against extracellular and intracellular exonuclease activity. These vectors have been developed by different groups and showed promising results both *in vitro* and *in vivo*. The first generation of these vectors, named "MIDGE" (Minimal Immunological Defined Gene Expression)", were generated through a multi-step enzymatic reaction (Figure 9-a) that included *in vitro* digestion of the standard plasmid DNA vector at two sites flanking the transgene expression cassette, and addition of two short hairpin sequence by ligation reaction to close the ends (*91*). Mammalian cells transfected by "MIDGE", expressing luciferase, were reported to

express the GOI up to 17 times higher than that seen by conventional CCC vectors, although this disparity in efficiency was shown to occur in a tissue-dependent manner (*92*). In addition, the mean numbers of "MIDGE" vector molecules per cell was significantly higher, suggesting that LCC DNA vectors transfect cells more efficiently. "MIDGE" technology has already been applied, with promising results, to the development of a *Leishmania* DNA vaccine and a colon carcinoma treatment. In this regard, IL-2 was delivered to a specific tumor cell line and two to four fold higher expressions was observed compared to the routine control plasmid vector (*91*). Moreover, "MIDGE" vectors have been applied fruitfully in several studies as new DNA vaccine candidates *via* local *in vivo* administration. Some examples include: 1) intradermal injection of vectors encoding glycoprotein gp140 from feline immunodeficiency virus (FIV) in cats by gene gun (*93*), 2) intradermal injection of "MIDGE" vectors encoding bovine herpesvirus-1 glycoprotein D (*94*), and 3) gene gun-mediated intradermal injection or intramuscular administration of "MIDGE" vectors containing the gene for hepatitis B surface antigen (HBsAg) in mice (*94*). In addition, nucleofection was applied to introduce "MIDGE" into human mesenchymal stromal cells (hMSC), where higher cell viability and stable and high protein expression levels were achieved even 55 days post-nucleofection, which indicates that regardless of nonviral transfection approach, mini LCC DNA vectors result in extended and stable transgene expression (*95*).

PCR amplification of the transgene expression unit from standard plasmid vector was another approach used to generate mini LCC vector (Figure 9-b). The two free ends of the PCR product were then capped by ligation of two short hairpin loops. This technology produced "MiLV" that contained only the transgene expression cassette. When testing "MiLV" *in vitro* and *in vivo* in Epstein-Barr virus (EBV) positive tumors, lower cytotoxicity, higher transfection efficiency, and more sustained expression of the GOI was observed (*96*).

The bacteriophage N15-derived cleaving-joining system has also been exploited for *in vitro* production of bacterial sequence-depleted LCC DNA vectors (Figure 9-c). Vectors, engineered to encode the TelN target site, *telRL*, were employed to transfer EGFP or IL-12 cistrons to a mouse model of melanoma to confer anti-metastatic activity (*47*). They showed that EGFP expression and the anti-metastatic capacity of LCC DNA vectors and the respective parental pDNA are at least comparable. Next, this research group inserted two *telRl* sequences into the plasmids harbouring *eGFP* and *IL-12* genes to precisely remove the bacterial elements and produce mini LCC DNA vectors *in vitro* that eliminate the remaining prokaryotic amplification and resistance DNA. They demonstrated that the mini LCC vectors are functional in cell culture and in an *in vivo* metastasis model and that the presence of prophage telomeres does not reduce the expression of EGFP or IL-12. Also, the anti-metastatic capacity of the IL-12-expressing mini LCC DNA vector tends to be higher than the parental pDNA. They concluded that mini LCC vectors are more

efficient because of their increased availability or distribution and confer facilitated entry into the cells and nucleus.

LCC DNA vectors may be superior to conventional CCC DNA vectors in that they are more efficient and likely safer. The obvious drawback to LCC DNA vectors is the multi-step approach to production that adds expense and makes them less desirable in clinical trials due to the fact that pDNA needs to be purified from bacterial cells, digested, chemically modified to produce the LCC DNA, and then finally purified as LCC vector. In the work described herein, we have developed an *in vivo*, cost effective, one-step, and robust technology for production of LCC transgene expression cassettes, called "DNA ministring" vectors. This technology exploits the phage PY54 recombination system in comparison to N15 (*10*).

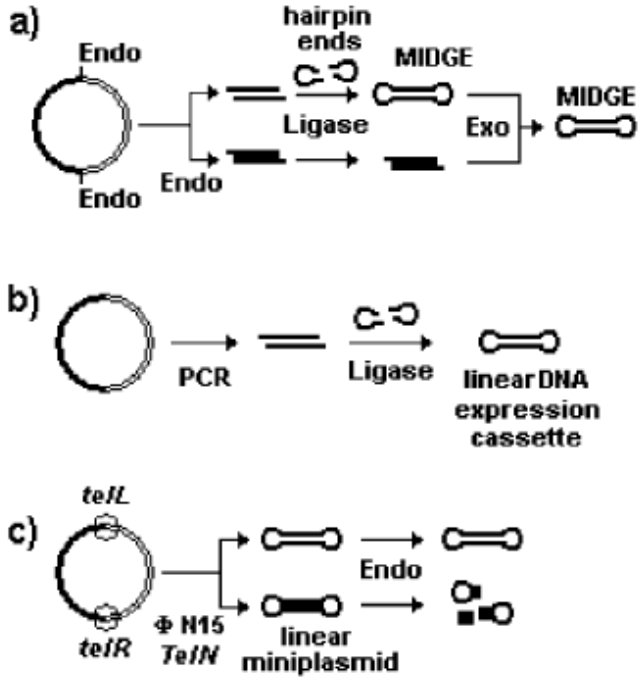


Figure 9- Methods of *in vitro* production of bacterial sequence depleted LCC DNA vectors.

a) Minimalistic immunogenic defined gene expression vectors (*"MIDGE"*) is generated by digestion of purified pDNA vector by restriction enzymes (*Endo*) to isolate the GOI expression cassette from bacterial sequences, followed by ligase addition for end-refilling with hairpin end oligonucleotides. **b)** PCR amplification of the GOI expression cassette from pDNA construct using specific primers. The PCR product is end-protected as in the "MIDGE" method, preventing its further degradation when administered *in vivo*. **c)** *In vitro* application of recombinant phage N15-derived TelN protelomerase and its cutting-joining reaction on its target site *telRL* flanking the GIO expression cassette [Reproduced by permission from (*37*)].

The PY54-derived Tel-*pal* system relies only upon protelomerase activity and the palindromic target site, offering the major advantage of a single enzyme and a single-step cleaving-joining enzymatic reaction that can be manipulated for unlimited *in vivo* production of mini LCC DNA vectors. We showed that addition of DTS sequences to ministring vectors dramatically enhances cytoplasmic diffusion and nuclear uptake, resulting in higher transfection efficiencies in slow-dividing cells, even compared to minicircle counterparts in the same mole-to-mole comparison. Furthermore, for the first time, we show that the integration of LCC DNA vectors into the mammalian target genome results in chromosomal breakage, loss of genome integrity, and induction of apoptosis. Thus, LCC integrants are eradicated from the transfected cell population, thereby eliminating the danger of undesired vector integration events (Nafissi *et.al*. in progress).

1.2.7 Future of gene therapy

DNA vectors have the potential to prevent or treat a great variety of diseases and showed promising results in nonviral gene delivery with different applications including DNA vaccinology and gene therapy of several types of disease such as genetic disorders and cancer. Optimized DNA vectors can lead to increased and prolonged expression of the GOI and plays an important role in improving the number of application of these vectors with nonviral carriers in clinical trials. Considering the recent improvements in design, synthesis, production, and purification of advanced, safe, and effective DNA vectors, perhaps, by designing novel engineered viral capsids, more effective and safe nonviral biological vectors, or safe and effective nano-scaled chemical compounds, GT would be replaced with many traditional treatments. The effort of research and clinical studies must be focused on manufacturing superior vectors and radical understanding of interactions between the vector and human body. GT in combination with recently reported successful achievements in stem cell therapies create the great platform for personalized therapies, regenerative medicine, and personalized genomic medicine.

Chapter 2

Construction and characterization of a robust *in vivo* technology for the production of DNA ministring vectors

While safer than their viral counterparts, conventional nonviral gene delivery DNA vectors offer a limited safety profile. They often result in the delivery of unwanted prokaryotic sequences, antibiotic resistance genes, and the bacterial origins of replication to the target, which may lead to the stimulation of unwanted immunological responses due to their chimeric DNA composition. Such vectors may also impart the potential for chromosomal integration, thus potentiating oncogenesis. We sought to engineer an *in vivo* system for the quick and simple production of safer DNA vector alternatives that were devoid of non-transgene bacterial sequences and would lethally disrupt the host chromosome in the event of an unwanted vector integration event.

We constructed a parent eukaryotic expression vector possessing a specialized manufactured multi-target site called Super Sequence (SS), and engineered *E. coli* cells (R-cell) that conditionally produce phage-derived protelomerase Tel (PY54), TelN (N15), or recombinase Cre (P1). Passage of the parent plasmid vector through R-cells under optimized conditions, resulted in rapid, efficient, and one step *in vivo* generation of mini LCC—linear covalently closed (Tel/TelN-cell), or mini CCC—circular covalently closed (Cre-cell), DNA constructs, separated from the backbone pDNA. Site-specific integration of LCC plasmids into the host chromosome resulted in chromosomal disruption and 10^5-fold lower viability than that seen with the CCC counterparts.

We offer a high efficiency mini DNA vector production system that confers simple, rapid and scalable *in vivo* production of mini LCC DNA vectors that possess all the benefits of minicircle DNA vectors and virtually eliminate the potential for undesirable vector integration events.

2.1 Introduction

The utility of any gene therapy strategy is defined by its balance between safety and effectiveness. While virus-derived vectors offer exceptional potential to target and deliver DNA cargo with high efficiency into the target cell, viral strategies often suffer in their safety profiling. Recent viral gene therapy-related patient mortalities in clinical trials highlight some of the safety issues attributed to the use of viral gene transfer

systems that include, but are not limited to unwanted immune responses to viral capsid proteins, regeneration of virulent viruses, and insertional mutagenesis (*97*). In contrast, nonviral strategies based on naked, lipoplexed or polyplexed plasmid DNA (pDNA) vectors generally offer safer gene therapy, vaccine design, and drug delivery approaches. DNA vectors are relatively easy to generate and store and offer tremendous design capacity. Several major barriers need to be considered in order to develop nonviral gene delivery systems as a therapeutic product to be safely administered *in vivo*. A successful transgene delivery system depends on the entrance of the DNA vector into the mammalian host nucleus and expression of the encoded transgene(s). While simple in theory, several cellular barriers must be overcome in practice. While travelling in the extracellular surroundings, vectors must be bio- and immuno-compatible and avoid degradation by serum nucleases and immune detection by phagocytes. Plasma nucleases digest the unprotected DNA within just a few minutes, so DNA vectors need to rapidly cross the plasma membrane of target cells. This is further complicated by the fact that the plasma membrane is composed of dense lipoprotein barriers that intrinsically inhibit efficient DNA translocation. Strategies to overcome this barrier include complexing DNA vectors with synthetic nanoparticles to form a structure similar to the plasma membrane (*70*), or receptor-mediated endocytosis; *i.e.* targeted liposomes (*72*). However, while nonviral gene delivery techniques work toward efficiency of DNA delivery, they generally prove poor in the delivery of pDNA vectors to the nuclear compartment. Many techniques are currently being investigated to enhance levels of nonviral gene transfer by targeting vectors to the nucleus. These techniques include modification of plasmids with DTS, covalent linkage of NLS to the plasmid DNA constructs, and attachment of import receptors such as karyopherins, to vectors that promote uptake through the nuclear membrane pore complex (NPC) (*70*, *89*, *98*). Modification of DNA with NLS-conjugates seems to result in highly efficient expression of linear, but not circular DNA, in combination with liposomal delivery vectors (*74*). This difference may be attributed to charge per unit ratio of linear versus supercoiled circular DNA and provides yet another intriguing opportunity for LCC vectors (*99*).

In addition to the aforementioned challenges, conventional nonviral gene delivery approaches may lead to unwanted immunological responses and oncogenesis, imparted by the presence of bacterial genetic elements in pDNA constructs. These include prokaryotic origins of replication, antibiotic resistance genes, as well as high-frequency immunostimulatory CpG motifs that activate Toll-like receptors in mammalian hosts (*100*). In order to improve the immuno-compatibily and durability of pDNA vectors, a new generation of DNA vectors have been constructed that exploit the bacteriophage λ integrase (Int)-*attP* or P1-derived Cre-*loxP* site-specific recombination systems to generate mini CCC DNA vectors (*101*).

These minicircles provide safer minimized transgene vectors by removing unwanted prokaryotic elements, thus enhancing bio- and immunocompatibility in the mammalian host (*37*). The smaller size compared to the

parental plasmid backbone also confers improved extracellular and intracellular bioavailability leading to efficient gene delivery and hence, improved gene expression (*102*).

A second group of modified vectors offering great promise are LCC pDNA vectors. Aside from the obvious topological differences, LCC dsDNA molecules are torsion-free as they are not subject to gyrase-directed negative supercoiling, and as such possess the properties of linear DNA (*92*). However, LCC DNA is not subject to ExoV exonuclease activity in prokaryotes due to covalent linkage of linear ends, preventing degradation of the LCC pDNA vector. LCC DNA vectors have been constructed by various *in vitro* strategies including the capping of PCR products, and the "minimalistic immunogenic defined gene expression (MIDGE)" vectors. "MIDGE" is generated by the digestion of both prokaryotic and eukaryotic backbones after isolation of plasmid from bacterial cells, followed by ligation of the therapeutic expression cassette into hairpin sequences for end-refilling (*91*). This technology has shown promising results in various applications including the development of a *Leishmania* DNA vaccine (*102*), and a colon carcinoma treatment (*91*). "MIDGE" vectors have also demonstrated up to 17 fold improved transgene expression profile *in vivo* in some tissues, compared to conventional pDNA vectors (*92*). Thus, LCC DNA vectors may in fact outperform their circular counterparts with respect to transgene expression efficiency and bioavailability. However, large scale production of LCC DNA vectors *via* existing multistep *in vitro* processes requires considerable time and financial cost.

E. coli phage N15 was the first discovered phage to exist in its lysogenic (prophage) state as a linear plasmid with closed ends (*43*) that is actively partitioned to daughter cells (*23*). The LCC conformation is conferred by the cleaving-joining activity of the protelomerase protein (Prokaryotic Telomerase), TelN (~72 kDa), acting upon the 56 bp *telRL* target sequence that is entirely sufficient to confer TelN-mediated processing and linearization both *in vivo* and *in vitro* (*47, 103*). Similarly, phage PY54, isolated from *Yersinia enterocolitica*, maintains its prophage as a linear, circularly permuted, and covalently closed plasmid with telomere hairpin ends and a genome size of 46 kb. The paralogous minimal protelomerase target site of PY54 is a 42 bp perfect palindrome that unlike N15, only partially functions *in vivo* in the absence of adjacent sequences (*49*). The paralogue of the N15 TelN protelomerase, Tel, encodes a 77 kDa protein with observably identical function, able to process recombinant plasmids containing the *pal*, 42 bp palindromic target site (*49*). The *tel* gene possesses 60% sequence identity to *telN* and the active recombinases are similar in size (~77 kDa). In addition, there is a partial homology between the 42 bp PY54 *pal* site and the 56 bp N15 *telRL* site, where the ten central palindromic nucleotides (5'-TACGCGCGTA-3') are identical (*49*). Despite obvious similarities between the two phages they are evolutionary quite distant, where N15 is more closely related to λ than to PY54. Purified TelN was shown to process circular and supercoiled pDNA containing the identified target site, *telRL*, to produce linear double-stranded DNA with covalently closed ends. The LCC and mini

LCC DNA vectors produced *in vitro* by recombinant TelN have been successfully applied in gene delivery experiments, and showed higher and more durable expression of the GOI in targeted human cells (*47*, *49*). In contrast, to the best of our knowledge, there are no reported applications of the Tel-*pal* system. Furthermore, current TelN-*telRL* applications are based on recombinant TelN production for *in vitro* LCC DNA vector generation (*104*).

In this study, we report for the first time the development and characterization of an optimized *in vivo* mini LCC DNA production platform, exploiting the Tel-*pal* and TelN-*telRL* protelomerase systems. This one-step production system combines the biocompatibility benefits of minicircles with the transfection efficiency and safety profile of "MIDGE".

2.2 Materials and methods

2.2.1 Strains and plasmids

E. coli K-12 strains were used in the generation of all recombinant cell constructs and DH5α and JM109, in particular, were employed as hosts for plasmid constructions and amplification. A list of bacterial and phage strains used in this study are shown in Table 3.

2.2.2 Construction of Recombinant Cells (R-cells)

W3110 was used for generation of R cells representing the *in vivo* platform for production of bacterial sequence depleted DNA vectors. These cells were employed for *in vivo* recombinase expression as follows. Protelomerase coding gene *tel* was amplified from bacteriophage PY54 lysate using the following primers: Tel-F 5′-GC*GGATCC*TGGGTTACTTTAATTTGTGTGTT-3′ and Tel-R 5′-CG*CTCGAG*TTACTCCATATTTTCAGTCCATGCTTGT-3′ (annealing Tm 64°C). Protelomerase coding gene *telN* was amplified from bacteriophage N15 lysate using primers: TelN-F 5′-ATC*GGATCCC*GATATCCAGAGACTTAGAAACGGG-3′ and TelN-R 5′-ATATA*AAGCTT*CTTTTAGCTGTAGTACGTTTCCCATGCG-3′ (annealing Tm 62°C). As a positive control for *in vivo* production of modified DNA vectors, the recombinase encoding gene *cre* was amplified from bacteriophage P1 *rev6* lysate using primers: Cre-F 5′-G*GAATTC*CGGTCGCTGGCGTTTCTATGAC-3′ and Cre-R 5′-CG*CTCGAG*TGAATATTAGTGCTTACAGACAG-3′ (annealing Tm 66°C). Italicized regions denote restriction sites for BamHI, XhoI, HindIII, and EcoRI. PCR amplifications were conducted using the "Phusion Flash High-Fidelity PCR Master Mix" (New England Biolabs) for 30 s at 98°C for initial denaturation, 30 cycles of 5 s at 98°C, 10 s at annealing Tm, 45 s at 72°C, and 2 min at 72°C for final extension to generate *cre* (1.3 kb), *tel* (2.1 kb), and *telN* (2.3 kb) fragments. Constructs were tested and

confirmed by colony PCR and analytical digestion. PCR products were purified from 0.8% agarose gel (Qiagen Gel extraction kit), and digested with the listed enzymes (New England Biolabs). Protelomerase genes were cloned into the MCS of the inducible prokaryotic expression plasmid vector pPL451 (Accession #AB248919) to produce pNN1, pNN2, and pNN3 vectors. pPL451 (4.2 kb) imparts temperature-regulated expression of the cloned gene *via* CI[Ts]857- mediated repression of the λ *pL/pR* strong promoters. A list of plasmids used or constructed in this study is shown in Table 3 . All primers were designed using the Gene Runner 3.01 (Hastings Software, Inc) and synthesized commercially (Sigma-Aldrich, Inc). R-cells were constructed *via* insertion of recombinase genes into *E. coli* W3110 chromosome using the pBRINT-*cat* integrating plasmids, which facilitate the homologous recombination and chromosomal integration of cloned sequence of interest into the *lacZ* gene of *E. coli* (*26*). For each plasmid construct encoding inducible expression of a cloned recombinase in pPL451, the *cI857-*P_L*-X-*t_L cassette (where X = *cre, tel* or *telN*) was amplified from the pNN1 to 3 constructs by the cI857X-F 5′-TCCC*CGCGG*AGCTATGACCATGATTACGAATTGC-3′, cI857-telN/cre-R 5′-GG*ACTAGT*CCCCATTCAGGCTGCGCAACTGTTG-3′, and cI857-tel-R 5′-GC*TCTAGA*GCAGGCTGCGCAACTGTTGGGAAG-3′ primers with SacII, SpeI, and XbaI sites respectively. The amplified cassettes were cloned into the MCS of pBRINT (Cm^R) integrating plasmid to produce pNN4, pNN5, and pNN6 integrating pDNA constructs. Amplification have been performed by the "Phusion Flash High-Fidelity PCR Master Mix" (New England Biolabs) for 10 s at 98°C for initial denaturation, 30 cycles of 1 s at 98°C, 5 s at 68°C, 120 s at 72°C, and 1 min at 72°C for final extension to generate *cI857-cre* (2.8 kb), *cI857-tel* (3.2 kb), and *cI857-telN* (3.5 kb) fragments. Constructs were tested and confirmed by colony PCR and analytical digestion.

Table 3- Bacteria, phage and plasmids

Strain	Genotype [1]	Source
Bacteria		
BW23474	F-, Δ*(argF-lac)169, ÄuidA4::pir-116, recA1, rpoS396*(Am), *endA9*(del-ins)*::FRT, rph-1, hsdR514, rob-1, creC510*	*E. coli* Genetic Stock Center (CGSC) # 7838 (*105*)
DH5á	F-, Δ *(argF-lac)169,* ö80d*lacZ58*(M15), *ÄphoA8, glnV44*(AS), λ- , *deoR481, rfbC1, gyrA96*(NalR), *recA1, endA1, thi-1, hsdR17*	CGSC # 12384 ;
DH5αλpir	F-, Δ *(argF-lac)169,endA1, pir*$^{+}$, *recA1*	Gift from Dr. T. Charles; (*106*)
JM109	F', Δ *(gpt-lac)0, glnV44*(AS), λ-, *rfbC1*?, *gyrA96*(NalR), *recA1, endA1, spoT1*?, *thi-1, hsdR17*, pWM5, F128-x	New England Biolabs
W3101	F-, λ-, *galT22, IN(rrnD-rrnE)1, rph-1*	CGSC # 4467;
W3110	F-, λ-, *IN(rrnD-rrnE)1, rph-1*	CGSC # 4474; (*107*)
W3110-Cre (W1NN)	F-, λ-, *IN(rrnD-rrnE)1, rph-1 lacZ::Cm-cI857-cre*	This study
W3110-TelN (W2NN)	F-, λ-, *IN(rrnD-rrnE)1, rph-1 lacZ::Cm-cI857-telN*	This study
W3110-Tel (W3NN)	F-, λ-, *IN(rrnD-rrnE)1, rph-1 lacZ::Cm-cI857-tel*	This study
Phages		
N15	Wild type (wt) (*telN*$^{+}$, *tos*$^{+}$)	Gift from Dr. S. Hertwig;(*49*)
P1	wt (*cre*$^{+}$, *loxP*$^{+}$)	Gift from Dr. B. Funnell; (*108*)
PY54	wt (*tel*$^{+}$, *pal*$^{+}$)	Gift from Dr. S. Hertwig; (*49*)
Plasmids		
pAH120	*attP λ* integration plasmid (Kn^{R})	NBRP (*26*)
pAH123	*cI857-pL-int* Φ80 (Ap^{R})	NBRP (*26*)

pAH153	*attP* Φ80 integration plasmid (Kn^R)	NBRP (*26*)
pBRINT	*lacZ::cat-MCS::lacZ* (Cm^R)	NBRP;
pGL2	SV40P-Luc-PolyA-SV40 intron	Promega
pInt(ts)	*cI857-pL-int λ* (Ap^R)	NBRP (*26*)
pNN1	*cI857-pR-pL-cre-tL* (Ap^R)	This study
pNN2	*cI857-pR-pL-tel-tL* (Ap^R)	This study
pNN3	*cI857-pR-pL-telN-tL* (Ap^R)	This study
pNN4	*lacZ::cat- cI857-pR-pL-cre-tL::lacZ* (Cm^R)	This study
pNN5	*lacZ::cat- cI857-pR-pL-tel-tL::lacZ* (Cm^R)	This study
pNN6	*lacZ::cat- cI857-pR-pL-telN-tL::lacZ* (Cm^R)	This study
pNN7	*pGL2-egfp* switched for *luc*	This study
pNN8	pNN7 + SS (upstream of SV40 promoter)	This study
pNN9	pNN8-SS (2XSS) (second SS downstream of SV40 polyA sequence)	This study
pNN10	pAH120 (SS^+)	This study
pNN11	pAH153 (SS^+)	This study
pPL451	*cI857-pR-pL-MCS-tL* (Ap^R)	Accession# AB248919, National Bioresource Project (NBRP); (*109*)

[1] sequences of interest confirmed by PCR and/or sequencing

E. coli cells were grown in Luria–Bertani (LB) medium and plated on LB-Agar plates composed of 1.0% Tryptone, 0.5% Yeast Extract, 1.0% NaCl, pH 7.0. Antibiotics (Ab) (Sigma-Aldrich, Inc) were used at the following concentrations for the growth of cells carrying multi-copy plasmids: ampicillin (Ap, 100 μg/ml in H_2O), chloramphenicol (Cm, 25 μg/ml in isopropanol), gentamycin (Gm, 15 μg/ml in H2O), and kanamycin (Km, 50 μg/ml in H2O). H2O used for dilution of primers, plasmids, antibiotics, and production of competent cells is nuclease-free sterile molecular grade water (Sigma-Aldrich, Inc). To achieve chromosomal integration of the pNN4 to 6 constructs into the Rec+ W3110, a 1:100 dilution of fresh overnight cells was grown on SOB media (2.0% Tryptone, 0.5% Yeast Extract, 2.0% NaCl, 1.0% KCl, 1.0% MgCl2 , and 1.0% MgSO4) at 37°C to A600 = 0.4 - 0.6 and cells were harvested by centrifugation at 5K RPM for 5 min. Cells were washed in water three times and W3110 cells were transformed by 1 μg of pNN4, 5, or 6 integrating vectors via electroporation at 800 v. Cells were recovered in SOC (SOB with 2.0% Glucose) at 30°C for 1 h, then spread onto selective media on 12.5 μg/ml of chloramphenicol, 100 μg/ml of 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal) (Promega), and 0.1 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, Inc) added to LB agar plates and incubated overnight at 30°C. To make a 50 mg/ml stock solution, 500 mg X-Gal were dissolved in 10 ml dimethylformamide and protect plates from light. White colonies were selected and further screened for sensitivity to ampicillin and chloramphenicol. White Ap^S colonies indicated loss the pNN integrating plasmids after disruptive insertion of the *cI857-PL-X-tL* cassette into the lacZ gene, generating recombinant derivatives, W1NN, W2NN, and W3NN (Table 3). W3110 [*lacZ::cat-cI857*(cre/tel/telN)] recombinants were confirmed for presence of the *cI857-PL-X-tL* cassette and temperature-regulated, conditional expression of recombinanse, by colony PCR using Taq polymerase enzyme (NEB), sequencing (Sigma), and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) (BioRad) at various temperatures between 30°C and 42°C. For the selection of cells carrying the antibiotic selection markers integrated in the chromosome, the following concentrations were used: Cm (12.5 μg/ml), Gm (5 μg/ml), and Km (20 μg/ml).

2.2.3 Construction of modified / new generation of pDNA vectors

The multi-purpose target site, named SS was designed to carry Cre, Flp and TelN minimal targets sites (*loxP-frt-telRL*) respectively, all within the Tel 142 bp target site, *pal*. SS also carries a 78 bp SV40 enhancer sequence that flanks each side of the *pal* sequence to facilitate nuclear translocation and enhancing transfection efficiency (Figure 12). The SS fragment was synthesized by the GeneScript and cloned into the pUC57 by EcoRI and HindIII. Commercial eukaryotic expression plasmid vector, pGL2-promoter (5.8 kb) (Promega), was modified by replacement of the *luc* gene (1.65 kb) with *egfp* (790 bp) from pGFP (Clontech, Inc.) to form pNN7 (Genescript, Inc.) (4.9 kb) Next, SS was cloned immediately upstream of the SV40 promoter + intron site of pNN7 to form pNN8 (5.3 kb). Then the SS fragment was cloned downstream of the

poly A site of pNN8 to form pNN9 (5.6 kb). The multi-copy pDNA vector pNN9 carries 2 SS sites that flank the *egfp* gene cassette and can be converted to a minicircle DNA vector (mediated by Cre-*loxP;* Flp-*frt*), or a mini LCC DNA, which we called DNA ministring vector (mediated by TelN-*telRL;* Tel-*pal*). R-cells were transformed by 1 μg of pNN7 to 9 DNA constructs on LB + Ap (50 μg/ml) to A_{600} = 0.6 at 30°C with aeration. To induce recombinase expression and plasmid conformational conversion, transformed R-cells were heat shocked to induce the recombinase expression at 42°C for 30 min at mid-logarithmic phase of bacterial growth, before being transferred to 30°C overnight. Cells were then harvested and plasmid extracted (Omega kit, VWR). Plasmid topology was assayed by agarose gel electrophoresis (AGE) and digestion. Standard recombinant DNA techniques were performed as described by Sambrook *et al.* (1989).

2.2.4 Chromosomal integration assays of LCC pDNA into bacterial chromosome

"CRIM (conditional-replication, integration, and modular)" integrating plasmids (*26*) that possess a R6K origin of replication and a phage attachment (*attP*) site were modified to carry the SS fragment. SS was cloned into the pAH120 (accession number AY048723) and pAH153 (accession number AY048735) constructs (from NBRP; Table 3) by ClaI and BamHI (New England Biolabs) to generate the pNN10 (3.3 kb) and pNN11 (2.6 kb) constructs, respectively. Plasmids were integrated into the host bacterial attachment (*attB*) site by supplying phage integrase (Int) from the helper plasmids. Plasmid pNN10 and pNN11 constructs were amplified in DH5α (λ*pir*) or BW23474, and the successful clones were confirmed by restriction pattern digestion and colony PCR. Int helper plasmids pINT-ts (λ *int*) and pAH123 (Φ80 *int*) that express *int* from λ *pL* under CI[Ts]857 control and carry a temperature sensitive pSC101 *ori* were used for integration of CRIM, pNN10 and pNN11 plasmids into their corresponding chromosomal *attB* sites of *pir*$^{-}$ hosts that are non-permissive for plasmid replication.

R-Cells W1NN, W2NN, and W3NN (Table 3) W3110 [*lacZ::cat- cI857(cre/tel/telN*)] were grown in 2 ml of SOB cultures at 30°C to an optical density of A_{600} = 0.6 and then electroporated and transformed by 50 ng helper plasmids pINT-Ts (λ *int*) or pAH123 (Φ80 *int*) and selected on LB + ampicillin agar at 30°C. R-Cells carrying helper plasmids were grown in 50 ml of SOB + ampicillin at 30°C to A_{600} = 0.6 then transformed by 1 μg of pAH120, pNN10 (pAH120+SS), pAH153, pNN11 (pAH153+SS) DNA, suspended in SOC at 37°C for 1 h for recovery and Int expression and at 42°C for 30 min for loss of helper plasmid, then selected on LB + antibiotic (15 μg/ml Km for pAH120, pNN10 and 5 μg/ml Gm for pAH153, pNN11) and incubated overnight at 37°C. Positive bacterial growth on selective media (15 μg/ml Km, or 5 μg/ml Gm) and negative bacterial growth on ampicillin media represents stable integration of the GOI and loss of the helper Int expression plasmid. Single-copy integrants W4NN to W15NN represent the SS^{+} pAH120 (pNN10), SS^{-} pAH120, SS^{+} pAH153 (pNN11), and SS^{-} pAH153 pDNA integrated into the W3110 or W3110 *lacZ::cat-cI857(cre/tel/telN*)]) were screened and selected by PCR using predesigned primers (*26*).

2.2.5 Viability assays of LCC pDNA integration

Single copy pAH120, pNN10 (pAH120+SS), pAH153, pNN11 (pAH153+SS) DNA integrants W4NN to W15NN were isolated on selective media. Integrants were grown in 2 ml LB media + selective antibiotic at 30°C to an optical density of A_{600} = 0.4 and then divided into two groups of 1 ml each and grown to A_{600} = 1. The first group was grown at 30°C with repressed *cre/tel/telN* expression and the second group was grown at 42°C, induced for *cre/tel/telN* expression. Cells were spread on selective plated and grew overnight at 30°C and 42°C, respectively. Viability was assayed by colony counting and size of the colonies grew at 30°C versus 42°C.

2.2.6 Visualization of bacterial cells

Cells were visualized by gram staining as previously described (*110*). Briefly, integrated cells were grown in 2 ml of LB media + selective antibiotic from freshly grown cells at 30°C to early log phase A_{600} = 0.2 and then were divided in two tubes and grown at 30°C and 42°C to late log phase, A_{600} = 0.8. Bacterial smears were then prepared on the slide and heat fixed and gram stained. Pictures of bacteria were taken at 1000X magnification. From pictures, 400 random cells were chosen for measurement under all tested conditions.

2.3 Results

2.3.1 R-cells exhibit temperature-regulated protelomerase expression

Recombinant *E. coli* cells (R-cells) were constructed that place *tel* or *telN* protelomerase genes under control of the bacteriophage λ-derived *pR* and *pL* promoters that are regulated by the temperature-sensitive λ repressor, *cI*[Ts]857 (Figure 10). We also similarly constructed *cre*-expressing cells to serve as a positive control, where *in vivo* Cre-*loxP* activity is well documented (*20, 31*). We examined total cellular protein in *tel* and *telN* R-cells under repressed (30°C) and fully induced (42°C) conditions (Figure 11). As expected, both R-cells demonstrated minimal recombinase protein levels, identified at 72 kDa for both TelN and Tel at 30°C, where CI[Ts] actively binds the *oL* operator and represses transcription of the downstream recombinase gene. Upon shifting cells to 42°C, where repressor activity is completely abrogated and occlusion of *pR* and *pL* promoter activity is relieved, prominent recombinase expression was observed. These results confirm that the constructed Tel^+ and $Tel\text{-}N^+$ cells are temperature inducible for recombinase production.

In combination with the R-cell system, we next constructed a eukaryotic *egfp*-expression pDNA vector that carries two specialized 343 bp sequences placed upstream of the SV40 promoter and downstream of the polyadenylation signal of the minimal *egfp* expression cassette (pNN9; Figure 12-A). This construct, termed SS, carries a modified *pal* target sequence of Tel, with integrated *telRL* (TelN), *loxP* (Cre) and *frt* (Flp) sequences in non-binding regions of *pal* sequence, and SV40 enhancer sequences that flank *pal* on either side

to enhance nuclear translocation (Figure 12-B). The rationale behind the multi-target sequence composition of SS was to allow the same parent plasmid vector to be passed through different R-cells to generate either mini LCC or mini CCC conformation of the minimal expression cassette. We employed the pNN9 (5.6 kb) plasmid to confirm that controlled production of recombinase from R-cells was associated with controllable recombinase enzymatic activity, by examining the ability of R-cells to convert plasmids carrying the SS sites to the appropriate mini LCC (Tel or TelN) or mini CCC (Cre) conformation. The SS^{+}-derivative (pNN9; Table 3) was passaged through Tel/$TelN^{+}$ or Cre^{+} R-cells at optimized conditions and assessed formation of mini LCC (ministring) or mini CCC (minicircle) DNA vectors during the one-step *in vivo* processing and amplification protocol (Figure 13-A). By flanking the GOI expression cassette with two SS sites, we expected to excise the GOI (2.4 kb) for easy separation from the remaining prokaryotic backbone (3.2 kb) parental DNA, including the origin of replication and Ap^{R} marker at temperatures inducing recombinase expression (Figure 13-B). Recombinase activity on pNN9 was evident when the plasmid was processed at 42°C where R-cell recombinase expression was "on". No SS^{+} plasmid processing was evident at 30°C when expression was "off", or with the SS^{-} control plasmids under all tested conditions (data is not shown). Optimal linearization conditions for pNN9 were conducted in both Tel^{+} and $TelN^{+}$ cells and pNN9-processed products were extracted and analysed. During the optimization process, different thermal conditions were tested to determine the ideal induction stage during R-cells growth and the induction duration that generates maximum recombinase production and plasmid processing. We found that shifting the temperature at late logarithmic phase to 42°C for 30 min to induce R-cells conferred optimal recombinase expression and plasmid processing. In all cases, extracted mini LCC DNA vectors behaved with equivalent stability in storage and manipulation to their CCC counterparts or parental pDNA (data not shown). Linearization of pNN9 and extraction of the mini LCC vector was also much more efficient in Tel^{+} than to the $TelN^{+}$ counterpart (Figure 13-C). These findings indicate that Tel is active upon pal^{+} constructs *in vivo* and that the modification of *pal* target site through the addition of *loxP*, *frt*, and *telRL* minimal target sequences into non-binding regions of the full 142 bp *pal* target site does not abrogate Tel-*pal* functionality.

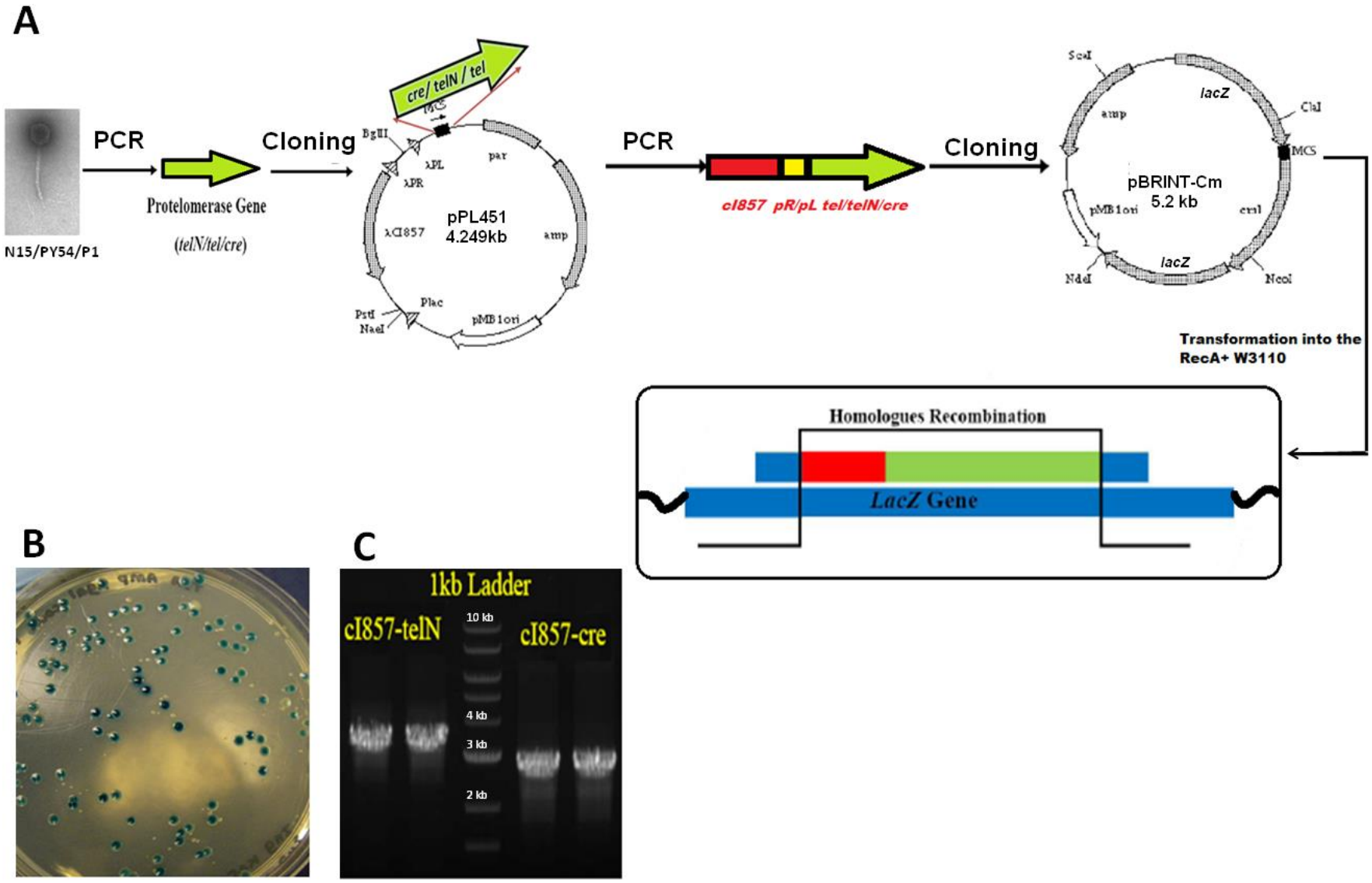

A
N15/PY54/P1
PCR
Protelomerase Gene
(telN/tel/cre)
Cloning
cre/ telN / tel
lacS
BglII
λPL
λPR
par
λCI857
pPL451
4.249kb
amp
PstI
NaeI
Plac
pMB1ori
PCR
cI857 pR/pL tel/telN/cre
Cloning
ScaI
lacZ
amp
ClaI
MCS
pBRINT-Cm
5.2 kb
pMB1ori
cmR
lacZ
NdeI
NcoI
Transformation into the
RecA+ W3110
Homologues Recombination
LacZ Gene
B
C
1kb Ladder
cI857-telN
cI857-cre
10 kb
4 kb
3 kb
2 kb

Figure 10- Construction of R-cells

A) Schematic representation of methodology for construction of R cells. Protelomerase coded genes were amplified from bacteriophage lysates and cloned into the MCS of the inducible prokaryotic expression plasmid vector pPL451 to impart temperature-regulated expression of the cloned gene *via cI857*-mediated repression of the λ *pL* and *pR* strong promoters. The *cI857(cre/tel/telN*) cassette were amplified from modified pPL451 plasmids and moved into the pBRINT-*cat* integrating plasmid, which facilitate the homologous recombination and chromosomal integration of cloned sequence of interest into the *lacZ* gene of *E. coli via* homolohous recombination. New constructs were tested and confirmed by blue-white screening and colony PCR. **B)** Blue-white screening and antibiotic selection. White Ap^S Cm^R colonies indicated loss the integrating plasmids after disruptive insertion of the *cI857-*P_L*-X-*t_L cassette into the *lacZ* gene of *recA*$^+$ W3110 cells, generating recombinant derivatives, W1NN, W2NN, and W3NN. **C)** New W3110 [*lacZ::cat- cI857(cre/tel/telN*)] recombinants were confirmed by colony PCR. Primers were used to amplify bacterial genomic DNA over the site of insertionfor to confirm presence of the *cI857-*P_L*-X-*t_L cassette. From left to right: *cI857-telN* (3.5 kb), 1 kb DNA ladder, and *cI857-cre* (2.8 kb) fragments.

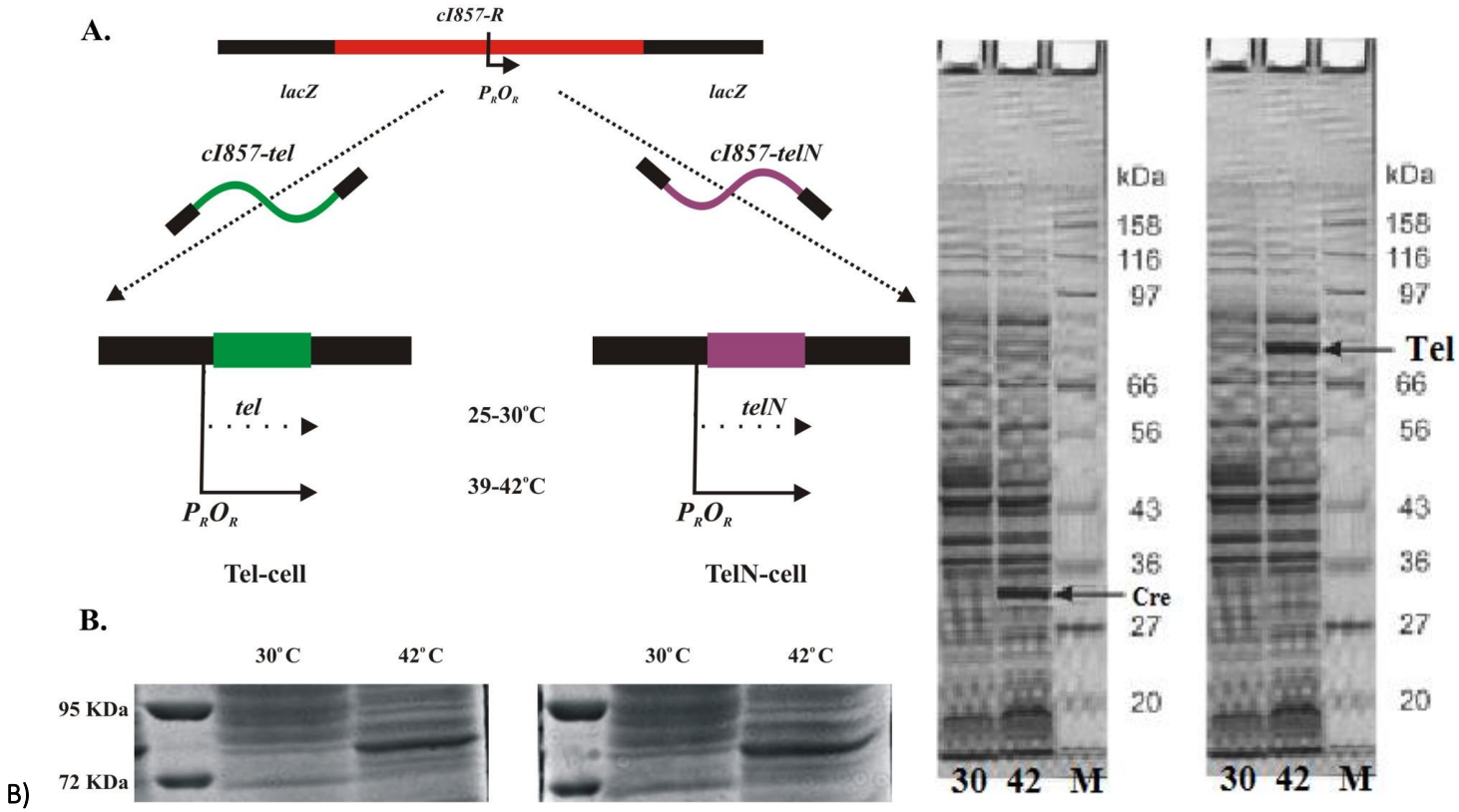
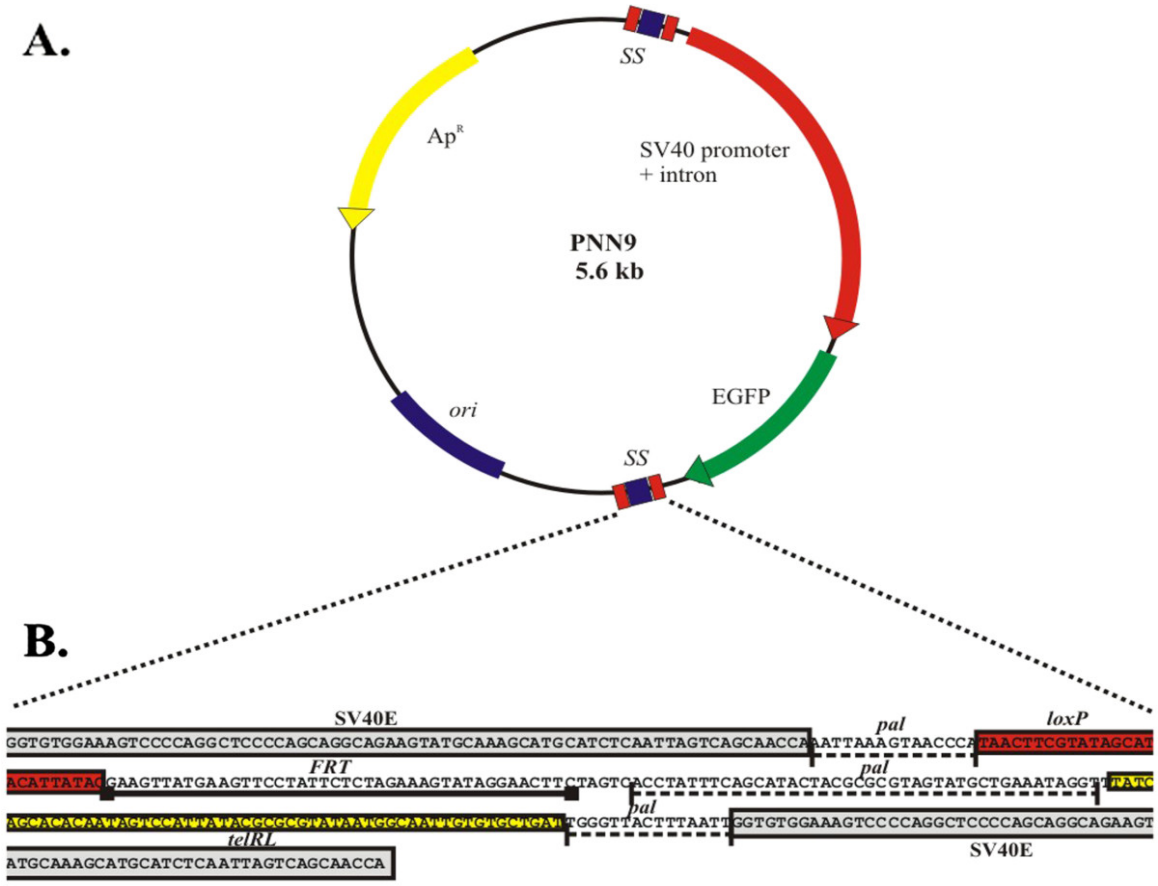


Figure 11- R-cell construction and temperature-regulated expression of Tel and TelN.

A) R-cell construction and temperature-regulated expression of recombinases, Tel and TelN. The *cI857-R* cassette was inserted into the *lacZ* gene of *E. coli* by homologous recombination. Under repressed (25-30°C) conditions, the λ temperature-sensitive CI857 repressor binds to λ operators to inhibit transcription of the recombinase gene, but upon shifting to 40-42°C, the repressor is denatured, falling off the operators and induces expression of the recombinase from the strong λ *pL/pR* promoter. **B)** Controllable Tel and TelN expression from R-cells. Expression of the recombinase proteins Tel, TelN, and Cre under repressed (30°C) and induced (42°C) conditions from total R-cell extract was confirmed by SDS-PAGE qualitative analysis. From left to right: Lane 1: proteinweight marker; Lane 2: no protelomerase expression at 30°C, and Lane 3: protelomerase expression at 42°C.



Target sequence	Start	End
SV40E	1	72
pal	73	87
loxP	88	120
FRT	121	154
pal	160	201
telRL	203	256
pal	257	271
SV40E	272	343

Figure 12- Parent plasmid pNN9 and "Super Sequence" multi-target site.

A) Map of the parent pNN9 construct. The map denotes location of primary genetic elements, including the designed and integrated SS. **B**) Map of the SS construct. The map includes relative locations of SV40 enhancer sequences as well as the *telRL*, *loxP* and *frt* sequences integrated into non-binding regions of the 142 bp *pal* target site (Tel).

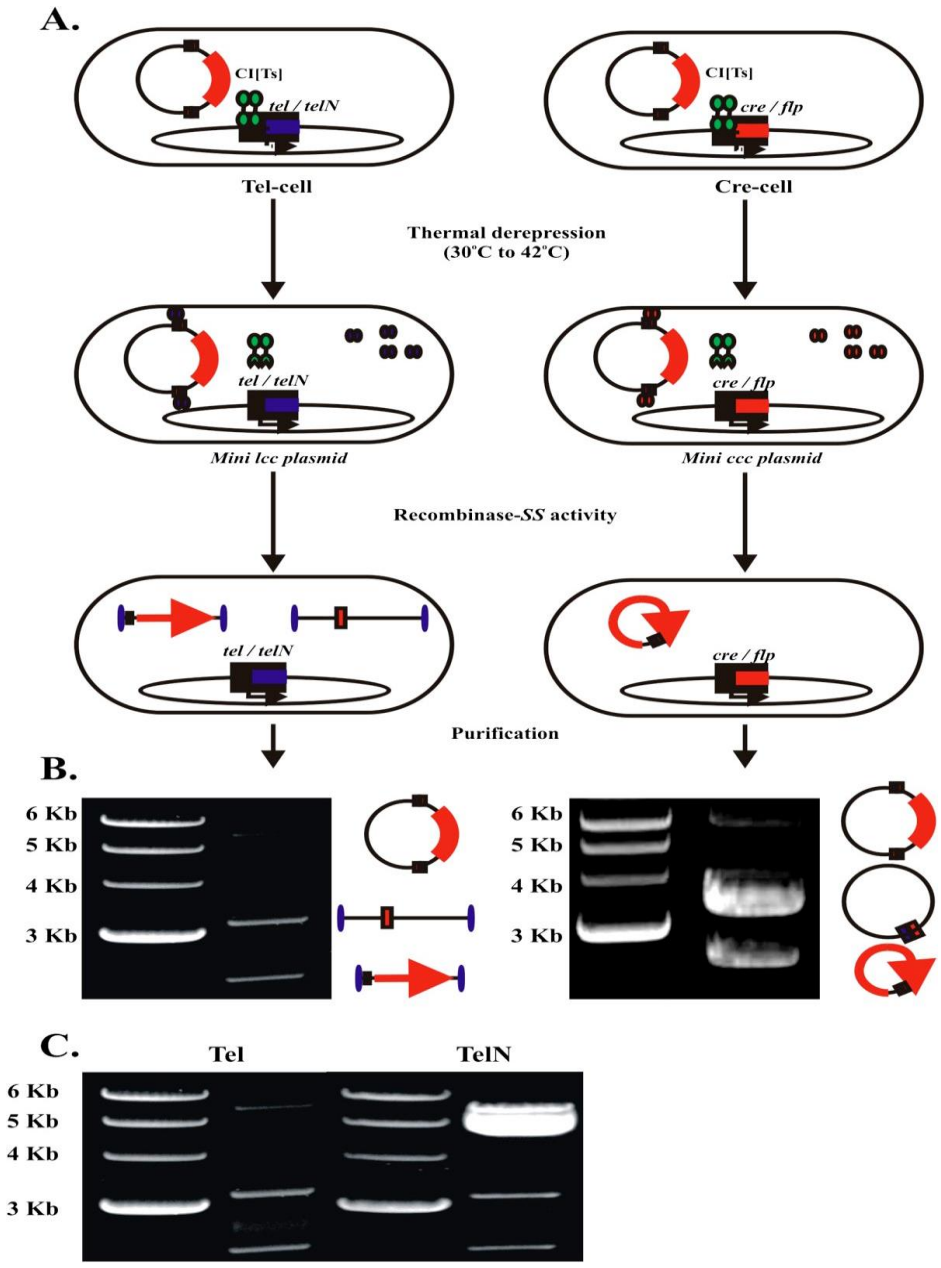


Figure 13- Conditional processing of the parent plasmid DNA vectors.

A) R-cell conditional processing of the parent pNN9 plasmid into mini vectors. Under induced conditions, R-cells lead to the production of mini LCC (TelN- or Tel-cell) and mini CCC (Cre-cell) DNA vectors by recombinase activity on its target site encoded within the 2 SS sites on pNN9. Processing of the parent plasmid DNA results in production of two species—the mini DNA vector and the mini plasmid backbone. **B)** Processing of the parent plasmid construct into mini LCC and mini CCC vectors. Efficiency of processing of the pNN9 plasmid into DNA Ministrings (Tel) and minicircles (Cre) after plasmid extraction from R-cells under induced (42°C) conditions. Schematics adjacent to each bands show the DNA constructs and expected conformation. **C)** *In vivo* Tel-*pal* cleaving-joining efficiency versus TelN-*telRL*. Efficiency of protelomerase-mediated processing of the pNN9 parent plasmid into mini LCC DNA vectors in Tel^+ versus $TelN^+$ R-cells.

2.3.2 Integration of LCC DNA into the bacterial chromosome results in loss of cell viability

We hypothesized that a single crossover recombination event integrating a linear pDNA vector with covalently closed ends into a host cell chromosome would disrupt the chromosome and kill the cell (Figure 15-D). We employed an *in vivo* approach by exploiting λ and Φ80 Int-*att* site-specific recombination systems (*26*) to assess the outcome of linearizing the *E. coli* chromosome by LCC DNA integration in a Rec^+ background. To target the chromosome, an integrating plasmid that possesses the λ *attP* target site of the λ integrase and an R6K origin was used as it is only capable of replication in the presence of π (encoded by R6K *pir* gene), which is absent from all R-cells. As such, the plasmid is either integrated or rapidly lost from the growing cell population (Figure 14).

An SS was incorporated into the plasmid to change plasmid conformation from CCC to LCC in the presence of TelN or Tel in R-cells. Using this system we sought to assess integration efficiency following transformation of parent, Tel or TelN-cells by $SS^{—}$ and SS^+ plasmids under conditions induced or repressed for recombinase expression in R-cells. A CCC λ or Φ80 *attP*$^+$-SS^+ plasmid that is taken up by the cell expressing λ or Φ80 *int*, respectively, and producing Tel or TelN, should be altered from CCC to LCC conformation. Upon Int-dependent directed chromosomal integration, the recombination of LCC should result in a disrupted chromosome. In addition, as unintegrated plasmids are incapable of replication, they would be rapidly lost from the cell population.

The integration frequency (IF) of *attP* λ SS^+ plasmids into induced Tel-cells was more than 10^5-fold lower than that seen for the $SS^{—}$ counterpart or the SS^+ plasmids in parent W3110 cells (Table 4). TelN-cells did not demonstrate nearly as strong an effect, whereby IF compared to the wild type cells was 50-fold lower, and only 25-fold lower versus integration of the $SS^{—}$ derivative. We further examined between 5 to 20 survivor isolates from both TelN and Tel-cells by PCR for evidence of chromosomal linearization, and found that 100% of tested survivors possessed intact (unlinearized) plasmid. These findings are likely due to the a higher efficiency of Tel-*pal* system in formation of a LCC product as compared to TelN acting on the minimal *telRL* target site encoded within the SS. We attempted to repeat this experiment using a constructed *attP* Φ80-SS^+ system, but found that integration frequencies were lower across all tested cells and the effect of SS integration into the chromosome in presence of the Tel activity on cell viability was far too low to reasonably assess differences in safety, maybe because of the distance of the Φ80 *attB* site to the origin of replication of R-cells.

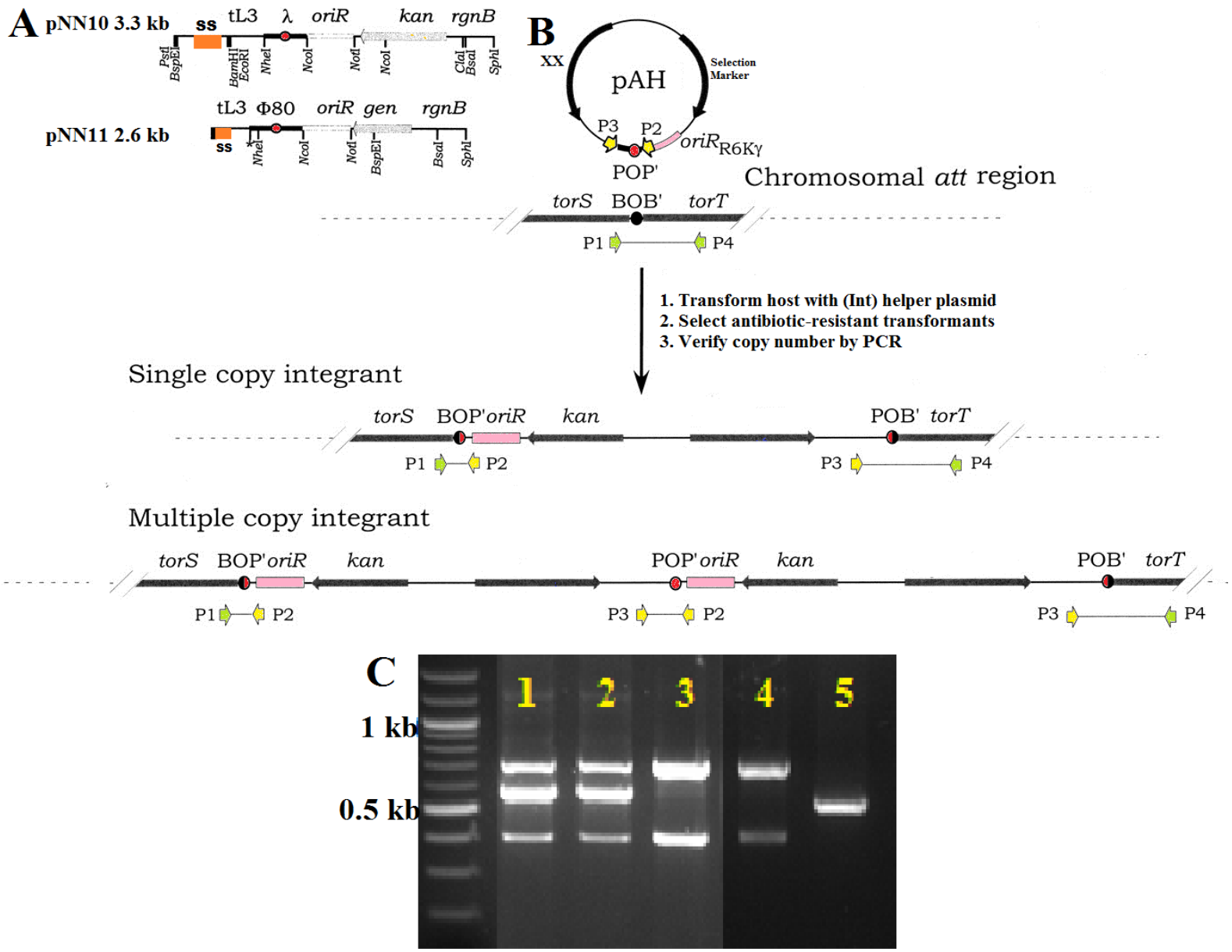

A
pNN10 3.3 kb
ss
tL3
λ
oriR
kan
rgnB
PstI BspEI
BamHI EcoRI
NheI
NcoI
NotI
NcoI
ClaI BsaI
SphI
pNN11 2.6 kb
tL3 Φ80
oriR gen
rgnB
ss
NheI
NcoI
NotI
BspEI
BsaI
SphI
B
XX
pAH
Selection Marker
P3
P2
oriR R6Kγ
POP'
Chromosomal att region
torS BOB' torT
P1
P4
1. Transform host with (Int) helper plasmid
2. Select antibiotic-resistant transformants
3. Verify copy number by PCR
Single copy integrant
torS BOP' oriR kan POB' torT
P1
P2
P3
P4
Multiple copy integrant
torS BOP' oriR kan POP' oriR kan POB' torT
P1
P2
P3
P2
P3
P4
C
1 kb
0.5 kb
1
2
3
4
5

Figure 14- Schematic representation of integration methodology.

A) Schematic representation of integration plasmid vectors pNN10 and pNN11 that possess a R6K origin of replication, a phage attachment (λ-*attP* and Φ80-*attP*, respectively) site, and the SS fragment.

B) Plasmids were integrated into the host bacterial attachment (*attB*) site by supplying phage integrase (Int) from the helper plasmids. Int helper plasmids pINT-ts (λ *int*) and pAH123 (Φ80 *int*) that express *int* from λ *pL* under CI[Ts]857 control and carry a temperature sensitive pSC101 *ori* were used for integration of CRIM, pNN10 and pNN11 plasmids into their corresponding chromosomal *attB* sites of *pir*⁻ hosts that are non-permissive for plasmid replication. R-Cells carrying helper plasmids were transformed by pNN10 and pNN11 integrating vectors and positive integrant were selected by 15 μg/ml Km, or 5 μg/ml Gm, and negative bacterial growth on ampicillin media represents stable integration of the GOI and loss of the helper Int expression plasmid.[Modified from (*26*)].

C) Single-copy integrants were screened and selected by colony PCR at the site of integration using predesigned primers P1, P2, P3, and P4. All four primers were used to amplify integrated colonies. From left to right: Lane 1 & 2 representing multiple copy integrants, Lane 3 & 4 representing single copy integrants, and Lane 5 representing no integration, in comparison with the ready to use 100 bp ladder from NEB.

Table 4- Reduced integration frequency conferred by LCC pDNA vectors.

R-cell[1]	Plasmid SS (+/-)[2]	Integration Frequency[3]
Cre	—	0.76
Cre	+	0.005
TelN	—	0.51
TelN	+	0.02
Tel	—	1.0
Tel	+	**1.03×10^{-5}**

[1] R-cells are all W3110 derivatives expressing recombinase gene at 42°C the recombinase gene is fully induced. R-cells carry the λ *int* expression plasmid pAH153.
[2] pAH120 plasmid carrying λ *attP*.
[3] Mean of minimum 3 trials. IF is expressed as fraction of integration frequency of parent strain, W3110.

We next sought to investigate chromosomal linearization by constructing SS^+ or SS^- integrants using the same λ and Φ80 Int-*attP* plasmid integration system, instead this time, rather than assessing the number of integrants formed in the presence of the linearizing recombinase, we first stably integrated the plasmid into the chromosome before inducing recombinase expression. Cells carrying the integrated SS^+ or SS^- vector were maintained in Tel and TelN "off" position and several isolates were assessed for integration of single versus multiple copies of plasmid by PCR. In all cases, single integration events represented the majority of recombinants for both plasmids (56.5-100%; Table 5). Multiple copy integrants were not studied further and discarded.

Table 5- Single integration event was dominant in R-cells. [1]

	JM101		**W3110**		**W3110::*telN***		**W3110::*tel***		**W3110::*cre***	
	SS-	SS+	SS-	SS+	SS-	SS+	SS-	SS+	SS-	**SS+**
No integration		48	0	5.6		0	37.5	0		**37.5**
Single integration		59.4	100	83		50	62.5	70		**56.5**
Multiple integration		0	0	31		30	0	76		**18.8**

[1] The numbers were recorded as percent based on the PCR results collected from integration experiments.

To determine the fate of cells upon linearizing/disrupting the *E. coli* chromosome, we incubated λ site-specific $SS^{—}$ and SS^{+} integrants under conditions that provide no to very low (30ºC) or full (42ºC) expression of the recombinase, then measured cell viability (Table 6). Under repressed conditions (30ºC), all recombinant cells retained near full viability regardless of the presence or absence of the SS integrated in the chromosome (Figure 15-A, B). However, upon shifting cells to 42ºC and inducing expression of Tel or TelN, recombinants that carried SS^{+} showed dramatically reduced viability, and in both systems, Tel-cells resulted in approximately 5 fold greater killing than that seen in TelN-cells (Figure 15-B, D). Interestingly, the killing effect of SS^{+} plasmid in phi80 *attB* site was about 10 fold lower than when integrated into λ *attB* site, suggesting that positioning of *attB* site may influence the viability of cells with linear chromosome.

Table 6- Protelomerase-mediated linearization of the bacterial chromosome results in cell death.

R-cell[1]	Integrated plasmid SS (+/−)[2]		Cell viability following induction [3]	
			30ºC	**42ºC**
TelN	—	λ	0.8	0.6
TelN	+	λ	1.0	5.7×10^{-4}
TelN	—	Φ 80	0.8	0.4
TelN	+	Φ 80	1.0	5×10^{-3}
Tel	—	λ	1.0	1.0
Tel	+	λ	1.0	1.3×10^{-4}
Tel	—	Φ 80	1.0	0.8
Tel	+	Φ 80	1.0	1.1×10^{-3}

[1] R-cells are all W3110 derivatives cured of pAH153 plasmid during construction stage and confirmed for single integration event by PCR.
[2] pAH120 plasmid carrying λ *attP* grown and prepared under minimal inducing conditions (30°C).
[3] Average of minimum 3 trials. Viability is expressed as a fraction of colonies counted under non-induced (25°C) conditions.

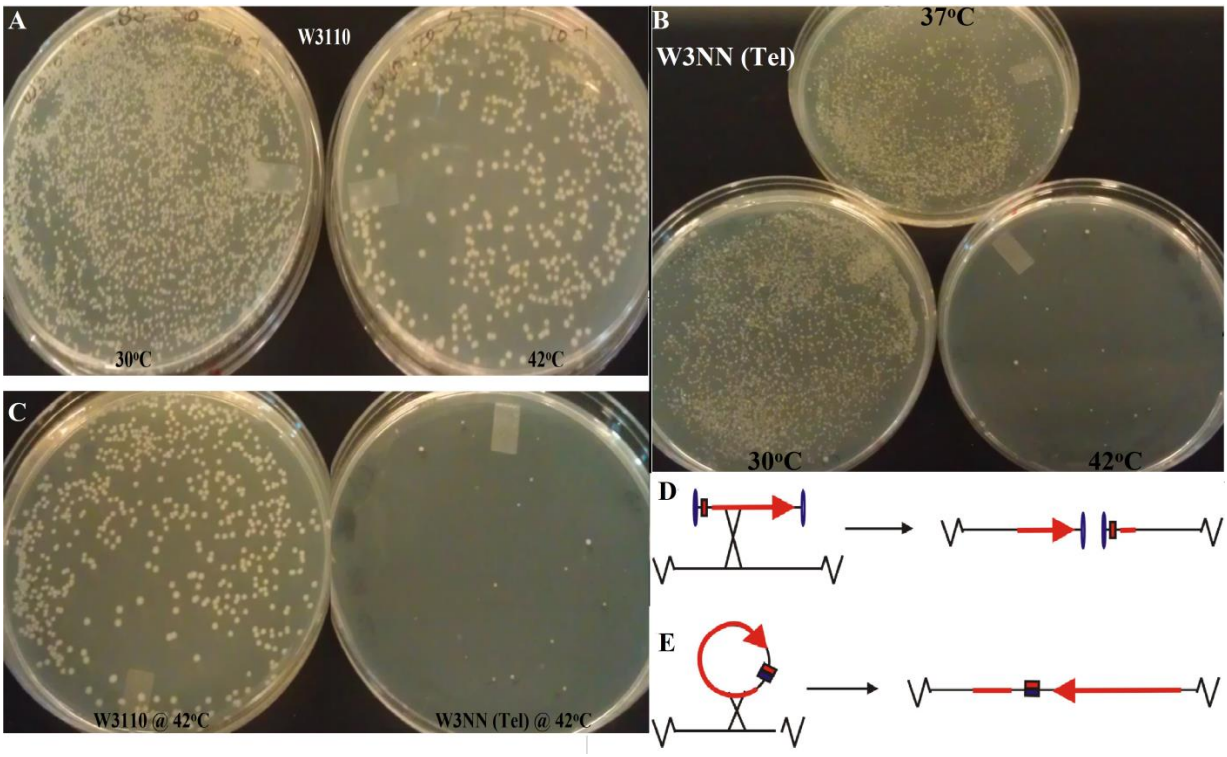


Figure 15- LCC pDNA-mediated integration into bacterial chromosome results in cell death.

Effect of Tel-mediated chromosomal disruption on colony numbers and morphology. To determine the fate of cells upon linearizing/disrupting the *E. coli* chromosome, we incubated λ site-specific SS^{-} and SS^{+} integrants under conditions that provide no (30°C) or full (42°C) expression of the recombinant protelomerase, then recorded cell numbers and cell morphologies. **A)** Control cells with no protelomerase expression showed normal colony morphology and viability at both 30°C and 42°C. **B)** In W3NN (Tel^{+}) cells, under repressed conditions (30°C), all SS^{+} integrants retained near full viability with presence of SS in their chromosome. However, upon shifting cells to higher tempratures at 37°C to 42°C and inducing expression of Tel, SS^{+} integrants show dramatically reduced viability at 42°C. **C)** In Tel^{+} W3NN cells at 42°C, upon Tel/*pal* linearization of host chromosomal DNA. SS^{+} integrants show dramatically reduced colony number/size compared to their Tel^{-} W3110 counterparts. **The schematic representations of single cross over integration events. D)** The LCC pDNA vector integration event would result in chromosomal ”break” at the site of integration, whereby the chromosome cannot be replicated or segregated and the integrated cells cannot divide due to the broken chromosome and genome integrity, which either initiate the repair mechanism or induce natural elimination of integrants, **E)** The CCC pDNA vector integration event results in stable integration and no chromosomal DNA “break” at site of integration, whereby the cell is able to continue dividing with the insertion.

2.3.3 Visualization of bacterial cells upon protelomerase induction

Wild type and Tel-cells carrying chromosomally inserted SS^+ or SS^- integrating plasmid vectors were Gram stained and visualized under repressed (30°C) versus induced (42°C) conditions for *tel* recombinase expression to investigate cell morphology as a result of chromosomal disruption (Figure 16). At 42°C, only SS^+ Tel^+ integrants demonstrated a highly contracted and irregular morphology compared to SS^- cells, or SS^+ cells grown at 30°C, repressed for *tel* expression. We screened 10 Tel^+/SS^+ colonies that grew at 42°C, for linearization by PCR and found that all of the colonies showed an unlinearized SS^+ plasmid insert (data not shown). Surviving colonies were however, very small compared to their SS^- counterpart or the wild type parent, and retained this morphology whether grown under inducing or repressed conditions. We averaged the length of 400 randomly selected cells. We noted that the SS^- controls were similar at both 30° and 42°C with a normal average length for log phase *E. coli* in rich medium, measuring 3.9 ± 0.4 and 3.9 ± 0.6 μm, respectively. In contrast, cells, possessing the chromosomally integrated SS site, were much smaller at 42°C when their chromosomal DNA was linearized by Tel/*pal* processing, where *tel* expression was fully induced, and they were averaging only 1.2 ± 0.2 μm in length. In contrast, a small proportion of these cells (1.5%) were filamentous. This may be attributed to replication delay or inhibition in these cells. Under the conditions where we would expect chromosomal disruption, we also observed a great deal of cellular debris. At 30°C, where *tel* expression was repressed, cells were considerably larger at 3.0 ± 0.8 μm but there was a much greater variability in size between cells ranging from 1.2 to 4 μm. This variability could be the consequence of leaky expression of *tel* due to incomplete repression of the CI[Ts]857 repressor at 30°C.

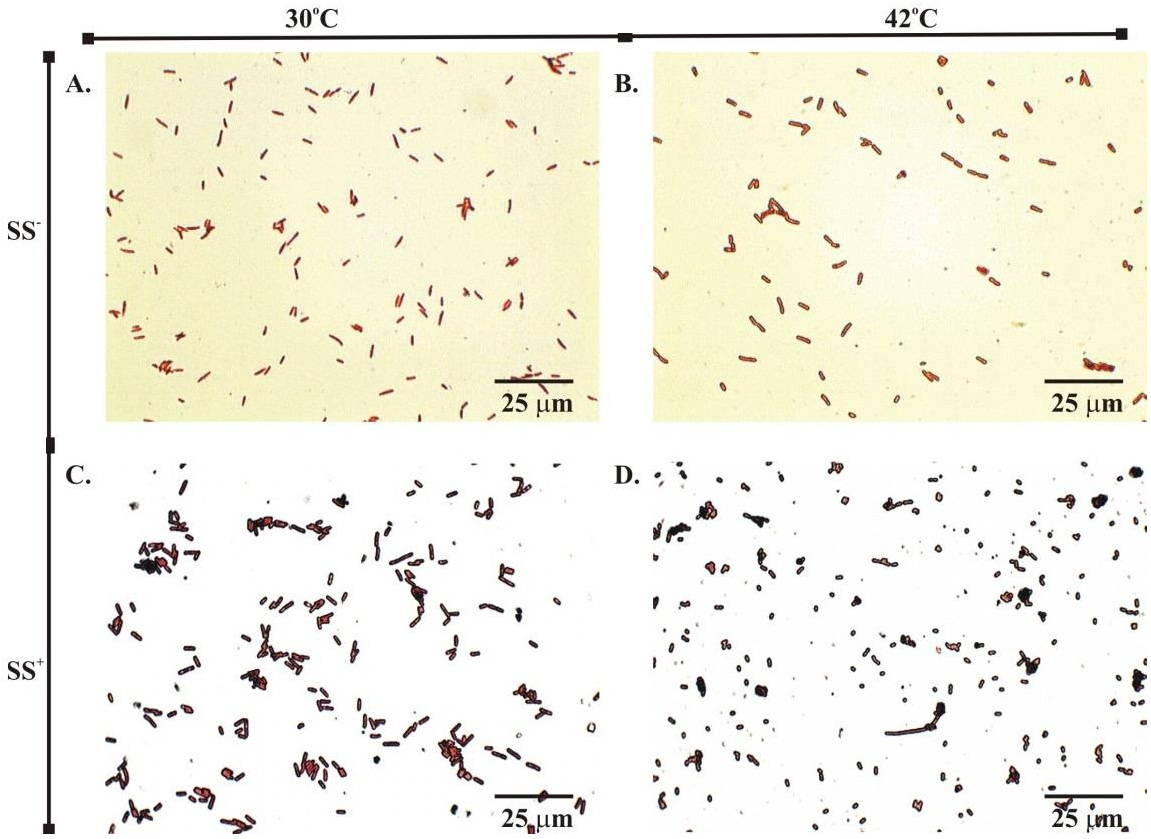


Figure 16- Effect of Tel-mediated chromosomal disruption on cell morphology.

Tel-mediated disruption of the host chromosome results in a contracted cellular morphology. Tel^+ R-Cells possessing an integrated plasmid that was SS^+ (*pal*$^+$) or SS^- (*pal*$^-$) were grown in LB media at 30°C (no Tel production) to early log phase A_{600} = 0.2 , then were divided in two tubes and grown separately at 30°C or 42°C to reach to late log phase A_{600} = 0.8. Bacterial smears were then prepared on the slide and heat fixed and gram stained. Pictures of bacteria were taken at 1000X magnification. **A**. SS^- integrants at 30°C where *tel* expression is repressed; **B**. SS^- integrants at 42°C where *tel* is induced but the *pal* target site is absent; **C**. SS^+ integrants at 30°C where *pal* target site is present but *tel* expression is repressed; **D**. SS^+ integrants at 30°C where *tel* is induced and present to act on the chromosomally integrated *pal* target site.

2.4 Discussion

We demonstrate that mini DNA vectors in form of LCC DNA devoid of bacterial DNA sequences can be produced by using the bacteriophages N15 and PY54 derived telomerase TelN and Tel -mediated recombination in *E. coli* in high quantities. The product is a unique linear expression cassette with closed ends that was used to establish a more robust alternative nonviral vector expression cassette that could be used for gene therapy and vaccination. Application of pDNA in forms of naked nucleic acids, lipoplex, or polyplex for vaccination and therapy is based on pDNA vectors designed either to administer genes coding a therapeutic proteins, antigens, or antibodies into a given organism, or to introduce a correct gene into a host cell and replace the mal- or non-functional gene (*63*). Derived from conventional pDNA vectors, bacterial sequence-free mini DNA vectors provide a promising improvement over traditional vectors and are currently being tested in clinical trials (*90*). Conventional pDNA vectors are subdivided into a bacterial backbone and a gene of interest transcription unit. The GOI transcription unit usually carries the target gene or sequence along with necessary regulatory elements. The bacterial backbone includes the necessary elements for production and maintenance of plasmid in bacterial cells like an antibiotic resistance gene, an origin of replication, and unmethylated CpG motifs. However, the bacterial backbone of plasmid DNA vectors can cause serious biological safety problems. Bacterial sequence-free DNA vectors show better bioavailability due to their smaller size compared to conventional pDNA vectors, and higher immunocompatibily due to the absence of an unintended immune response against unmethylated CpG motifs. It was previously shown that the antibiotic resistance gene in the plasmid backbone which are used for selection of pDNA during bacterial growth is undesirable for administration to human body due to the adverse effects such as 1) the potential transfer of antibiotic resistance genes to normal microbial flora by endogenous horizontal gene transfer; and 2) the adverse effect of antibiotic resistant genes on the expression of the gene of interest under control of mammalian promoters (*111*). Also, it was shown that the immune response through toll-like receptors against unmethylated CpG dinucleotides present in bacterial backbone of plasmid vectors cause decrease or in some cases loss of transgene expression (*111*).This property makes bacterial sequence-free pDNA an excellent vector for nonviral gene therapy (*87*).

However, to date, production of mini vectors is being conducted *in vitro*, adding expense and complexity to the developmental process more particularly in industrial scale. Using the novel linearization methods described in this paper, for the first time, high quality bacterial sequence-free mini DNA vectors in both mini CCC and mini LCC topology can be produced and purified directly from *E. coli* using standard plasmid isolation methods without the need for digestion, ligation, and gel purification.

In order to optimize output we sought to engineer *E. coli* cells (R-cells) which chromosomally encode specific protelomerase under tight control of thermally-regulated promoter system based on phage λ CI[Ts]857 allele conditional repression of the *pL* and *pR* promoters to circumvents any potential toxicity, metabolic stress or recombinase interference that might arise from the use of a chemically inducible system. In this study we successfully optimized the recombinant telomerase expression applying the *pL* and *pR* promoters from the lambda phage. Below a temperature of 35°C, association with the *cI* repressor suppresses the activity of the promoter. Any temperature above 37°C, the repressor is inactivated and the promoters recover their activity to the maximum level at 42°C (*112*).

Conventional pDNA vectors are extra chromosomal circular, covalently closed, dsDNA molecules that often possess elements or sequences such as viral promoters or cloned coding sequences that could subject them to unwanted recombination events. A major safety concern associated with gene delivery strategies whether by viral or nonviral means is the possibility for integration of pDNA into the host chromosome, activating proto-oncogenes and/or deactivating tumor suppressor genes resulting in oncogenesis or silencing of adjacent genes. We show here for the first time that the frequency of viable integrants of LCC pDNA into the circular prokaryotic *E. coli* genome is reduced at least five orders of magnitude compared to the CCC counterparts. As such, LCC vectors present the promising option for targeted double cross over recombination events conferring site-specific gene replacement, where any unwanted recombinants arising from vector integration results in unviable products. We reasoned to investigate the extension of our prokaryotic results in human cells. We anticipate that chromosomal disruptions in eukaryote will result in even lower viability due to the separation of the telomere from its centromere, preventing propagation of the vector-integrated cell and its natural elimination from the tissue. By this mean, mini LCC vectors could be potentially the best vector for knock-in and gene replacement studies in stem cell and pluripotent stem cells for generation of transgenic plant and animal models, transgenic studies, and regenerative medicine avoiding damage and side effects of integration.

2.5 Conclusions

In this study we used the two bacteriophages λ and Φ80 *attB* sites to break apart and linearize the bacterial genome and we showed that the lethal effect of SS^+ disruption on λ *attB* is stronger than Φ80 *attB* site, which is more further to the origin of replication. In addition, microscopy showed dramatic change in cell size after Tel and TelN induced genome disruption, more particularly in case of SS integration into the λ *attB* site. Also the few surviving cells generate filamentous cells in part due to the unbalanced replication of a pair of chromosome arms with different lengths (*113*).

Our results in bacterial system agree with the previous study. By applying the TelN cleaving-joining reaction on its palindromic target site, which was inserted at different regions of the *E. coli* chromosome, Cui *et.al.* showed that the closer the linearization of the chromosome occurred to the *E. coli* origin of replication, the stronger the growth defect is observed relative to wild-type cells (*113*).

The results of this study would serve to significantly expand basic scientific knowledge with potential for future development of a novel technology that could drive the design of virtually limitless innovations with applications to health, agriculture and industry.

Chapter 3

Optimization of a one-step heat-inducible *in vivo* mini DNA vector production system

The temperature inducible expression systems utilized in our recombinase production system relies on the bacteriophage λ strong *pL* and/or *pR* promoters regulated by the thermolabile CI[Ts]857 repressor. This temperature-sensitive expression system has been widely used to produce recombinant proteins in prokaryotic cells. The expression system was also used to express recombinant bacteriophages PY54, N15, and P1-derived recombinase enzymes Tel, TelN, and Cre, respectively, by increasing the culture temperature, generally above 37°C. The increase in temperature along with the over expression of recombinant enzymes lead to different intercellular stress responses and enhanced pDNA replication rate. In this study, we sought to optimize our novel one-step *in vivo* mini DNA vector production system by process engineering and genetics. We assayed outcomes under varied temperature schedules and *via* various genetic modifications affecting pDNA vector replication and processing rates, and cellular heat stress responses.

3.1 Introduction

We have previously described an *in vivo* mini DNA vector production system, whereby vectors are the result of an *in vivo* site-specific recombination process. The parental plasmid (PP) carries the GOI expression cassette flanked by two recognition sites of a site-specific recombinase and the clinically undesirable bacterial sequences that are necessary for the maintenance and propagation of plasmids in bacterial cells. The *in vivo* expression of the respective recombinase results in the excision of the recognition site, dividing the PP into two smaller molecules: 1) a replicative mini plasmid carrying the unwanted bacterial backbone sequences, and 2) a mini DNA vector carrying the therapeutic expression unit (Figure 17).

The recombinases exploited to generate bacterial-sequence-free DNA vectors in the forms of supercoiled DNA or minicircle were derived either from the tyrosine family of recombinases including, but not limited to, λ integrase (Int), P1 derived Cre and Flp from the yeast plasmid 2-μm circle; or the serine family such as the highly efficient *Streptomyces* bacteriophage ΦC31-derived integrase (*114*, *115*). We previously showed (Chapter 2) that the phage N15 and PY54-derived recombination systems also separate the therapeutic expression unit from the PP DNA backbone, but in forms of linear DNA with closed ends, which we called ministring DNA vectors (*10*). The benefits of LCC DNA vectors were reviewed in Chapter 1.

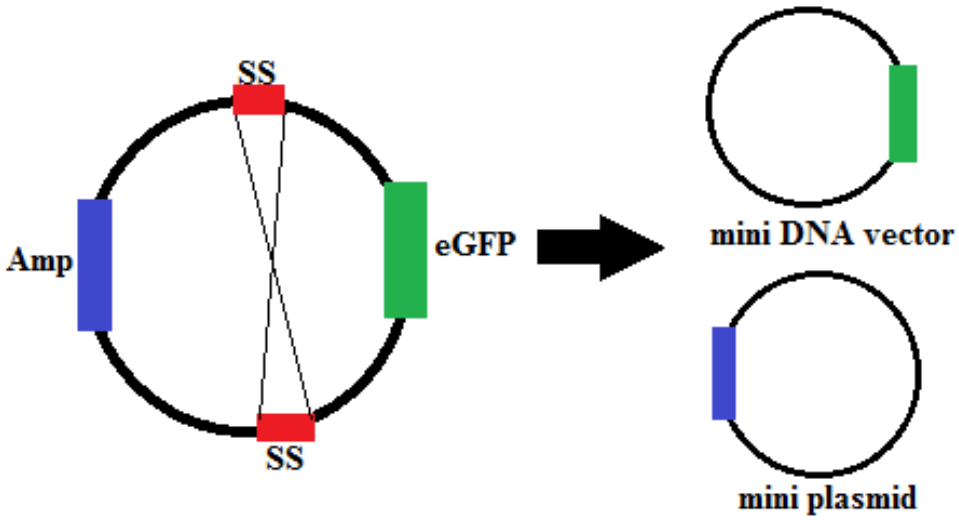


Figure 17- Generation of mini DNA vector by site-specific recombination.

Mini DNA vectors are generated by site-specific recombination *via* two excision sequences (red boxes), placed flanking the mammalian expression cassette (eGFP) and the bacterial amplification unit (Amp) in a recombinant plasmid vector. Arrows indicate the recombination reaction.

In this study, the strong λ *pR* promoter, governed by the temperature-sensitive CI857 repressor, was employed to yield an inducible expression system whereby, high level expression of the TelN, Tel, or Cre recombinase occurs under induced conditions, but expression is prevented, or minimal, under repressed conditions. A high ratio of recombinase to pDNA vector (each carrying two recombinase target sites) is necessary to yield high rates of plasmid processing. Our approach to integrate a single copy of the telomerase gene under control of the λ expression system into *E. coli* chromosome produced an effective, but imperfect plasmid processing efficiency (70%) which was in agreement with similar studies that obtained 50–90% DNA minicircle recovery at maximum according to agarose gel electrophoresis (AGE).

The *E. coli* K-12 host was selected for this study due to its widespread application, relative ease of modification and management, well studied use in the production of a wide breadth of recombinant proteins, and cost-effective scalability. Additional factors favoring the selection of *E. coli* as the expression host of choice were the availability of a variety of strong inducible promoters and its well-recognized suitability for the maintenance and propagation of various pDNA vectors (*116, 117*). Constitutive expression of a recombinant protein in the bacterial host results in high stress levels on cells because the metabolic load of the recombinant protein production is constant while the source of nutritional factors and energy are limited,

which in turn negatively impacts the growth rate of the host cell (*118, 119*). In many situations, the best conditions for cell growth are different from those for recombinant protein production. As such, stringent control of expression of the selected recombinase is needed to impart an efficient recombination process. First off, the expression system must efficiently repress recombinase expression prior to induction to avoid premature plasmid processing that would reduce plasmid propagation and hence yield. Most promoters commonly employed for heterologous protein expression, require the addition of an inducer molecule, the depletion or addition of a nutrient, or a shift in a physical or physicochemical factor, such as pH (*118*). In theory and in small laboratory settings, each of these options is practical and can promote efficient control. However, in large-scale settings requiring industrial level production of the final product, each option may present processing disadvantages. For example, chemical inducers, such as IPTG, are expensive and introduce significant processing costs, can also be toxic at industrial level quantities, and their presence either in final or in waste product can cause environmental hazards and added costs for suitable containment and elimination (*120, 121*). In addition, additional controls and downstream operations may be required to remove chemical inducers, particularly from pharmaceutical-grade products intended for clinical application, which does not only complicates the bioprocess, but also dramatically increases production costs (*122*). High level protein production may also impart nutrient fatigue, whereby exhaustion of an amino acid from the culture broth results in starvation, compromising cell metabolism and synthesis of the recombinant enzyme, and/or plasmid replication rate (*123*). This drawback is exacerbated by the challenge that precise control of the induction time in large scale cultures can be very difficult. At present, not many pH-inducible expression systems are available for exploitation, characterization studies are still insufficient, and pH-mediated induction of gene expression could interfere with the optimal pH for protein folding, function, and physiological conditions (*124*). In consideration of these system limitations, a thermo-regulated expression system was used to express phage-derived recombinase enzymes and produce mini DNA vectors.

Thermo-regulated expression systems circumvent many of the drawbacks aforementioned including the use of special media, toxicity or expensive chemical inducers. On the other hand, there are advantages associated with thermo-regulated expression system including the relatively ease of handling, minimized contamination risks, and scalability. Our system of choice employs the bacteriophage λ strong *pL* and *pR* promoters, regulated by the thermolabile λ CI[Ts]857 repressor that has been extensively exploited for the regulated production of many recombinant proteins and peptides (*125*). This system is based on inserting the GOI into different vectors that contain the strong major leftward (*pL*) and/or rightward (*pR*) promoters. The gene cloned downstream of the λ promoter(s) are efficiently regulated by CI857, with an increasing expression profile as temperature rises from 30^{o} to 40^{o} C (*126*). Thus, gene expression is inhibited at culture temperature below 35°C (normally in the range of 28-32°C for full suppression), whereas optimal repressor inhibition occurs at 37^{o}C, and completely abrogated over 37^{o}C, permitting efficient transcription of the GOI by host

RNA polymerase above 37°C with the maximum level at 42°C (*127, 128*). However, temperatures of 40°C and above that confer full depression and high level gene expression, also yield an adverse host metabolic environment for recombinant protein production. Heat induction triggers the heat-shock response (HSR) that is controlled by the alternative heat shock sigma factor, σ^{32} (*rpoH* gene product) (*129, 130*). The σ^{32} factor alters the expression of different genes, including transcription factors, by delivering RNA polymerase to gene promoters that are part of the heat shock regulon; it regulates the activity of the transcriptional apparatus itself and executes various roles in cell homeostasis (*131*). The σ^{32} governed heat shock regulon is formed by a large family of genes, particularly those encoding proteins involved in protein folding and degradation. These include chaperones such as ClpB, DnaK/J and GroEL/S and proteases such as Lon, ClpP, ClpC, HsIV (ClpY) HsIU, ClpQ, and FtsH (*132*). The heat shock response (HSR) includes a rapid and selective synthesis of heat-shock proteins (HSPs) soon after heat induction. This is followed by an adaptation period characterized by a lower rate of protein synthesis that eventually reaches a new steady-state level. The HSPs and proteases are involved in folding, degradation, and proper feedback regulation of the HSR (*130, 133*). In addition to the synthesis of HSPs following heat shock, the physiological response of *E. coli* includes the temporary decrease in growth rate, which directly impacts the replication and segregation of plasmid DNA. Furthermore, heat shock does not only induce genes encoding HSPs such as DnaK, DnaJ, ClpP, and FtsH; but also activates the genes coding for proteases such as the *ompT* and *ftsH* genes as well as *recA* gene that is involved in recombination (*134*).

Among many different variables that may affect the heat-inducible systems, we sought to improve plasmid copy number and mini DNA vector production efficiency *via* our *in vivo* λ CI857/*pL*/*pR* system, by optimizing bacterial growth media composition, host cell genotype, and induction scheduling.

3.2 Materials and methods

3.2.1 Strains and plasmids

E. coli K-12 strains were used to generate all recombinant cell constructs and JM109 were particularly employed as hosts for plasmid constructions and amplification. A list of bacterial strains and plasmids used in this study are shown in Table 7.

Table 7- Strains and plasmids

Strain	Genotype [1]	Source
Bacteria		
JM109	F', Δ*(gpt-lac)0*, *glnV44*(AS), λ⁻, *rfbC1*, *gyrA96*(NalR), *recA1*, *endA1*, *spoT1*?, *thi-1*, *hsdR17*, pWM5, F128-x	New England Biolabs
W3110	F-, λ⁻, *IN(rrnD-rrnE)1*, *rph-1*	CGSC # 4474;(*107*)
W3110-Cre (W1NN)	F-, λ⁻, *IN(rrnD-rrnE)1*, *rph-1 lacZ::Cm-cI857-cre*	(*10*)
W3110-TelN (W2NN)	F-, λ⁻, *IN(rrnD-rrnE)1*, *rph-1 lacZ::Cm-cI857-telN*	(*10*)
W3110-Tel (W3NN)	F-, λ⁻, *IN(rrnD-rrnE)1*, *rph-1 lacZ::Cm-cI857-tel*	(*10*)
Plasmids		
pBRINT	*lacZ::cat-MCS::lacZ* (Cm^R)	NBRP; (*11*)
pGL2	*SV40P-Luc-PolyA-SV40 intron*	Promega
pUC57-SS	*Multi-target site "SS"* (Ap^R)	GeneScript
pcDNA5/FRT	*Flp-In Integrating vector frt-hyg-pCMV::MCS-BGHpA* (Ap^R Hyg^R)	Invitrogen
pNN7	*pGL2-egfp switched for luc*	(*10*)
pNN8	*pNN7 + SS (upstream of SV40 promoter)*	(*10*)
pNN9	*pNN8-SS (2XSS) (second SS downstream of SV40 polyA sequence)*	(*10*)
pNN12	*pBRINT* (Cm^R) *(SS^+)*	This study
pNN13	*pcDNA5/FRT (SS^+)*	This study

[1] sequences of interest confirmed by PCR and/or sequencing

3.2.2 Construction of Recombinant cells (R-cells)

W3110 was used to generate R cells representing the *in vivo* platform for production of bacterial sequence depleted DNA vectors in the two forms of isogenic CCC DNA minicircles and mini LCC (DNA ministring) vectors. These cells were employed for heat-inducible *in vivo* protelomerase expression as previously described in Chapter 2.

3.2.3 Construction and linearization of high and medium copy pDNA vectors in R-cells

The multi-purpose target site, named SS was designed as previously explained in Chapter 2. The SS fragment was synthesized by the GeneScript and cloned into the pUC57 by EcoRI and HindIII. To construct modified DNA vectors, the SS fragment was moved from pUC57 into the MCS of pBRINT (Cm^R) vector (National Bioresource Project; NBRP) by BamHI and EcoRI to generate pNN12 and moved from pNN12 by BamHI and XhoI into MCS of the high copy pcDNA5/FRT vector (Invitrogen) to produce pNN13 (5.35 kb). The medium copy plasmid vector pNN8 (5.3 kb) was constructed as previously described in Chapter 2. New constructs were tested and confirmed by colony PCR and analytical digestion.

W3NN (F-, λ^-, *IN (rrnD-rrnE)1, rph-1 lacZ::Cm-cI857-tel)* cells (*10*) were transformed with 0.1 μg of pNN8 and pNN13 DNA constructs and selected on LB agar + Ap (50 μg/ml). A single colony was grown overnight in 5 ml LB + Ap (50 μg/ml) in repressed telomerase conditions at 30°C with aeration. 1 : 100 dilution of fresh cells were prepared in 10 ml and grown in a 50 ml flask at 30°C with aeration to mid log phase A_{600} = 0.6. To induce recombinase expression and plasmid conformational conversion, transformed R-cells were heat shocked to induce the recombinase expression at 42°C for 30 min at mid logarithmic phase of bacterial growth, before being transferred to 30°C overnight. Cells were then harvested and plasmid extracted (Omega kit, VWR). Plasmid topology was assayed by agarose gel electrophoresis (AGE) and digestion by NdeI. Standard recombinant DNA cloning and transformation techniques were performed as described by (*135*). Qualitative analysis was performed by ethidium bromide (EtBr) staining and AGE.

3.2.4 Assessing effects of volume on the processing and production of mini DNA vectors

W3NN (F-, λ^-, *IN (rrnD-rrnE)1, rph-1 lacZ::Cm-cI857-tel)* cells were transformed by 1 μg of pNN9 DNA constructs (Table 3) and selected on LB agar + Ap (50 μg/ml) described previously in Chapter 2. A single colony was grown overnight in 5 ml LB+ Ap (50 μg/ml) in repressed telomerase conditions at 30°C with aeration. 1 : 100 dilution of fresh cells were prepared in 10 ml and started growing in a 250 ml flask at 30°C with aeration to late-logarithmic phase A_{600} = 0.8. To induce recombinase expression and plasmid conformational conversion, cells were heat induced at 42°C for 15 min and transferred to 30°C. Cells were next diluted 1 : 5 by addition of 40 ml fresh media and grown at 30°C with aeration to A_{600} = 0.8 and heat induced at 42°C for 15 min and transferred to 200 ml fresh media (1 : 5 dilution) at 30°C in a 1-liter flask following the same growth/induction pathway, and then transferred into 750 ml fresh media in a 2-liter flask

to a final volume of 1 liter. Cells were then grown at 30°C with aeration to late log phase and heat induced at 42°C for 30 min before placing them in ice for 15 min. Next, cells were harvested and DNA vector was extracted using Endotoxin-free Maxi prep kit (Omega kit, VWR). The plasmid topology was assayed by AGE and digestion analysis. Qualitative analysis was performed by ethidium bromide (EtBr) staining and AGE.

3.2.5 Assessing the effects of heat induction variations on the processing and production of mini DNA vectors

W3NN (F-, λ^-, *IN (rrnD-rrnE)1, rph-1 lacZ::Cm-cI857-tel)* cells were transformed by 1 μg of pNN9 DNA and selected on LB agar + Ap (50 μg/ml). A single colony was grown overnight in 5 ml LB + Ap (50 μg/ml) at 30^o C with aeration under repressed recombinase expression conditions. A 100 ml 1 : 100 dilution of fresh cells was grown with aeration in a 500 ml flask at 30°C to mid log phase A_{600}= 0.6. Cells were then divided into two groups (A and B) and each group was further divided into five subgroups (I, II, III, IV, and V) into 10 flasks, each with 10 ml of the culture. Flasks A-I and B-I were heat shocked for 5 min, A-II and B-II for 15 min, A-III and B-III for 30 min, A-IV and B-IV for 45 min, and A-V B-V for 60 min. After heat induction, the flasks in group A were shifted to 37°C for 4 h, while the flasks in group B were shifted to 30°C for 4 h. Cells were then harvested and DNA vector extracted with Endotoxin-free Maxi prep kit (Omega, VWR). Resultant plasmid topologies were then analyzed by AGE and restriction analysis.

In a follow-up experiment, W3NN[pNN9] cells were exposed to fast or slow temperature shifts to induce recombinant recombinase expression in R-cells, by growing 1 : 100 dilutions of fresh cells in 20 ml LB broth (250 ml flask) at 30°C with aeration to late log phase A_{600}= 0.8. Cells were then divided into two flasks, each with 10 ml of the culture. The first flask (A) was gradually incremented to 42°C, while the other flask (B) was rapidly shifted to 42°C. After 30 min, flask A was gradually reduced back to 30°C, while flask B was quickly shifted back to 30°C. Cells in both flasks were grown to stationary phase A_{600}= 1.0. Cells were then harvested and DNA vector was extracted by the miniprep kit (Omega, VWR). DNA vector sizes and topologies were determined by AGE and restriction digest analysis. Qualitative analysis was performed by ethidium bromide (EtBr) staining and AGE.

3.2.6 Assessing the effects of bacterial culture media and bacterial growth rate on the processing and production of mini DNA vectors

Fresh overnight W3NN [pNN9] cells were diluted 100-fold in 10 ml of minimal M9 media (1X M9 salt composed of mixed solution of the $(NH_4)_6Mo_7O_{24,}$ H_3BO_3, $CaCl_2$, 1 mM $MgSO_4$, 1 μM $CaCl_{2,}$ 0.2% Carbon source, pH 7.0), or enriched LB media (1.0% Tryptone, 0.5% Yeast Extract, 1.0% NaCl, pH 7.0). Cells were grown in a 50 ml flask at 30°C with aeration to reach corresponding phase of bacterial growth A_{600}= 0.2, 0.4, or 0.8. To induce recombinase expression and plasmid processing, R-cells were heat induced at 42°C for 30

min then transferred to 30°C and grown overnight. Cells were then harvested and DNA vectors extracted by the miniprep kit (Omega, VWR). Plasmid topology was assessed by AGE and restriction analysis.

In a follow-up experiment, W3NN [pNN9] cells were exposed to ciprofloxacin to synchronize plasmid replication rate with telomerase expression. A single colony was grown overnight in 5 ml LB + Ap (50 μg/ml) at 30°C under repressed conditions. Cells were diluted 100 fold into 40 ml of fresh LB + Ap in a 250 ml flask and grown with aeration at 30°C to late log phase A_{600}= 0.8. Cells were then shifted to 42°C for 30 min to induce telomerase expression. The culture was then divided into four flasks, each with 10 ml of the culture but a different concentration of ciprofloxacin (0, 0.1, 1, and 10 μg/ml). Cells were grown with aeration at 30°C overnight. Cells were then harvested and plasmid extracted by the miniprep kit (Omega, VWR). DNA vector sizes and topologies were determined by AGE and restriction digest analysis. Qualitative analysis was performed by ethidium bromide (EtBr) staining and AGE.

3.2.7 Qualitative analysis of protelomerase mediated production of mini DNA vector

All DNA ministring production efficiencies were calculated qualitatively as a comparison between the total amounts of the DNA ministring over the total amount of parent pDNA by EtBr staining and AGE.

3.3 Results

3.3.1 Plasmid copy number influences efficiency of parent plasmid processing to LCC DNA vector

The mini DNA vector production system involved thermo-regulated protelomerase expression accompanied by subsequent protelomerase mediated processing of parental CCC pDNA into LCC DNA ministrings. The first step in optimizing this process was to examine the effect of parental pDNA copy number on the productivity of the CI857/*pL*/*pR* system in generating DNA ministrings. To do so, the high copy pNN13 vector (500-700 copies per cell) and medium copy pNN8 vector (30-100 copies per cell) were assessed for plasmid processing following protelomerase induction. Following pDNA extraction, restriction analysis was employed to assess DNA ministring production efficiency from parental pDNA. Qualitative analysis was performed by ethidium bromide (EtBr) staining and agarose gel electrophoresis (AGE). In all cases, following heat induction of the R-cell recombinase, the medium copy number plasmids carrying the target SS exhibited a remarkably improved LCC conversion efficiency from the parent plasmid. Interestingly, the mid copy plasmid exhibited an improved efficiency of LCC DNA production relative to the high copy number pDNA vectors.

3.3.2 A fed-batch growth approach improves LCC mini DNA vector production efficiency

We attempted to generate/mimic the process of a fed-batch system on a small scale to assess the influence of bacterial growth systems on mini DNA vector processing and production. Tel^+ R-cells were transformed by the mid copy (pNN9) parent plasmid (Table 7). The fed-batch system was generated by growing transformed cells under repressed conditions at 30°C to late log phase of bacterial growth before inducing cells at 42°C to express recombinant protelomerase briefly for 15 min, and shift it back to 30°C. This heating schedule was tightly controlled while fresh media continued to be added after each induction at 42°C for a total of five induction events to increase the culture volume to 1 L. This process supported the plasmid stability, where cells were initially grown at low temperature ensured an "off" recombinant enzyme production state to confer high plasmid stability and cell density. By controlling heat induction and replenishing fresh media during exponential growth phase, the efficiency of DNA ministring vector production was dramatically enhanced (Figure 18).

3.3.3 Heat induction schedules strongly influence the production efficiency of DNA ministring vectors

A variety of heating strategies were assessed to examine the effect of induction times and maximum parent plasmid processing to DNA ministrings (mini LCC) by minimizing adverse effects of high temperature on growth rate, cell damage, decreased viability and productivity, and plasmid instability. In so doing, a single colony of W3NN [pNN9] was grown overnight in 5 ml LB + Ap (100 µg/ml) under repressed conditions at 30° C with aeration. Fresh cells were grown from the overnight culture at 1:100 dilution at 30°C with aeration to late log phase A_{600} = 0.8. Protelomerase expression was induced upon temperature up-shift to 42°C for various timed durations (5, 15, 30, 60 min). After induction, the cultures were subjected to various post-induction temperatures either at 30 or 37°C (either fully repressed or partially repressed conditions, respectively) for 4h prior to overnight incubation at 30°C.

We found that Tel-processing of parent plasmid in the production of DNA ministrings was highest when heat induction at 42°C scheduled for 60 min following post-induction state at fully repressed conditions (30°C) (Figure 19). Interestingly, partial repression and post-induction temperature at 37°C was not as effective as exposing cells to post-induction tempratures at 30°C, and shorter induction periods of 15 or 30 min did not provide adequate protelomerase level regardless of the subsequent resting temperatures. As expected, heat shock induction at 42°C above 90 min would result in loss of protelomerase acitivity and eventual plasmid DNA instability. Furthuremore, the uninduced control conferred no observable mini LCC vector production.

In a follow-up experiment, we exposed cells to fast or slow temperature shift to induce recombinant telomerase expression in order to assess the productivity of our system during a temperature change. We

found that the R-cells that have been slowly exposed to temperature shift from 30°C to 42°C and back to 30°C showed a higher mini DNA vector production compared to the R-cells that were quickly exposed to temperature shift. This suggests that cells subjected to a graduale temperature shift to 42°C may adapt to thermal stresses better than those exposed to a fast temperature increase (Figure 20).

3.3.4 Bacterial culture media and bacterial growth rate strongly influence the production efficiency of DNA ministring vectors

We attempted to synchronize plasmid replication rate with telomerase expression to enhance the efficiency of DNA ministring production in our one-step *in vivo* system. To do so, a variety of culture media, heating induction at different bacterial growth stages, and effect of ciprofloxacin were assessed. Tel+ R-cells were transformed by the mid copy (pNN9) parent plasmid (Table 7) and grown at two different rich and simple media. Telomerase expression was induced at different growth stages. We found that Tel/ palprocessing of parent plasmid carring the SS site into bacterial sequence depleted DNA ministring vectors was highest by growing cells in an enriched culture media and induce recombinase expression when cells are at late log phase once the highest number of cells and plasmids and enough source of nutritional factors and energy were available (Figure 21). Interestingly, growing and inducing heat on transformed cells in minimal M9 media was not as effective and did not provide adequate source of energy to plasmid replication and recombinase induction regardless of the subsequent bacterial growth stages. Even though additional carbon source was added prior to recombinase induction, the simple M9 media did not provide adequate nutritional factors to cells to grow to late log phase and could not support processing of parent plasmid into DNA ministring vectors.

In a follow-up test, we evaluated different concentrations of ciprofloxacin after recombinase induction to arrest DNA replication, thereby maximizing telomerase processing and DNA ministring vectors production. However, there was not a significant difference in efficiency of *in vivo* processing when ciprofloxacin was added (Figure 22).

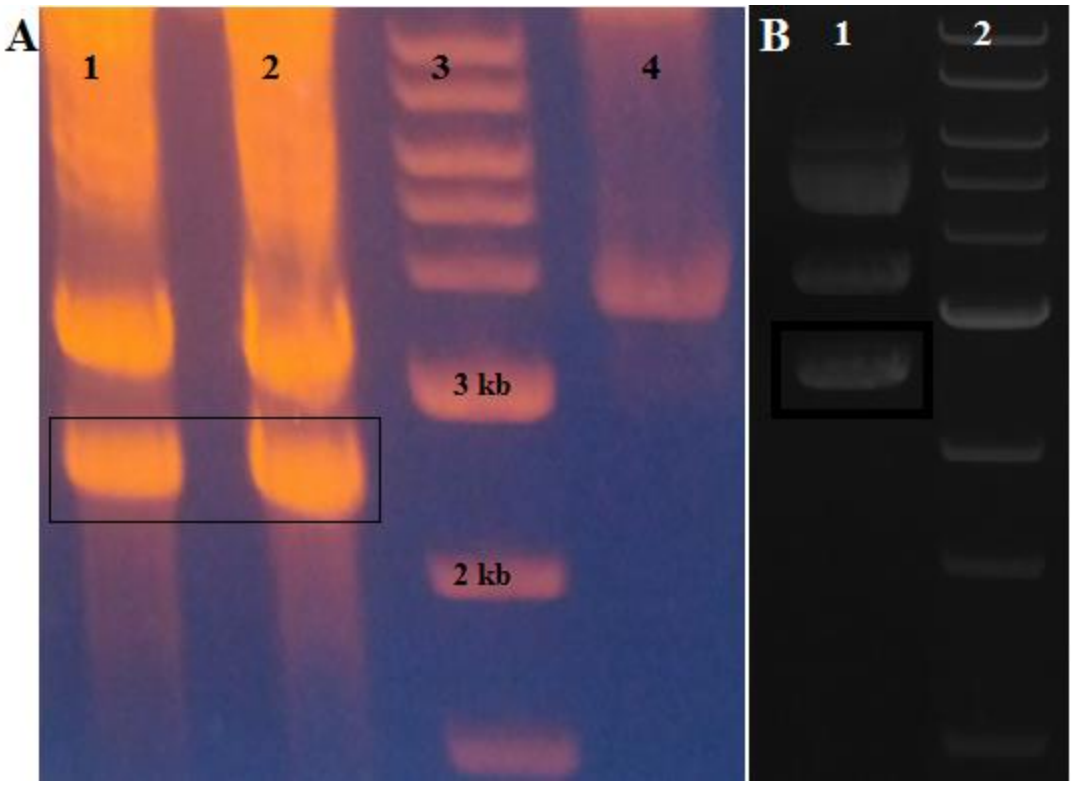


Figure 18- Influence of bacterial growth system on DNA ministring vector production.

A) Fed-batch-like bacterial growth system. Medium copy pNN9 vector possessing two SS site were transformed into *tel* expressing W3NN cells. Following heat induction at 42°C, fresh media was added to each batch; this cycle was repeated for five times until culture volume increased from 10 ml to 1 L, yielding 500 ug of total DNA (50 ug of total DNA per lane). From left, **L1**-**L2**) fed-batch induced cells, DNA ministring vectors are separated from parental pDNA (black box), **L3**)1 kb ladder, **L4**) uninduced control. **B)** Batch control. **L1**) processed DNA vectors extracted from 50 ml batch culture. DNA ministring derived from parent plasmid after heat induction (black box) and **L2**) 1 kb DNA ladder.

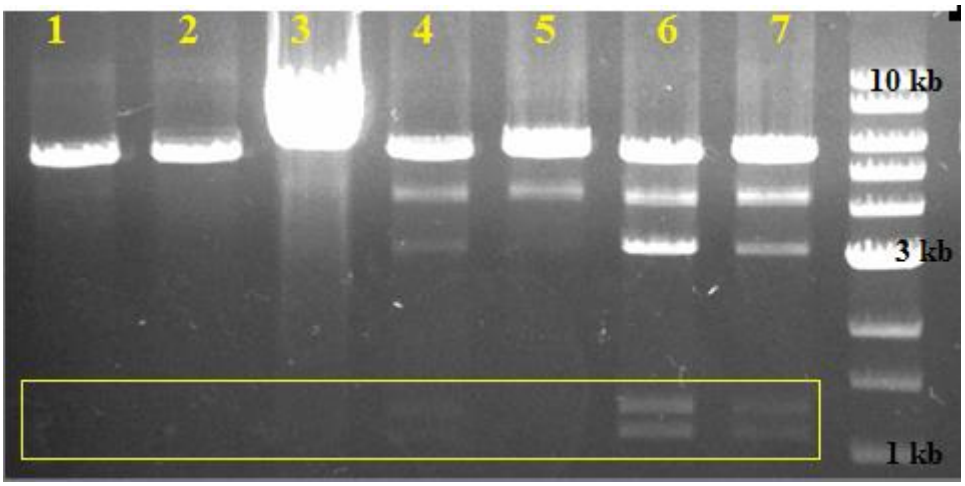


Figure 19- Effect of duration of heat induction in CI857/*pL*/*pR* system on *tel* expression and activity.

Medium copy pNN9 vector possessing two SS site were transformed into *tel* expressing W3NN cells. Following heat induction at 42°C for a series of induction durations, cells were exposed to either 30°C (fully repressed) or 37°C (partially repressed) post induction tempratures. DNA vectors were extracted from cells and digested by a single cutter restriction enzyme. An equal volume of extracted DNA was analyzed by AGE and EtBr staining. The larger single band (5.6 kb) represents residual circular parent plasmid; while the medium band (3.2 kb) represent the LCC mini plasmid and the two smaller bands (yellow box) represent successfully processed LCC mini DNA vector. **L1**) uninduced control; **L2)** 15 min induction, back to 30°C; **L3)** 15 min induction back to 37°C (overloaded) ; **L4)** 30 min induction back to 30°C; **L5)** 30 min induction back to 37°C; **L6)** 60 min induction back to 30°C; **L7)** 60 min induction back to 37°C; and **L8)** 1 kb DNA ladder.

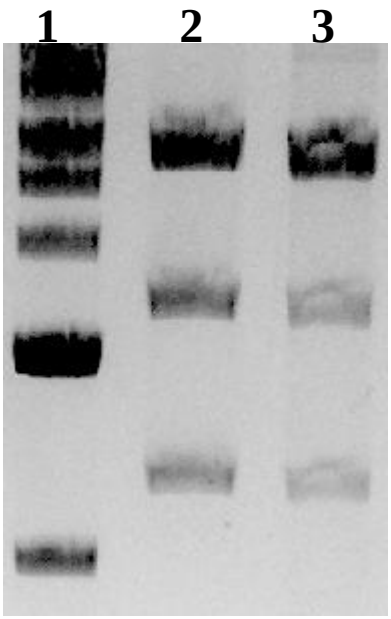


Figure 20- Effect of fast or slow temperature shift in CI857/*pL*/*pR* system on *tel* expression and activity.

Medium copy pNN9 vector possessing two SS site were transformed into *tel* expressing W3NN cells. Cells were grown to late log phase and exposed to fast or slow temperature shift from 30°C to 42°C for 30 min to induce recombinant telomerase expression following fast or slow temperature shift back from 42°C to fully repressed conditions at 30°C. DNA vectors were extracted from cells and an equal number of extracted DNA was analyzed by AGE and EtBr staining. The larger single band (5.6 kb) represents residual circular parent plasmid, while the medium bands represent the processed LCC mini plasmid and LCC mini DNA vector. From left **L1**) 1 kb DNA ladder; **L2)** gradually shifted; **L3)** rapidly shifted.

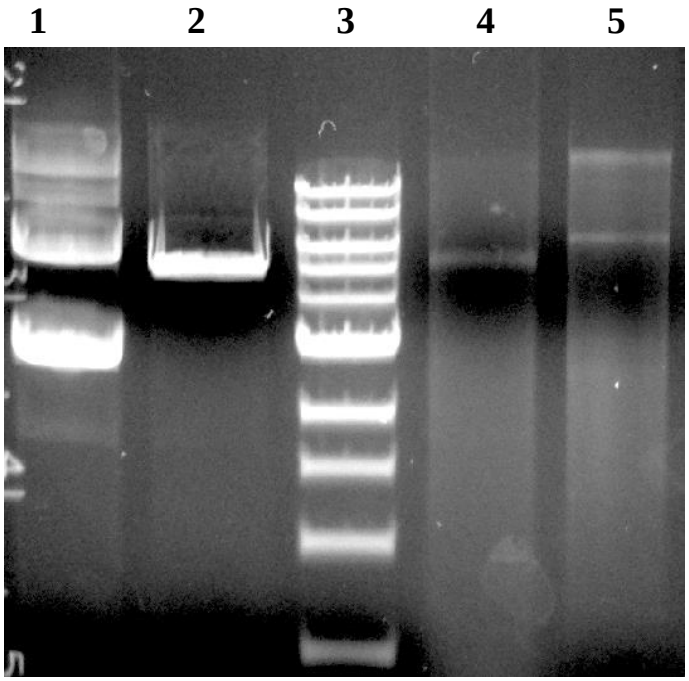


Figure 21- Effect of culture media on cell growth and plasmid processing in CI857/*pL*/*pR* system.

Medium copy pNN9 vector possessing two SS site were transformed into *tel* expressing W3NN cells and grown at two different rich (LB) and simple (M9) media. DNA vectors were extracted from cells and digested by a single cutter restriction enzyme. An equal volume of extracted DNA was analyzed by AGE and EtBr staining. From left **L1**) uninduced and undigested pNN9 extracted from LB; **L2)** uninduced and digested pNN9 extracted from LB; **L3)** 1 kb DNA ladder; **L4)** uninduced and digested pNN9 extracted from M9; **L5)** uninduced and undigested pNN9 extracted from M9.

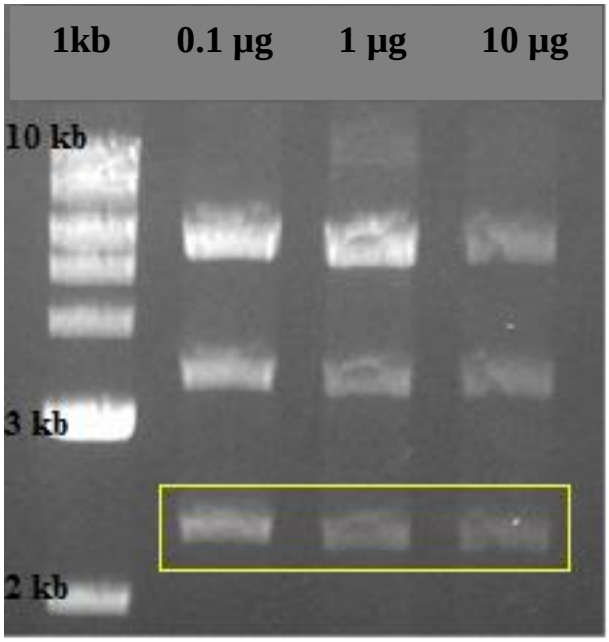


Figure 22- Influence of Ciprofloxacin on DNA ministring vector production.

Medium copy pNN9 vector possessing two SS site were transformed into *tel* expressing W3NN cells. Following heat induction at 42°C at late log phase in LB media, cells were returned to fully repressed (30°C) conditions and different concentrations of ciprofloxacin (0.1, 1, and 10 µg/ml) were added. DNA vectors were extracted at stationary phase. An equal volume of extracted DNA was analyzed by AGE and EtBr staining. The larger single band (5.6 kb) represents residual circular parent plasmid; while the medium band (3.2 kb) represent the LCC mini plasmid and the smaller bands (yellow box) represent successfully processed LCC mini DNA vector.

3.4 Discussion

In this study, we examined how an increase in temperature can affect certain variables including the bacterial growth rate, duration of induction, effect of culture media formula, and the temperature after induction in order to synchronize the recombinase expression and pDNA vector replication to maximize the efficiency of our temperature-regulated mini DNA vector production system.

As mentioned earlier, site-specific recombination is the technique used for production of mini DNA vectors. However, in case of the well-known Cre/*loxP* and Flp/*frt* recombination systems for generating minicircle DNA vectors, it was shown that the genetic crossover between the recombination sites regenerates a site of identical or highly similar sequences and thus the mediated recombination event is bidirectional and fully reversible, giving rise to intramolecular and intermolecular recombination events, which leads to production of unwanted dimeric and trimeric minicircles, residual parent plasmid, mini plasmids and mixed concatemers. Even one mutated recombination site, which favors unidirectional recombination, does not eliminate the concatemers (*136*). Bacteriophage Φ31 derived integrase belongs to the large serine recombinase family, which provides the only strict unidirectional recombination event between an *att*P and an *att*B site and the reverse reaction is not active in *E. coli* (*36*).

Likewise, the broad host range plasmids RK2 and RP4 derived ParA resolvase belongs to the small serine recombinase family directs the recombination event only in one direction and only mediates intramolecular and not intermolecular recombination events between the corresponding resolution sites and thus is not able to mediate reverse events. As a conclusion, DNA minicircle production can be driven to a high percentage without generation of multimers or other concatemers applying Φ31 integrase and ParA resolvase.

We previously reported application of bacteriophages N15 and PY54 recombination systems to *in vivo* production of bacterial-sequence-free LCC DNA vectors, which we called DNA ministrings (*10*). We inserted single copy of the recombinase gene into the *E. coli* genome to avoid some of the drawbacks of overproduction and episomal expression of the recombinant enzyme and resultant imperative stresses, metabolic unbalances, and/or accumulation of recombinant recombinase in inclusion bodies post induction (*119*). During prolonged periods of heat induction (>40ºC), by using CI857/*pL*/*pR* systems, the propagation of plasmid-free cells is favored since plasmid partitioning and stable plasmid inheritance at high temperature is not fully functional and about 50% of the dividing cells lose the plasmid expressing recombinant protein as the temperature shifts to 42°C. However, this complication can be alleviated by incorporating selective antibiotics into the growth media (*125*) or inserting recombinant protein gene and its *cI857*/*pL*/*pR* regulatory cassette into bacterial genome. Hence, in large-scale production systems, in which no IPTG or other selective chemicals are preferred, the latter approach is favored. Furthermore, maintenance and replication of the recombinant plasmid overloads the host metabolic pathways and consumes important cellular resources.

The essential resources of energy required for replication of recombinant plasmids in heterolougous enviromet such as pentose phosphate (PP) pathway effects the generation of NADPH and amino acids necessary for host cell methabolic pathways. Such metabolic burden and energetic drain is maximized by the induction of the heterologous enzyme which leads to the overconsumption of nucleotides and amino acids in order to provide necessary material for synthesis of the recombinant protein and cause several changes in the metabolism of the host cell (*118*). This changes would in particular effect the tricarboxylic acids (TCA) pathway and consequently and imbalanced central carbon metabolism. Therefore, the imbalanced methabolic pathways in host cells carrying recombinant plasmid and expressing heterolougous enzyme would result in biosynthesis stress, impaired cell growth and protein synthesis, and accumulation of undesired by-products. These changes would induce the proteolysis of proteins, loss of plasmid, or in some conditions cell death (*137*). We found that pDNA vectors with 20-100 copy per cells were processed in to mini DNA vectors by the cleaving-joining activity of recombinant protelomerase with more efficacy compared to their high copy counterparts. We could dramatically increase the productivity of our system to yield higher quantity of mini DNA vectors *via* using a fed-batch-like production system by tight control over heating induction cycles and constant fresh media supplementation. Our results are in agreement with previous studies that generated recombinant interferon and T4 DNA ligase in *E. coli* cells using heat induction in a fed-batch system and offered significantly improved production compared to a batch system (*138*, *139*). Indeed, fed-batch systems and two continuous cultures connected in series, offer the advantage of linearly or exponentially growing rate to maximize cell concentration and expression of the recombinant enzyme while avoiding loss of plasmid (*140*).

Induction temperature, duration of induction, and the specific growth rate before or after induction are valuable factors that can be controlled and optimized to improve the productivity of thermal inducible systems (*141*). We could optimize our system to maximize the Tel-processing of parent plasmid into DNA ministring vectors in our novel *in vivo* temperature-regulated mini DNA vector production system. Unlike the previous study, which obtained recombinant protein degradation when the induction phase at 42°C lasted 40 min (*142*), we showed that Tel-processing of parent plasmid in production of ministrings was highest in enriched culture media using a schedule whereby cells grow at repressed recombinase state to late log phase and induced for 60 min at 42°C before returning to fully repressed conditions at 30°C.

Chapter 4

DNA ministrings: superior gene delivery vectors

The pDNA vector is the current conventional technology driving therapeutic gene transfer, whether for use toward malfunctioned/nonfunctional gene replacement, DNA vaccination, or production of recombinant proteins in mammalian cells. The conventional pDNA vector suffers from several safety and efficiency limitations: 1) it imparts adverse immune responses to bacterial sequences required for maintenance and amplification in prokaryotes; 2) its bioavailability can be compromised due to size; and 3) it may be genotoxic due to its potential to integrate into the host chromosome and yield an oncogenic event. We have constructed an *in vivo* platform for the production of mini linear DNA vectors with covalently closed ends (LCC DNA), called DNA ministrings. DNA ministrings are devoid of unwanted bacterial sequences, encoding only the gene(s) of interest and necessary complementary eukaryotic expression/enhancement genetic elements. Transfection of DNA ministring vectors encoding the enhanced Green Fluorescent Protein gene (eGFP) into rapidly dividing and slow dividing cells results in significantly higher transfection efficiency, bioavailability, and cytoplasmic kinetics compared to the parental plasmid precursor and isogenic CCC DNA minicircle counterparts. As previously noted in prokaryotes, the integration of LCC DNA into the mammalian host genome will result in chromosomal disruption and subsequent apoptotic elimination of potentially oncogenic vector integrants from the cell population, thus improving the safety profile of ministrings.

DNA ministring (mini LCC) vectors are significantly safer and more efficient compared to pDNA and other circular DNA vectors.

4.1 Introduction

It was once noted that "nowhere in biotechnology has the promise been more tantalizing and the failures more devastating than in gene therapy."[1] Despite obvious therapeutic power of gene therapy (GT), advances to improve the safety of GT vectors is direly needed before the full clinical potential of this treatment modality can be realized". Gene delivery strategies can be categorized as either viral or nonviral in design, each

[1]Branca, M. "Gene Therapy: cursed or inching towards credibility?" *Nature Biotechnology* ***23*** *(2005)*519-512.

variably emphasizing effectiveness or safety. Viral delivery vectors, derived from engineered retrovirus, adenovirus, herpes virus, lentivirus or hybrid retro/adeno virus, are generally more effective at gene delivery, while nonviral (synthetic) vectors, comprised of various combinations of polymers and lipids, nucleic acids, or nucleic acid-protein conjugates, favor safety. Recent viral GT-related patient mortalities in clinical trials highlight some of the safety issues attributed to the use of viral gene transfer systems that include, but are not limited to unwanted immune responses to viral capsid proteins, regeneration of virulent viruses, and insertional genotoxicity (*97*). These systems also tend to be limited in the size of DNA cargo that they can carry and suffer from poor stability that impacts effective storage (*59*).

In contrast, nonviral transgene delivery strategies and in particular DNA vectors offer safer GT, vaccine design, and drug delivery approaches, including lower host inflammatory and immune responses than their viral counterparts, larger DNA cargo carriage, and superior stability in storage. However, among existing nonviral systems, a gap to provide highly safe, effective, and non-toxic gene delivery vectors still exists. The effectiveness of nonviral GT has been limited in particular by unimpressive transgene expression resulting fromhuman clinical applications (*66*).

A successful transgene delivery system depends on the entrance of the DNA vector into the mammalian host nucleus and expression of the encoded transgene(s), while simple in theory, several cellular barriers must be overcome in practice. Plasmid DNA encoding the GOI must be immune- and bio-compatible to survive in and pass the extracellular environment, blood, and other body fluids. Upon crossing the cell membrane, the DNA vector must escape the endosome and make its way through the cytoskeletal network in the cytoplasm before contending with the translocation of the nuclear membrane. Until recently, most efforts have focused on modifying DNA delivery techniques through improved synthetic carriers and physical methods. However, gene delivery efficiency can also be dramatically enhanced through the strategic modification of pDNA composition and conformation, thereby improving bioavailability, biocompatibility, durability, and safety.

Prokaryotic DNA sequences such as CpG dinucleotides motifs, origins of replication, and antibiotic selection markers are necessary to maintain and amplify pDNA vectors in bacterial host cells, but compromise vector bio-compatibility and safety. Unmethylated CpG motifs, characterized by CpG dinucleotides flanked by two 5′ purines and two 3′ pyrimidines, are present at 20-fold higher frequency in prokaryotic versus eukaryotic DNA and have been shown to induce inflammatory responses when applied in clinical studies (*37, 100*). In addition to compromising safety through unintended induction of immunostimulatory host responses, these elements also induce necrosis- or apoptosis-mediated cell death in target cells thereby resulting in short-lived and abated transgene expression (*143, 144*). Furthermore, upon cellular entry, bacterial sequences of pDNA vectors are rapidly associated with histone indicators and packed into a dense heterochromatin structure that when spread into the adjacent GOI is inaccessible by transcription

factors, leading to unsatisfactory transgene expression levels or even silencing the eukaryotic promoter and the GOI (*145*).

Mini DNA vectors including circular covalently closed (CCC) minicircles and linear covalently closed (LCC) "MIDGE" and "MiLV" are minimized GOI expression units devoid of bacterial backbone sequences and encoding only the therapeutic GOI with regulatory sequences (*87, 92, 96*).

The "MIDGE" vector, first introduced in 2001, is a LCC "dumbell-shaped" molecule, which was generated by *in vitro* digestion of parent plasmid vector and ligation of a hairpin cap to the open ends. "MIDGE" vectors have been successfully used as vaccine candidates with promising results both *in vitro* and *in vivo* (*92-94*). For example, the *in vivo* hydrodynamics-injection of MIDGE vectors confirmed significantly higher transgene expression in liver (2.5-fold), lung (3.5-fold), kidneys (3.9-fold) and heart (17-fold) as well as significantly higher mean numbers of MIDGE vector molecules per cell compared to parent plasmids (*92*). In various *in vitro* and *in vivo* studies, MIDGE vectors showed great improvement in transgene expression level and sustainability. Furthermore, exclusion of the prokaryotic backbone and antibiotic resistant genes of mini DNA vectors greatly enhanced the immunocompatibility and reduced the risk of spreading the antibiotic resistant gene into normal body microbial flora, and occupy a transcriptionally active heterochromatin structure pattern (*34, 85, 87-89, 95, 146*). Mini DNA vectors are also more likely to overcome obstacles during intracellular diffusion, thereby improving their bioavailability compared to their larger parent precursor counterparts (*96*). We previously reported a one-step *in vivo* prokaryotic platform for generating mini LCC DNA vectors that were processed to remove any unnecessary pDNA sequences. The scalable system exploits the bacteriophage-derived Tel/*pal* protelomerase system that separates the LCC DNA conformation of the minimal cistron, intact with GOI, promoter SV40, polyadenylation sequence and four copies of the SV40 enhancer (SV40E), from the unwanted plasmid backbone through one-step cleaving-joining reaction (*10*). The 72 bp SV40E sequence has been shown to increase pDNA nuclear import in mammalian cells thereby enhancing transgene expression efficiency (*70*). The SV40E sequence would bind to at least 10 transcription factors (TF) that vicariously confer NLS for nuclear import of the DNA-protein complex and can be seen as a scaffold for TFs and their bound importin proteins, resulting in nuclear import of the entire protein–DNA complex.

We hypothesized that LCC DNA ministring vectors should confer the benefits of DNA minicircles with the advantage of reducing or even eliminating the risk of insertional genotoxicity by killing any host cells that have undergone vector integration events, thereby preventing the potential activation of proto-oncogenes/deactivation of tumor suppressor genes, chromosomal DNA rearrangements and instability, or silencing of the GOI.

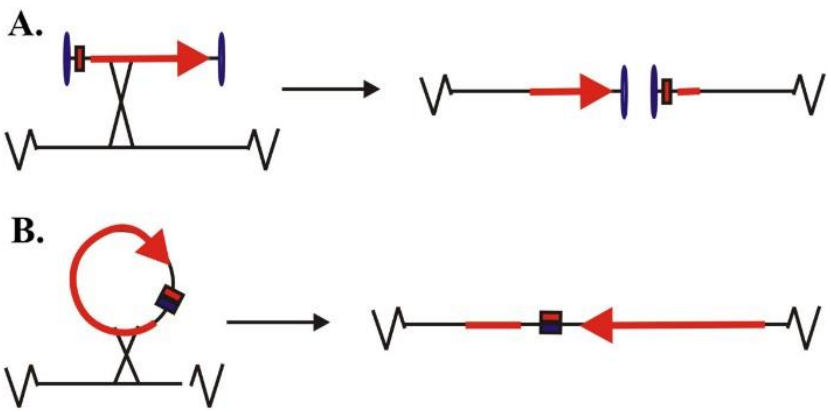


Figure 23- Schematic representations of linear and circular vector integration events.

A DNA minivector that undergoes a single recombination event with the host chromosome is rare due to the removal of all elements except the cistron containing the GOI expression cassette and the flanking SS sites. **A)** An LCC DNA ministring vector integration event would result in a chromosomal ”break” at the site of integration, whereby the chromosome cannot be replicated or segregated and the integrated mammalian cell cannot divide due to its separation of the centromere from the telomere. **B)** A DNA minicircle vector can integrate into a non-essential region of the host chromosome without “breaking” the chromosome, whereby the cell is able to continue dividing with the insertion.

Our previous study in prokaryotic cells showed chromosomal disruption and the lethal effect of single crossover of LCC pDNA vectors into bacterial host. In this study, we extended our analysis to compare the transfection efficiencies and intracellular distribution rates of isogenic mini DNA (ministring and minicircle) vectors and their parental plasmid precursor, as well as investigate the fate of human host cells following CCC and LCC pDNA integration into the chromosome.

4.2 Materials and methods

4.2.1 Strains and plasmids

E. coli K-12 strains were used to generate recombinant cell constructs and to employ JM109 as hosts for plasmid construction and amplification. A list of bacterial and phage strains used in this study are shown in Table 8.

Table 8- Strains and plasmids.

Strain	Genotype [1]	Source
Bacteria		
JM109	F', Δ*(gpt-lac)0*, *glnV44*(AS), λ⁻, *rfbC1*, *gyrA96*(NalR), *recA1*, *endA1*, *spoT1*?, *thi-1*, *hsdR17*, pWM5, F128-x	New England Biolabs
W1NN	F-, λ⁻, *IN(rrnD-rrnE)1*, *rph-1 lacZ::Cm-cI857-cre*	(*10*)
W2NN	F-, λ⁻, *IN(rrnD-rrnE)1*, *rph-1 lacZ::Cm-cI857-telN*	(*10*)
W3NN	F-, λ⁻, *IN(rrnD-rrnE)1*, *rph-1 lacZ::Cm-cI857-tel*	(*10*)
Plasmids		
pBRINT	*lacZ::cat-MCS::lacZ* (Cm^R)	NBRP; (*11*)
pGL2	*SV40P-Luc-PolyA-SV40* intron	Promega
pUC57-SS	*Multi target site "SS"* (Ap^R)	GeneScript
pcDNA5/FRT	*Flp-In Integrating vector frt-hyg-pCMV::MCS-BGHpA* (Ap^R Hyg^R)	Invitrogen
pcDNA5/FRT/CAT	*Flp-In positive control Integrating vector frt-hyg-pCMV::MCS::CAT-BGHpA* (Ap^R Hyg^R)	Invitrogen
pOG44	Flp-In Integrase vector *(FLP $^+$)*(Ap^R)	Invitrogen
pcDNA 3.1$^{(+)}$	*pCMV::MCS-BGHpA* (Ap^R Neo^R)	Invitrogen
pcDNA 3.1$^{(+)}$/CAT	*pCMV::MCS::CAT-BGHpA* (Ap^R Neo^R)	Invitrogen

pNN7	*pGL2-egfp* switched for *luc*	This study
pNN8	pNN7 + SS (upstream of SV40 promoter)	This study
pNN9	pNN8-SS (2XSS) (second SS downstream of SV40 polyA sequence)	This study
pNN12	pBRINT (Cm^R) (*SS^+)*	This study
pNN13	pcDNA5/FRT (*SS^+*)	This study
pNN14	pcDNA5/FRT/CAT *(SS^+)*	This study
pNN15	pcDNA5/FRT (*tel^+*)	This study
pNN16	pcDNA $3.1^{(+)}$(*tel^+*)	This study
pNN17	pcDNA $3.1^{(+)}$/CAT *(SS^+)*	This study

[1] sequences of interest confirmed by PCR and/or sequencing

4.2.2 Vectors and cell lines

Plasmids pcDNA5/FRT, pcDNA5/FRT/CAT integrating vectors, and pOG44 integrase expressing vector were obtained from Invitrogen (Carlsbad, USA) and pGL2 expression vector was obtained from Promega (Madison, USA). Restriction enzymes and DNA modifying enzymes were obtained from New England Biolabs (Beverly, USA). HEK 293 cells were grown in high Glucose Dulbecco's Modified Eagles Medium + sodium pyruvate + GlutaMAX supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 IU/ml penicillin. OVCAR-3 cells were grown in RPMI + GlutaMAX media supplemented with 20% fetal bovine serum, 100 μg/ml streptomycin, and 100 IU/ml penicillin. Flp-In 293 cells were grown in high Glucose Dulbecco's Modified Eagles Medium + sodium pyruvate + GlutaMAXsupplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 100 μg/ml zeocine. For transfection, exponentially growing cells were harvested by disassociation by TrypLE™ Reagents (Invitrogen) and seeded in 6-well culture plates where washing steps were done by phosphate-buffered saline (PBS). The viability of the cells was determined by trypan blue dye exclusion. Corresponding cells (more than 90% viable) were transfected using either lipid-based (Invitrogen) or polymer-based (Clontech, Mountainview, USA & VWR, Ontario, Canada). The GFP expressing pVGtelRL vector (a gift from Dr. Jochen Heinrich, Germany) was used as an internal control for transfection efficiency (TE). All cell culture reagents were provided by Life Technologies (Mississauga, Canada), cell culture equipment from VWR and Fisher, and chemical reagents from Fisher and Sigma.

4.2.3 Construction and characterization of DNA ministring vectors

The multi-purpose target site, named SS, was designed to carry two flanking 78 bp SV40 enhancer sequence to facilitate nuclear translocation and enhancing TE and moved to pGL2 vectors to generate plasmid vectors pNN7, pNN8, and pNN9 [Chapter 2 (Table 3)] as was explained previously (*10*). New constructs were tested and confirmed by colony polymerase chain reaction (PCR) and analytical digestion. The covalently closed supercoiled plasmid DNA (CCC pDNA) of pVGtelRL, and pNN9 (*eGFP* expressing vectors with four DNA translocation sequences; DTS) were converted to linear DNA with covalently closed ends (LCC DNA), DNA minicircles or DNA ministrings by passaging 1 μg of the conventional corresponding plasmids through R-cells. R-cells were then grew on LB + Ap (100 μg/ml) to A_{600} = 0.8 at 30°C with aeration. To induce recombinase expression and plasmid conformational conversion, cells were collected at late logarithmic phase of bacterial growth by centrifuge at 12,000 RPM for 2 min, then the media was removed and transformed R-cells were transferred to a new culture flask with media preheated at 42°C to conduct heat shock and to induce the recombinase expression at 42°C for 30 min at late logarithmic phase of bacterial growth, before being transferred to 30°C overnight. Cells were then harvested and plasmid was extracted (Endotoxin-free plasmid isolation maxi kit, Omega, VWR). DNA vector topology was assayed by AGE and digestion. Standard recombinant DNA techniques were performed as described by Sambrook *et al.* (*135*).

4.2.4 Transfection efficiency assay of enhanced mini DNA vectors

Cationic polymer reagents (jetPRIME) was obtained from VWR and cationic lipid reagents (Lipofectamine 2000, Lipofectamine LTX, and Plus reagents) was obtained from Invitrogen. For transfection using these reagents, 0.5 - 1 X 10^6 OVCAR-3 and 1-2 X 10^6 HEK 293 cells were seeded into six-well culture plates 24 h before transfection in complete media w/o antibiotic. One to two pmol DNA vector were mixed by lipid or polymer based carriers for each well. 1 h before transfection, culture medium was replaced to serum-free medium. DNA and transfection reagent were then mixed with 0.5 ml serum-free OptiMEM culture medium in separate tubes and incubated for 10 min at room temperature. Cationic complex jetPRIME or Lipofectamine solution was added to the DNA solution, mixed by vortexing, and incubated for an additional 30 min at room temperature. Medium culture was removed from the plate and the mixture of transfection-reagent and DNA was added drop wise. The culture was centrifuged for 5 min at 200 rpm at room temperature. The plates were incubated at 37^o C and after 2 h, six-well plates were filled up to 2 ml on complete medium w/o antibiotic. TE was controlled after 48 h. Ratios of DNA and transfection reagent were optimized for the two cell lines and for the LCC pDNA. Different combinations of transfection reagent (1–10 μl cationic complex, corresponding to 1–2 pmol pDNA) were tested.

4.2.5 Flow Cytometry

TE was determined 48 h after transfection by flow cytometry using an Epics XL (Biology, University of Waterloo). Cells were trypsinized 48 h after transfection, washed with PBS, and counted. Data were collected from 10^6 cells. Ten microliters of propidium iodide (PI), 20 mg/ml (Sigma–Aldrich, Canada) was added to measure toxicity by detecting necrotic cells. Cells with no transfection and cells transfected only with transfection reagent served as PI and mock transfection control, respectively. Cells transfected by 1 pmol of pVGtelRL served as GFP control. Indicator *eGFP* expression levels were calculated by multiplying the mean relative fluorescence values of transfected cells by the percentage of transfected cells. This parameter is considered to be directly proportional to the total amount of produced transgene product. Apoptosis was detected using the AnnexinV/PI staining assay, as previously described (see reference below). Annexin V binds to plasma membrane associated with phosphatidyl serine, and PI intercalates with DNA only by entering the cell through a broken plasma membrane. Flow cytometry readings of Annexin$^+$/PI$^+$, Annexin$^+$/PI$^-$ and ANN$^-$/PI$^-$ indicate a viable cell, early apoptotic cell and dead cell, respectively. Flow cytometry was performed using a "Guava EasyCyte Flow Cytometer" (Millipore, Billerica, MA, USA).

4.2.6 Statistical analysis

One way ANOVA was used to analyze statistical significance. A P value of ≤ 0.05 was considered significant. Each standard error bar represents a minimum of three separate transfections.

4.2.7 DNA vector labeling and real time assay of interacellular kinetics of enhanced mini DNA vectors

Modified pGL2 pDNA expressing eGFP (pNN7) with no DTS and (pNN9) flanking two SS and four DTS, ministring and minicircle *egfp* expression DNA vectors, and were labeled with the *Label* IT® Tracker Reagent Cy5 (Excitation wavelength 649 nm and Emission wavelength 670 nm from Mirus) at a weight ratio Cy5: DNA of 0.5 : 1 was incubated for 3 h at 37ºC. Unreacted Cy5 was removed from the labeled DNA by ethanol precipitation. Labeled DNA was resuspended in 10 µl sterile nuclease-free water. Concentration of the purified, labeled DNA was measured by NanoDrop spectrophotometer and the integrity of labeled DNA was quantified by AGE before and after labeling with and without EtBr staining in order to confirm the direct and non-destructive nature of the labeling reaction. Labeled DNA was protected from light. 10^4 OVCAR-3 or 10^5 HEK 293 cells were seeded on non-coated or collagen coated 35 mm glass bottom plates, respectively, in their complete media, such that their confluency was approximately 60% at the time of imaging and treated by 0.5 µM SYTO80 nuclear stain 1 h prior to transfection. These cells were transfected (Lipofectamine) by 0.5 µg of labeled DNA vectors pNN7, pNN9, ministring and minicircle *egfp* expression DNA vector, and pVGtelRL as an internal *eGFP* expression control. Lipoplexed DNA was prepared by diluting 3 : 1 ratio of Lipofectamine 2000: labeled DNA in serum-free OptiMem media, incubated for 30 min at room temperature prior to transfection. Transfected cells were monitored for DNA vector diffusion rate into the cytoplasm to nuclear memberane at 3 h and 5 h post-transfection and pictures were obtained by 2D Z stack with dual channel imaging of SYTO Orange and Cy5 scan to record localization of DNA vector with respect to the nucleus.

4.2.8 Construction and characterization of LCC integrating pDNA vectors

To construct modified integrating vectors, the SS fragment was moved from pUC57 into MCS of pBRINT (Cm^R) plasmid by BamHI and EcoRI to generate pNN12, and moved from pNN12 by BamHI and XhoI into MCS of pcDNA5/FRT and pcDNA5/FRT/CAT vectors (Invitrogen) to produce pNN13 and pNN14, respectively (Table 8). New constructs were tested and confirmed by colony PCR and analytical digestion. One microgram of supercoiled CCC pNN13 and pNN14 constructs were elecroporated into W3NN R-cells (*tel*$^+$) and grew on LB + Ap (100 µg/ml) to A_{600} = 0.8 at 30°C with aeration. To induce recombinase expression and plasmid conformational conversion, transformed cells were heat shocked to induce the Tel recombinase expression at 42°C for 30 min at late logarithmic phase of bacterial growth, before being transferred to 30°C overnight. Cells were then harvested and the plasmid was extracted (Omega maxi plasmid extraction kit, VWR). LCC pDNA topology was assayed by AGE and digestion. Standard recombinant DNA techniques were performed as described by Sambrook *et al.* (*135*).

4.2.9 Assessing fate of LCC DNA vector chromosomal integrant human cells

5 x 10^5 Flp-In 293 cells/well were seeded in a 6-well plate with 2 ml transfection media (high glucose DMEM + sodium pyruvate + GlutaMAX supplemented with 10% fetal bovine serum), such that their confluency was approximately 80% at the time of transfection. Cells were cotransfected by 0.3 µg of CCC and LCC forms of pNN13 (pcDNA5/FRT+SS) and CCC and LCC forms of pNN14 (pcDNA5/FRT/CAT+SS) with a 9 : 1 (integrase : pDNA) molarity ratio of pOG44:integrating pDNA vector. pDNA vectors were mixed by Lipofectamine LTX and Plus reagents to the 1 : 3 DNA:lipid and 1 : 0.5 DNA:plus helper lipid, respectively, and incubated for 30 min at room temperature to produce lipoplexed DNA in serum-free OptiMem media. We included the following controls: 1) untransfected cells as a negative control; 2) pcDNA5/FRT/CAT vector as a positive control; 3) pCDNA5/FRT/GOI transfected without pOG44 (Table 8) to see when the selection is complete; and 4) mock transfection. 48 h post-transfection, transfected cells were collected and expanded into 100 mm plates contained complete media with no zeocin (high glucose DMEM + sodium pyruvate + GlutaMAX supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin, 100 IU/ml penicillin), in such way that they were not more than 25% confluent. After cells were attached to the new plates, hygromycin was added to the final concentration of 200 µg/ml. Selection and expansion of stably integrated cells were performed in presence of hygromycin for 3 to 5 weeks post-transfection. Integrated cells were tested for lack of β-galactosidase activity followed B-gal staining kit protocol from Invitrogen. CAT expression level was measured following CAT ELISA protocol from Roche with slight modification. Zeocin sensitivity test was performed by growing cells in a gradient concentration of zeocin 0, 25, 50, 75, and 100 µg/ml. To further verify that the site-specific recombination was occurring at the *frt* site, genomic DNA isolated from Hyg^R, Zeo^S, B-Gal^- Flp-In:: *GOI* cells was subjected to PCR with pCMV- F and BGH-R specific primers that would only amplify at site of integration. New cells were expanded as Flp-In::*CAT*, Flp-In:: *SS-CAT*, and Flp-In:: *SS*.

4.2.10 Viability assay of LCC DNA integration

Integrant cells (10^6) Hyg^R, Zeo^S, B-Gal^- CCC and LCC were collected and washed two times by cold PBS. 250 uL of the Annexin V and PI solution was added and incubated in the dark for 15 min at room temperature and then measured by flow cytometry. Data were normalized by three controls: unstained non- integrated cells, non-integrated cells stained with only Annexin V, and non-integrated cells stained with only PI (ApoAlert Annexin V Apoptosis Kit from Clontech).

4.2.11 Assessing the functionality of the Tel/*pal* recombination system in human cells

In order to test the functionality of the PY54-derived Tel/*pal* protelomerase cleaving-joining reaction in human cells, the protelomerase coding gene *tel* was amplified by PCR from bacteriophage PY54 lysate using the following primers: Tel-F Invitro 5'-GC*GGATCC***GAT**ATG**G**TGGGTTACTTTAATTTGTGTGTT-3' and Tel-R

Invitro 5'-CG*CTCGAG*TCATTACTCCATATTTTCAGTCCATGCTTGT-3' (annealing T_M = 72°C). Italicized regions denote restriction sites for BamHI and XhoI. Underlined and bolded regions represent initiation codon and the Kozak sequence, respectively. PCR amplifications were conducted using the "Phusion Flash High-Fidelity PCR Master Mix" (New England Biolab) for 30 s at 98°C for initial denaturation, for 30 cycles of 5 s at 98°C, 45 s at 72°C for annealing and extension, and 2 min at 72°C for final extension to generate *tel* fragment for 2.1 kb. PCR products were purified from 0.8% agarose gel (Qiagen Gel extraction kit), and digested with the listed enzymes (New England Biolabs). The PCR product was ligated into the integrating pcDNA5/FRT vector (Invitrogen) to make the pNN15, and into the eukaryotic expression pcDNA3.1$^{(+)}$ vector (Invitrogen) to make pNN16, which expresses *tel* in mammalian cells under the control of the cytomegalovirus (CMV) immediate-early promoter. SS was moved from pNN12 by cutting the fragment with BamHI and KpnI and cloning into the pcDNA 3.1$^{(+)}$/CAT vector (Invitrogen) to produce pNN17. Plasmid constructs were tested and confirmed by colony PCR and analytical digestion. To perform the Tel/*pal* functionality assay in human cells, Flp-In 293 host cells (Invitrogen) containing *frt* site were grown to confluency in high glucose DMEM + sodium pyruvate + GlutaMAX supplemented with 10% FBS, 1% penicillin/streptomycin, and 100 μg/ml zeocine in a 75 ml culture flasks. Cells were trypsinized and split into 6-well plates (5 x 10^5 cells/well) and grown in the above medium without zeocin and penicillin/streptomycin for 24 – 48 h, until they were ~80% confluent. 0.3 μg of pNN15 (pcDNA5/FRT+ *tel*) with a 9 : 1 (pOG44 integrase: integrating pDNA) molarity ratio were mixed by 9 μl Lipofectamine LTX (1 : 3) and 1.5 μl Plus transfection reagents (1 : 0.5) and incubated for 30 min at room temperature in serum-free OptiMem media and transfected to cells. Both negative controls (untransfected cells, no DNA (mock), 3 μg pNN15 with no pOG44 Int, and 3 μg of pOG44 Int) and positive control (pcDNA5/FRT/CAT vector) were included to monitor integration efficiency. Transfected cells were trypsinized 48 h later and split into 100 mm plates in high glucose DMEM + sodium pyruvate + GlutaMAX supplemented with 10% FBS and 1% penicillin/streptomycin with no zeocin in a way where they did not exceed more than 25% confluent. After the cells were attached to the new plates, hygromycin was added to the final concentration of 200 μg/ml. Selection and expansion of stably integrated cells were performed in the presence of hygromycin 3 to 5 weeks post-transfection. Hyg^R cells were collected and expanded in several 35 ml plates in high Glucose DMEM + sodium pyruvate + GlutaMAX supplemented with 10% FBS and 1% penicillin/streptomycin with 200 μg/ml hygromycin for further analysis for the absence of β-galactosidase activity and zeocine sensitivity. To verify that site-specific recombination was occurring precisely at the Flp-In *frt* site, the genomic DNA isolated from Hyg^R, Zeo^S, B-Gal^- cells was subjected to PCR with pCMV- F and BGH-R specific primers that would only amplify at the site of integration. These experiments revealed the expected fragment only when GOI was integrated into the *frt* site (Figure 34-C). New cells were expanded as Flp-In::*tel* cells.

Flp-In:: *tel* and Flp-In:: *SS-CAT* cells were grown to confluency in high glucose DMEM + sodium pyruvate + GlutaMAX supplemented with 10% FBS, 1% penicillin/streptomycin, and 200 µg/ml hygromycin in a 75 ml culture flasks. Cells were trypsinized and split into 6-well plates (5 x 10^5 cells/well) and grown in the culture media without penicillin/streptomycin and hygromycin for 24–48 h until they were ~80% confluent. 3 µg of pNN16 (pcDNA3.1$^{(+)}$ + *tel*) and pNN17 (pcDNA 3.1$^{(+)}$/CAT +SS) were mixed by 9 µl Lipofectamine LTX (1:3) and 1.5 µl Plus transfection reagents (1 : 0.5) and incubated for 30 min at room temperature in serum-free OptiMem media. Flp-In::*tel* and Flp-In::*SS-CAT* cells were transfected by pNN17 and pNN16, respectively. As negative controls, untransfected cells and mock were included. 18 h after transfection, the media was changed to a high glucose DMEM + sodium pyruvate + GlutaMAX supplemented with 10% FBS, 200 µg/ml hygromycin and 100 µg/ml neomycin. 48 h post-transfection, transfected cells were tested for CAT expression. To generate a stable Flp-In::*tel* cell line that carried the SS^+ CAT expression cargo, pNN17- transfected Flp-In::*tel* cells were trypsinized 48 h later and split into 100 mm plates in high glucose DMEM + sodium pyruvate + GlutaMAX supplemented with 10% FBS, 1% penicillin/streptomycin with 200 µg/ml hygromycin yielding no more than 25% confluence. After the cells were attached to the new plates, neomycin was added to the final concentration of 100 µg/ml. The selection and expansion of stably integrated cells were performed in presence of neomycin 3 to 5 weeks post-transfection. Hyg^R and Neo^R cells were further analysed for CAT expression.

4.2.12 Assessing of CAT expression level by ELISA

The lysis buffer was prepared by adding one Protease Inhibitor Cocktail Tablet (Sigma) into 10 ml of lysis buffer (Roche). The culture media was removed and the cells were washed three times in pre-chilled PBS. The PBS was removed gently by pipetting and 1 ml of lysis buffer was added. Cells were incubated in lysis buffer for 30 min at room temperature. Cell extracts were collected and 0.5 ml was transferred to a new tube and centrifuged with the maximum speed in a pre-chilled centrifuge for 20 min to remove cellular debris. To measure the cell extract concentration, protein concentrations were normalized compared to a serially diluted concentration of BSA by Bradford Assay. A 1 : 1 ratio of cell extract samples was added to Bradford reagent (Fisher) and incubated for 10 min at room temperature with gentle rocking. Absorbance measurements were performed at 595 nm and protein concentrations were normalized according to a BSA standard curve. In order to prepare a serially diluted CAT enzyme standard, a 1 ng/ml CAT enzyme stock was prepared and a 1 : 2 dilution was prepared in 4 tubes. A 200 µl blank (sample buffer) was prepared, and standards and 10 µg of protein samples diluted in sample buffer were added to an ELISA plate and incubated at 37°C for 2 h. After removing the solution and washing the wells 5 times for 1 min each time with 200 µl washing solution, 200 µl anti-CAT-DIG solutions was added and incubated at 37°C for 1 h. The ashing steps were performed as described above. Next, 200 µl of anti-DIG-POD solution was added and plates were incubated at 37°C for 1 h followed by washing. Then, 200 µl POD substrate was added and

plates were incubated at room temperature for a minimum of 15 min to allow for a color to develop. Absorbance was measured at 405 nm and normalized for CAT expression level against a CAT enzyme standard curve.

4.3 Results

4.3.1 Enhanced mini DNA vectors exhibit higher transfection efficiencies

We previously constructed a pGL2 (Promega) expression vector derivative that expressed *eGFP* under control of a SV40 strong promoter (pNN7). Next, we made pNN8 by adding a specially designed target sequence for the Tel recombinase that is flanked on both sides by the 76 bp DTS, SV40 Enhancer sequence (SV40E), called SS, upstream of the promoter (*10*). We then added another SS downstream of the polyA sequence to construct pNN9 that in total carries 4 copies of the SV40E (Table 8). We examined the influence of the SV40E copy number on TE and found that TE continues to improve with the addition of SV40E sequences. TE of pNN9 (2 SV40E) was significantly greater than that of pNN7 (no SV40E) in both slowly dividing (HEK 293) and rapidly dividing (OVCAR-3) cells (Figure 24). These results demonstrate that enhanced TE can be conferred by increasing the units of SV40E DTS on the vector.

Next, parental pDNA and derivative bacterial sequence-free LCC (ministring) and CCC (minicircle) DNA vectors carrying the eGFP expression cassette were produced as previously described in Chapter 2 using a one-step *in vivo* linearization and separation R-cell system. In head-to-head experiments, these vectors were transfected into epithelial and cancer cells using cationic synthetic vectors, Lipofectamine (Invitrogen) or jetPRIME polymer (VWR), after measuring the *eGFP* expression by applying the same weight-to-weight ratios of mini DNA and parental pDNA vectors by flow cytometry. After complexing with either Lipofectamine or jetPRIME, both enhanced DNA ministrings and minicircles conferred a TE that was significantly higher than the parental pDNA controls ($p < 0.001$) in slowly dividing epithelial cells (Figure 25-A). However, lipid-complexed DNA ministrings also significantly outperformed isogenic enhanced minicircles ($p < 0.05$) in these cells. Lipid-complexed DNA ministring vectors transfect more than 80% of the relatively slowly dividing epithelial cells compared to isogenic DNA minicircles ($p < 0.05$). Similar results were observed in rapidly dividing cancer cells, although no significant difference in TE was noted between lipoplexed enhanced minicircles and ministrings (Figure 25-B). In both cell types, lipoplexing proved to be more advantageous in the transfection of mini DNA vectors, while the opposite was true for the parent vector that benefited from polyplexing. Even by using the same number of molecules, by applying the same mole ratios of mini DNA and parental pDNA vectors, after complexing with Lipofectamine, enhanced DNA ministrings conferred a TE that was significantly higher than the parental pDNA controls ($p < 0.05$) in slowly dividing HEK 293 cells (Figure 26).

The cytotoxicity of cationic synthetic carriers complexed with the DNA vectors was lower for enhanced DNA ministring and minicircle due to the lower molecular weight of mini vectors compared to their parental precursors.

As a result, carrier (transfection reagent) concentration was reduced, particularly for DNA ministring vectors due to our optimization results indicating that LCC conformation requires 2-fold lower cationic carrier to DNA ratio for higher TE compared to the CCC counterparts (Figure 27).

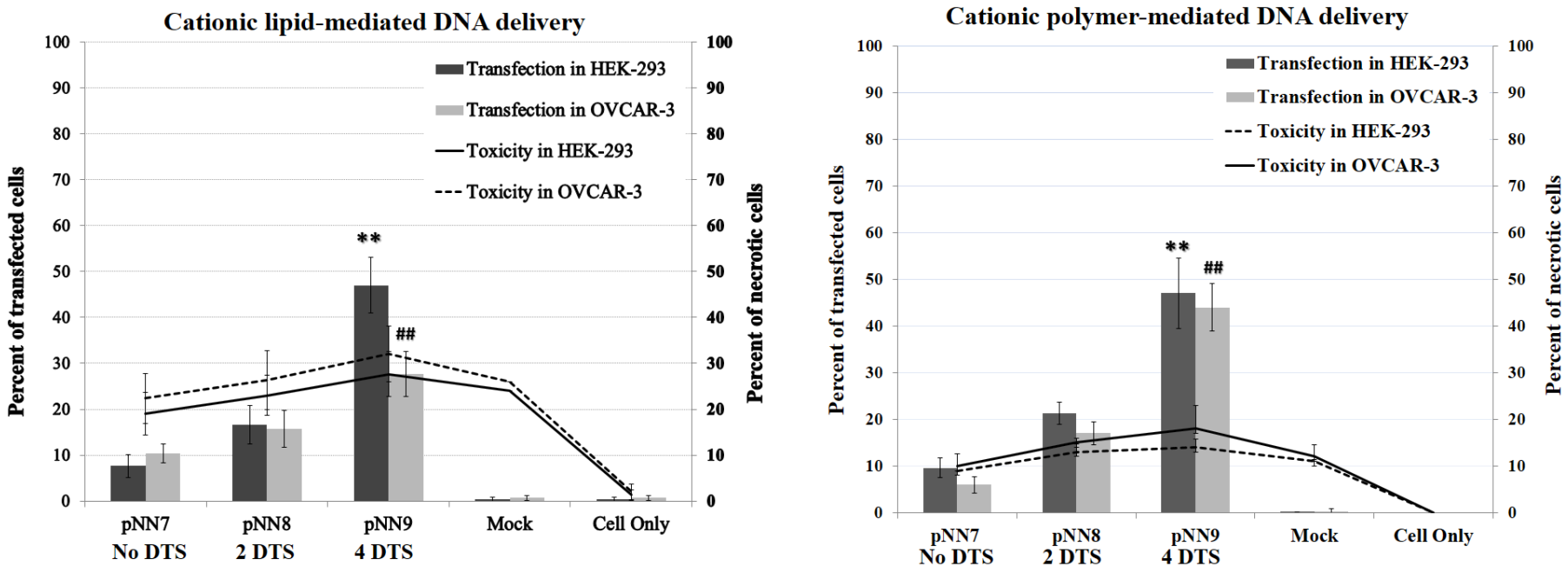
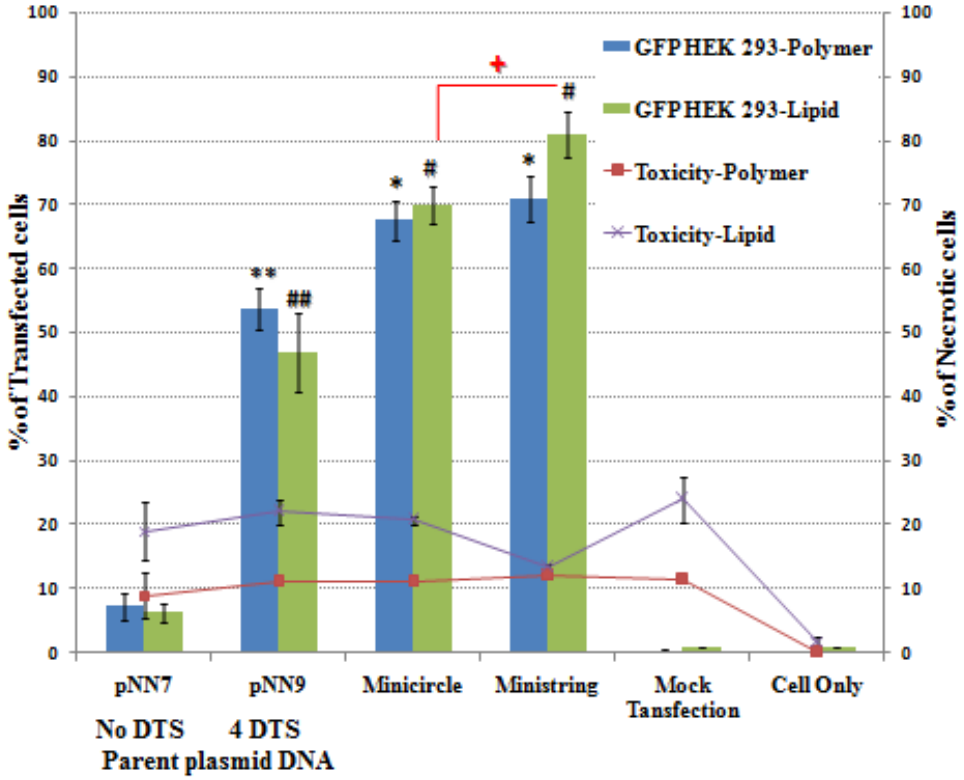


Figure 24- Effect of SV40 enhancer sequence on transfection efficiency.

5 µg of plasmid DNA vectors pNN7 (no SV40E), pNN8 (2 SV40E), and pNN9 (4 SV40E) were mixed with 10 µl Lipofectamine LTX and 2.5 µl plus transfection reagents (Invitrogen), or by 10 µl jetPRIME polymer (VWR). Lipoplexed/polyplexed DNA vectors were transfected into rapidly dividing cancer cells (OVCAR-3) and relatively slowly dividing epithelial cells (HEK 293). Transfected cells were collected 48 h post-transfection and analyzed by FACS. TE was measured as the number of GFP expressing cells divided to the total number of cells. PI was added to measure the toxicity of the transfection reagents. The left panel represents the cationic lipid mediated (lipoplexed pDNA) and the right panel represents cationic protein mediated (polyplexed pDNA) *egfp* delivery into both HEK 293 and OVCAR-3 cells. These results confirm that regardless of the synthetic carrier, both polyplexed and lipoplexed pNN9 vectors (carring 4 DTS) show significantly higher TE compared to their pNN7 (no DTS) counterparts $p \leq 0.001$ in either epithelial or cancer cells.


GFP HEK 293-Polymer
GFP HEK 293-Lipid
Toxicity-Polymer
Toxicity-Lipid
%of Transfected cells
%of Necrotic cells
pNN7
pNN9
Minicircle
Ministring
Mock Tansfection
Cell Only
No DTS
4 DTS
Parent plasmid DNA

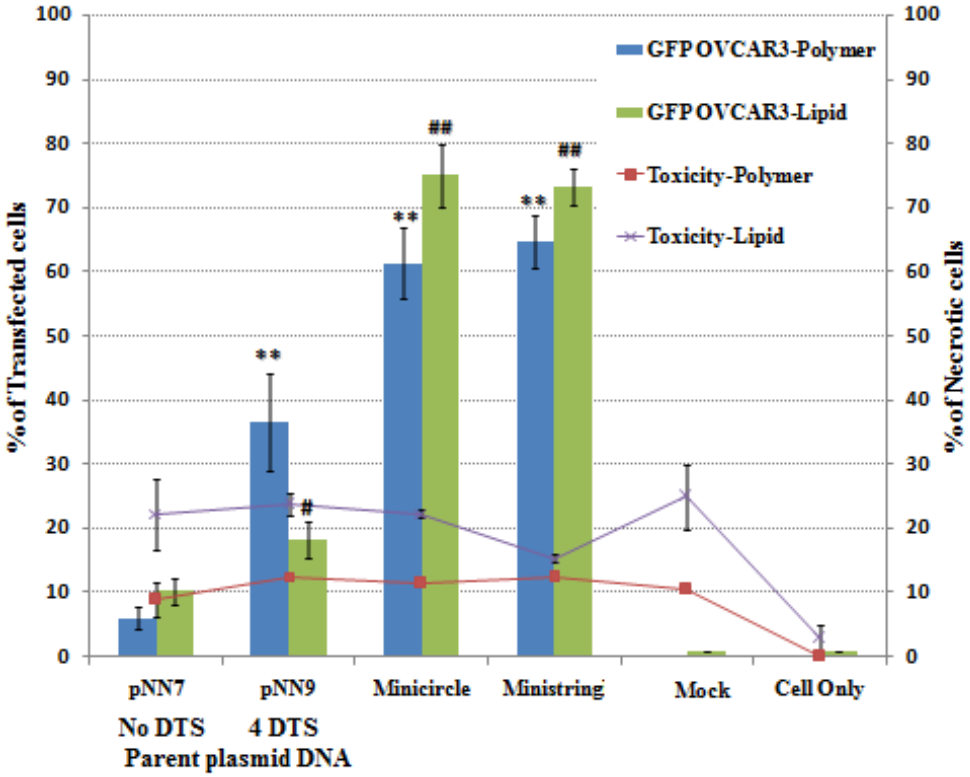

GFP OVCAR3-Polymer
GFP OVCAR3-Lipid
Toxicity-Polymer
Toxicity-Lipid
%of Transfected cells
%of Necrotic cells
pNN7
pNN9
Minicircle
Ministring
Mock
Cell Only
No DTS
4 DTS
Parent plasmid DNA

Figure 25- Isogenic enhanced minicircle and ministring DNA vectors confer superior transfection efficiencies.

Plasmid pNN9 (4 SV40E) was processed into isogenic enhanced DNA minicircle and ministring vectors by passaging through Cre and Tel R-cells, respectively. DNA vectors (5μg) were mixed with Lipofectamine LTX + Plus reagents (1 : 2 ratio for conventional vectors pGL2-2SS parent plasmid pNN9 and the "minicircle" derivative) and (1 : 1 ratio for DNA ministring vector) or with jetPRIME polymer (1 : 2 ratio). Cells (8 x 10^5 cells/well) were seeded into 6-well culture plates and polyplexed or lipoplexed by DNA vectors. Cells were collected 48 h post-transfection and analyzed by FACS for *eGFP* expression and cytotoxicity of cationic lipid/polymer. Parent plasmid pNN9 is a 5.6 kb DNA molecule carrying 2 SS sites flanking the minimal *eGFP* cistron. Unlike pNN9, pNN7 does not possess an SS (and hence, no SV40 enhancer sequences), limiting its nuclear translocation efficiency particularly in relatively slowoly dividing epithelial cells (HEK 293). TE was measured as the number of eGFP expressing cells divided by the total number of cells. PI was added to measure the toxicity of the transfection reagents. **A. (top)** slowly dividing HEK cells**, B. (bottom)** rapidly dividing OVCAR-3 cells**.** In both cell lines, mini DNA vectors show significantly higher TE ($P \leq 0.001$) compared to their parent plasmid. In HEK cells, DNA ministring showed significantly higher TE versus its isogenic minicircle counterpart ($P \leq 0.05$).

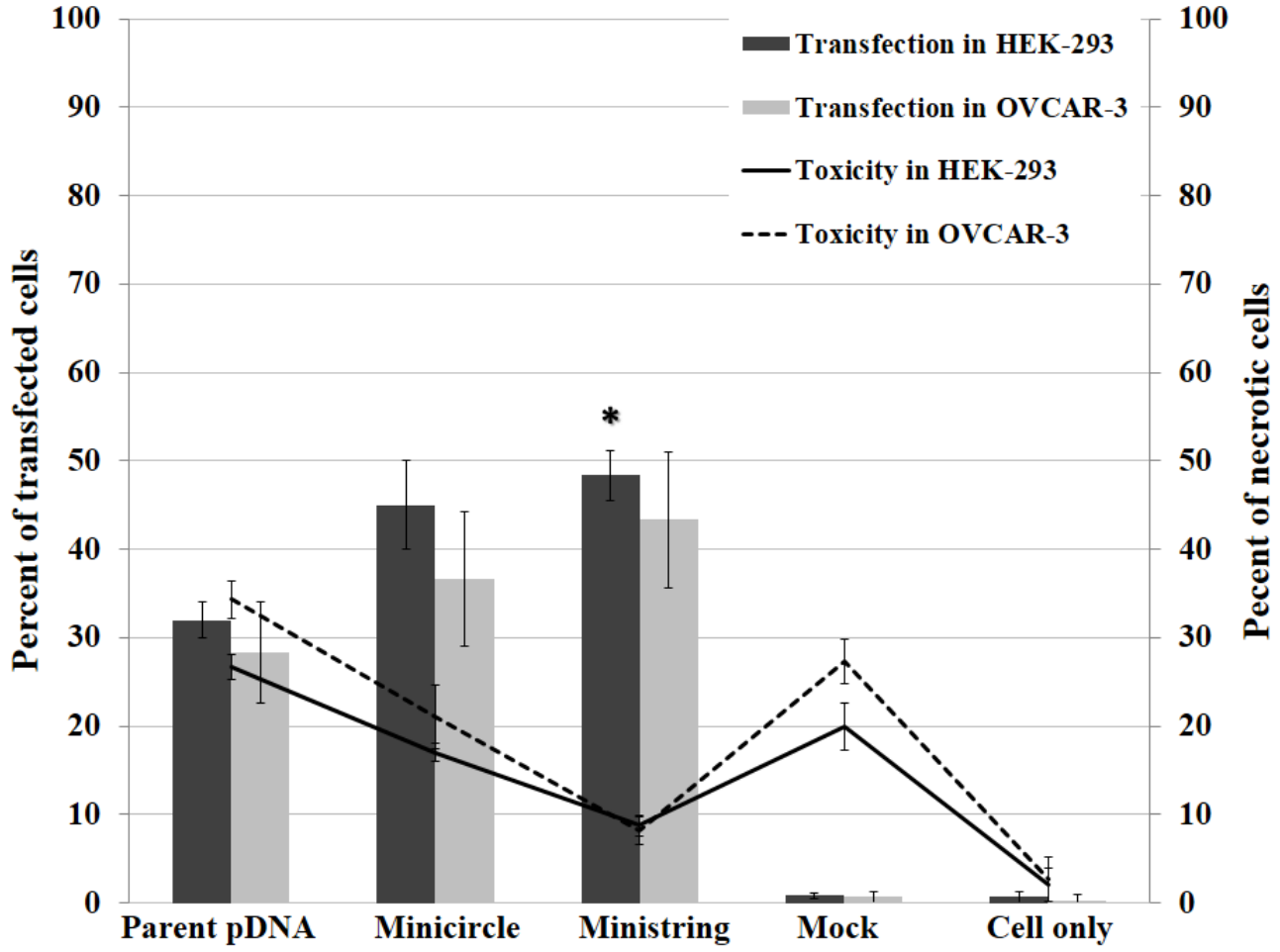


Figure 26- Transfection efficiency of DNA ministring vector.

Plasmid pNN9 (carring 4 SV40E sequence) was processed into isogenic enhanced DNA minicircle and ministring vectors by passaging through Cre and Tel R-cells, respectively. DNA vectors (2 pmol) were mixed with Lipofectamine 2000 (Invitrogen) at 1 : 3 ratio for conventional parent pNN9 and minicircle, and 1 : 1 for DNA ministring. 8 x 10^5OVCAR-3 and 10^6 HEK 293 cells were seeded into a 6-well culture plates and transfected by lipoplexed DNA vectors. Cells were collected 48 h post-transfection and analyzed by FACS. TE was measured as the number of eGFP expressing cells divided to the total number of cells. PI was added to measure cellular toxicity of the transfection reagents. In relatively slowly dividing epithelial cells (HEK 293) DNA ministring vectors showed significantly higher TE compared to parent pDNA based on identical copy number of DNA vectors transfected in to both cancer and epithelial cell lines ($p \leq 0.05$).

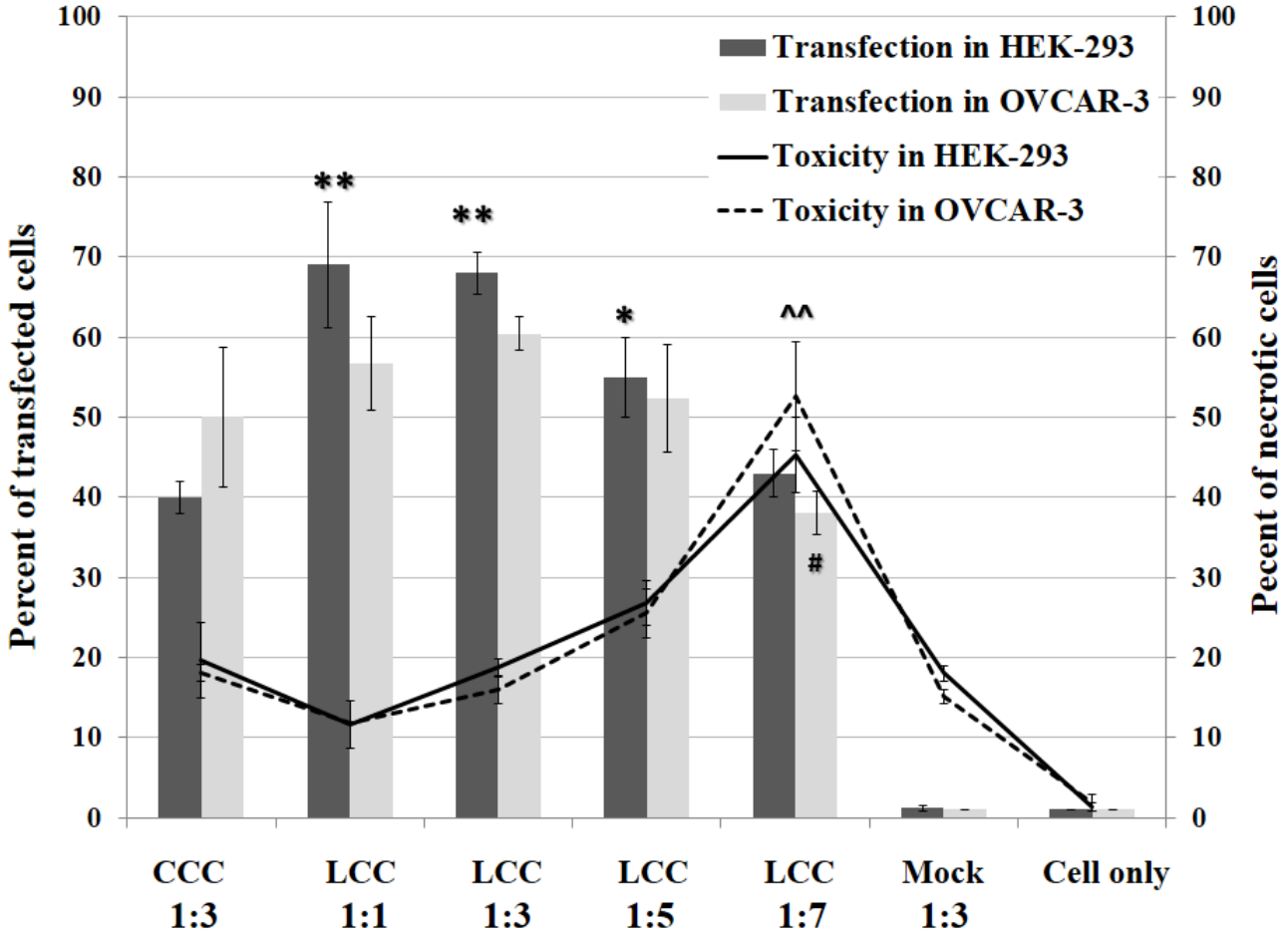


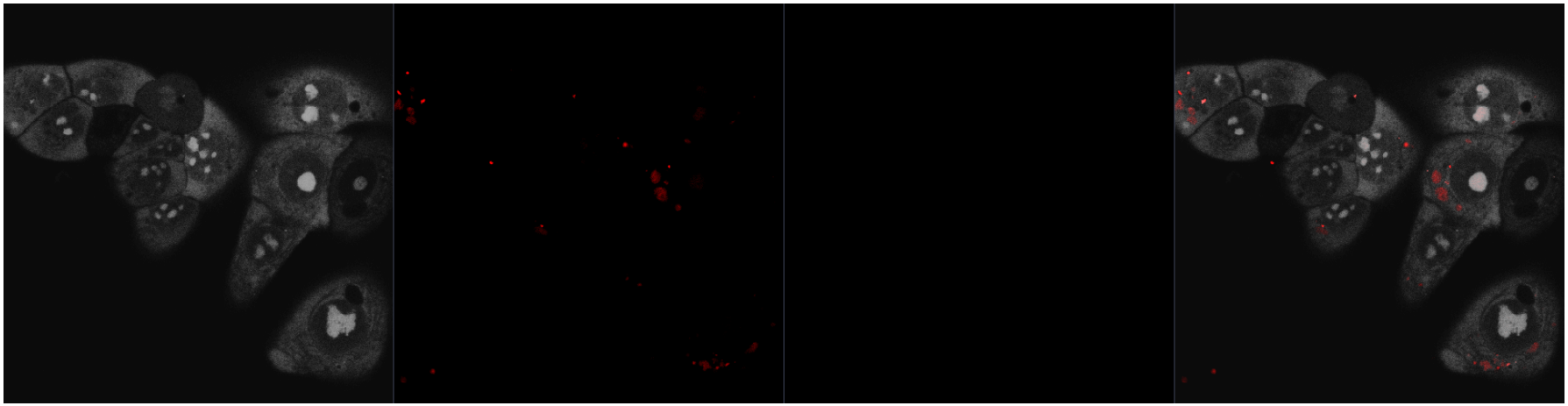
Figure 27- Optimization of LCC pDNA transfection.

1 pmol of pVGtelRL pDNA was complexed with Lipofectamine 2000 (Invitrogen) at 1 : 3 ratio for conventional CCC plasmid and at different ratios for isogenic LCC form of pVGtelRL (pDNA string) including 1 : 1, 1 : 3, 1 : 5, and 1 : 7. OVCAR-3 (8 X 10^5) and HEK293 (10^6) cells seeded into a 6-well culture plates were transfected by lipoplexed DNA vectors. Cells were collected 48 h post-transfection and analyzed by FACS. In HEK 293 cells, the highest TE and lowest Lipofectamine-mediated toxicity were observed at 1 : 1 ratio of LCC pDNA to lipid transfection reagents ($p \leq 0.001$). By increasing the volume of transfection reagents with a 1:7 ratio, TE in OVCAR-3 cells was significantly lower than that seen under standard conditions (1:3) ($p \leq 0.05$) and cytotoxicity was significantly higher $p \leq 0.001$).

4.3.2 Enhanced DNA ministring vectors exhibit efficient cytoplasmic diffusion

Unlike previous investigations that have only focused on assessing the efficiency of transgene expression by measuring the gene and protein expression levels of reporter genes more than 24 h post-delivery, by live cell confocal imaging, we further investigated the kinetics of DNA vectors cytoplasmic diffusion and localization to the nuclear membrane of the target cell at very early stages of gene delivery within few hours post transfection. We investigated the effect of DNA vector size and DTS availability on intercellular diffusion and distribution of enhanced DNA minicircles and ministrings by observing live vector localization *via* confocal microscopy in both repidally dividing cancer cells and relatively slowly dividing epithelial cells. The parental precursor (pNN9) and ministring/minicircle derivatives were labeled by fluorophore prior to transfecting epithelial and cancer cells (HEK 293 and OVCAR-3, respectively) and following vectors' intracellular location by laser confocal fluorescence microscope at variable time post-transfection. The pVGtelRL plasmid was used as a positive control, while carrier-only was employed as a negative/mock control. Assessment of vector intercellular kinetics revealed a remarkably quicker internalization and distribution of the pDNA vectors carrying DTS sequences (Figure 28-A) compared to the conventional plasmids lacking a DTS (Figure 28-B) or the isogenic enhanced mini DNA vectors compared to their parent plasmid in both cancer (Figure 29) and epithelial (Figure 30) cells. In addition, DNA ministring vectors, compared to isogenic minicircles and the parent plasmid precursor were far more efficient at accumulating toward nuclear memberane after 3 h suggesting improved and enhanced cytoplasmic diffusion and nuclear uptake associated with this DNA topology (Figure 30). In contrast, minicircles predominantly inhabited lysosomes at 3 h post-transfection with virtually no nuclear accumulation. Parent plasmid demonstrated the poorest diffusion capabilities with no nuclear circumscription by 3 h in addition for poor cellular internalization and cytoplasmic diffusion.

A



B

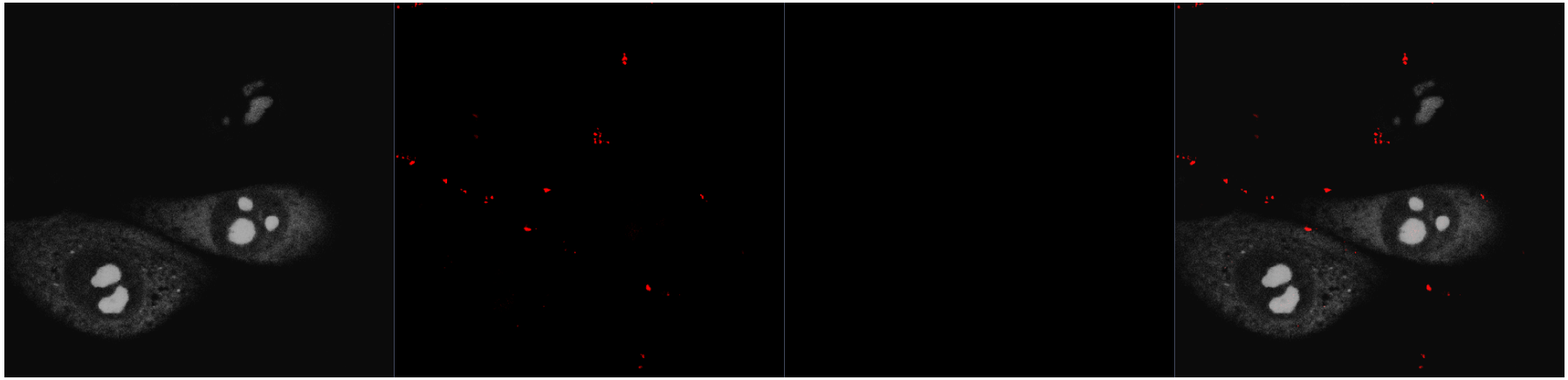
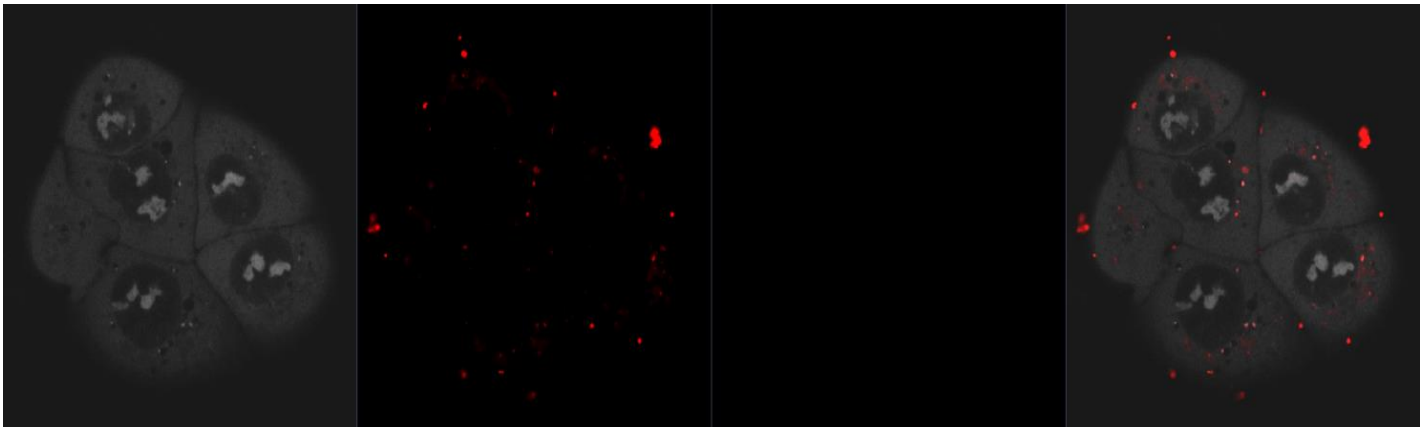


Figure 28- Effect of SV40 enhancer sequence on pDNA cytoplasmic difusion

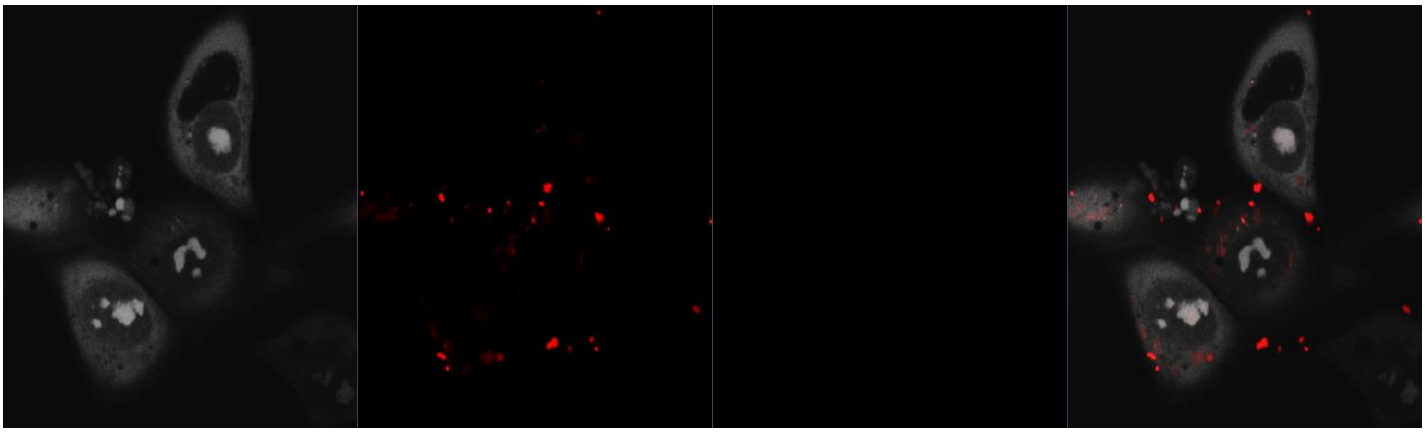
Fluorescence confocal photomicrographs of cells containing internalized labeled plasmid. Relatively slowly-dividing epithelial cells were transfected by Label IT Tracker Cy5 (Mirus)-labeled pNN9 and pNN7 complexed with Lipofectamine 2000 (Invitrogen). Cells were stained with SYTO80 nuclear stain reagent (Invitrogen) 1 h prior to transfection. Transfection was performed in serum-free media. Cells were prepared for microscopic analysis at 4 h post-transfection. Images show white fluorescence of the nucleus and cytoplasm (acquired to indicate cell location) as compared to red fluorescent DNA signal indicating labeled DNA. **A)** HEK 293 cells transfected by lipoplexed pNN9 vector (carring 4 SV40E sequences). B) HEK 293 cells transfected by lipoplexed pNN7 vector (carring no SV40E sequence). From left to right: SYTO80 signal showing only the nucleus and cytoplasm, Cy5 signal showing only the pladmid DNA localization, off signal, colocalization of SYTO80 and Cy5 to locate the plasmid DNA in respect to nucleus and cytoplasmic memberane. Internalization and accumulation of pNN9 vectors in cytoplasm occurs with greater efficiency compared to pNN7 vectors within 4 h post-transfection.

3 hours post-transfection

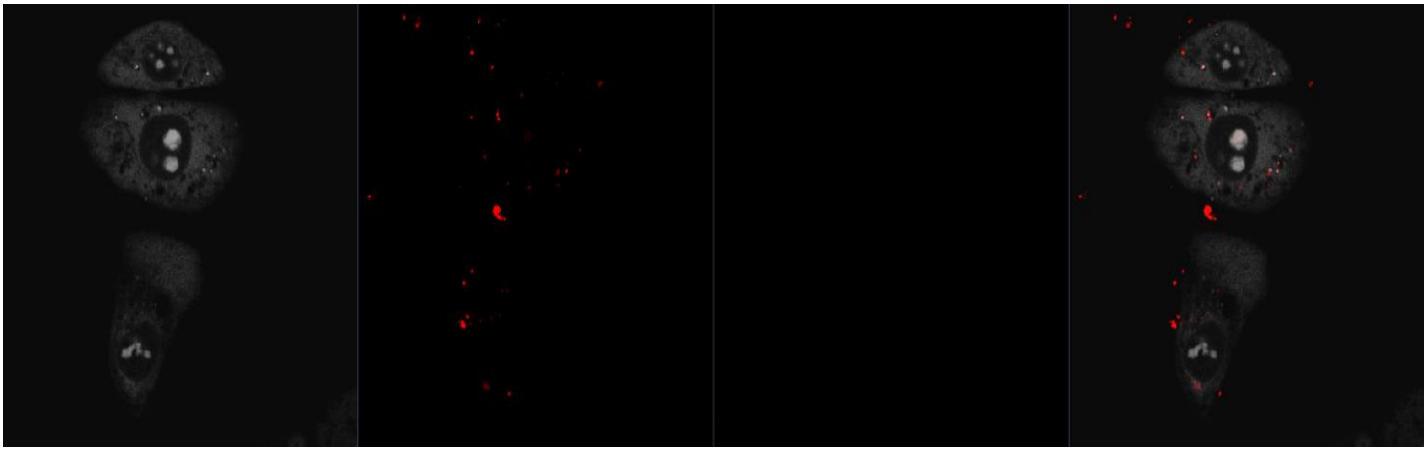
Enhanced DNA ministring vector



Enhanced DNA miniclrcle vector

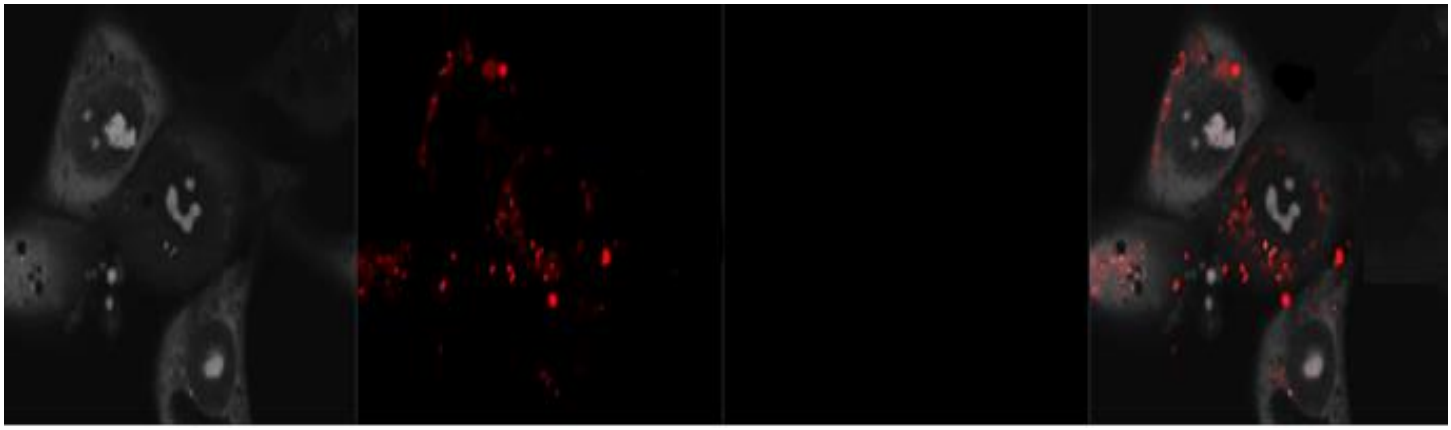


Parent pDNA vector

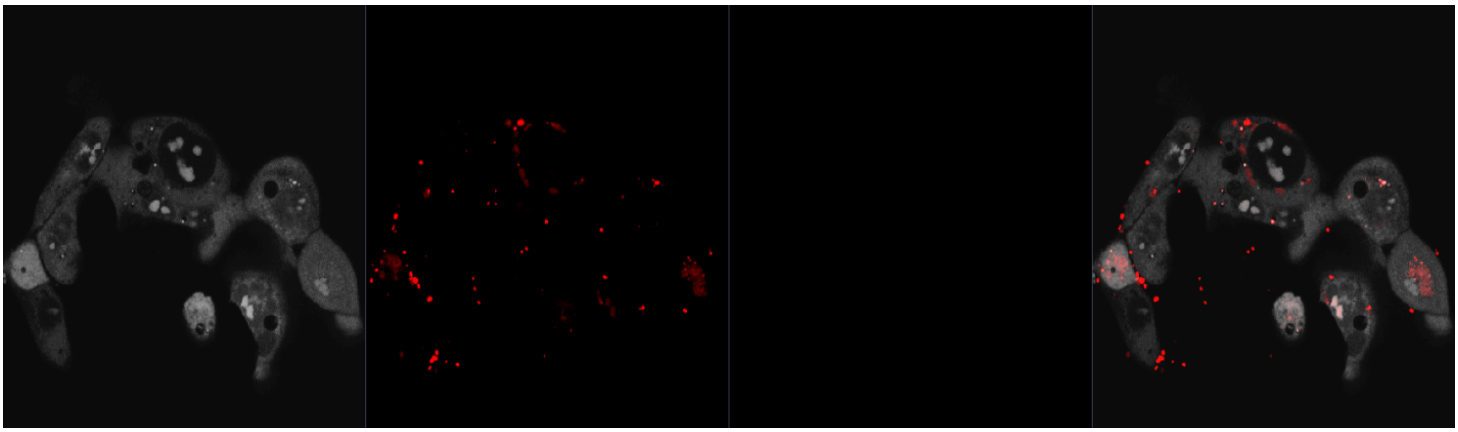


7 hours post-transfection

Enhanced DNA ministring vector



Enhanced DNA minicircle vector



Parent pDNA vector

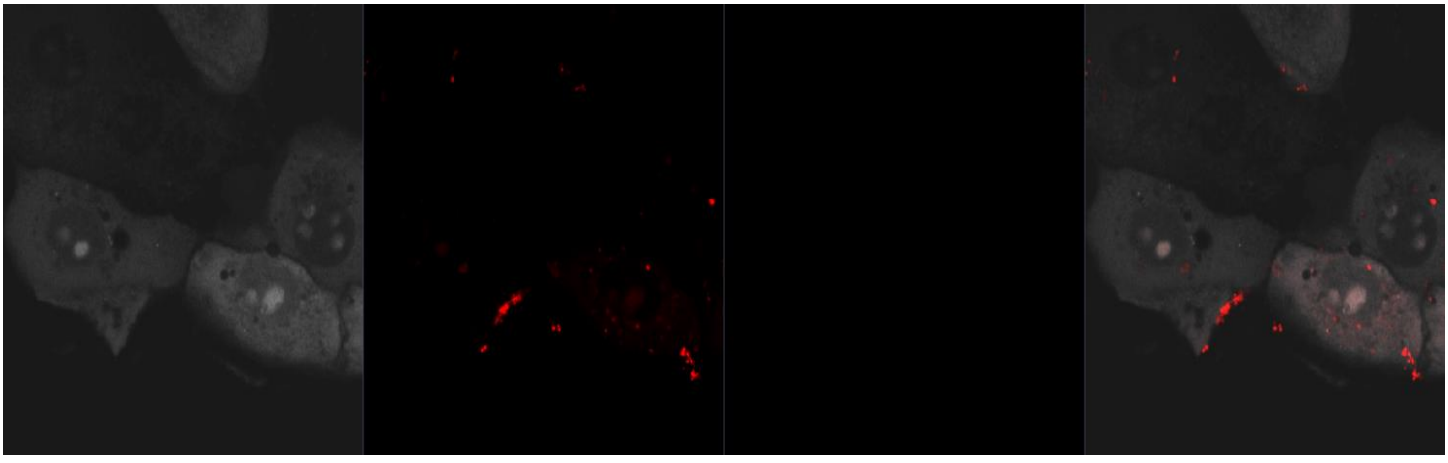


Figure 29- Effect of DNA vector size and topology on cytoplasmic diffusion in cancer cells

Fluorescence confocal photomicrographs of cells containing internalized labeled DNA vectors. Human ovarian cancer cells (OVCAR-3) were transfected with Label IT Tracker Cy5 (Mirus) labeled pNN9, isogenic enhanced DNA minicircle and ministring vectors complexed with Lipofectamine 2000 vector (Invitrogen). Cells were stained with SYTO80 nuclear stain reagent (Invitrogen) 1 h prior to transfection. Transfection was performed in complete media with no media change. Cells were prepared for microscopic analysis at 3 h and 7 h post-transfection. Images show red fluorescent DNA signal (indicating labeled DNA) with respect to fluorescent cytoplasm and nucleus. From left to right : SYTO80 signal showing only the nucleus and cytoplasm, Cy5 signal showing only the pladmid DNA localization, off signal, colocalization of SYTO80 and Cy5 to locate the plasmid DNA in respect to nucleus and cytoplasmic memberane.

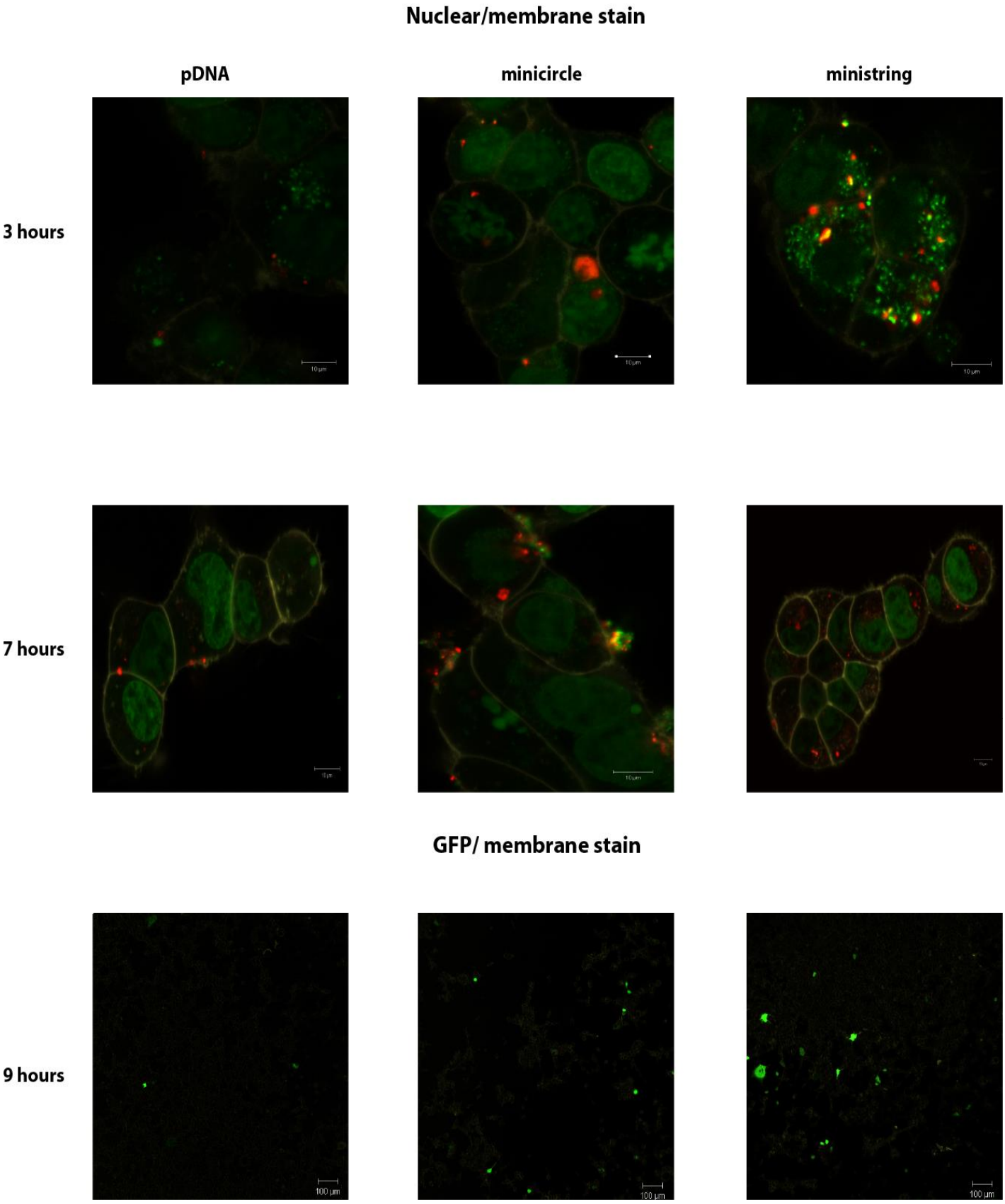

Nuclear/membrane stain
pDNA
minicircle
ministring
3 hours
7 hours
GFP/ membrane stain
9 hours

Figure 30- Effect of pDNA vector size and topology on cytoplasmic diffusion in epithelial cells.

Fluorescence confocal photomicrographs of cells containing internalized labeled DNA vectors. Epithelial human embryonic kidney cells (HEK 293) were transfected with Label IT Tracker Cy5 (Mirus) labeled pNN9, isogenic enhanced DNA minicircle and ministring vectors complexed with Lipofectamine LTX and Plus transfection reagents (Invitrogen). Cells were stained with SYTO-Green nuclear stain and Orange "Cell Mask" reagents (Invitrogen) for nuclear staining and cytoplasmic memberane staining, respectively, 10 min prior to imaging. Transfection was performed in complete media with no media change. Cells were prepared for microscopic analysis at 3 and 7 h post DNA delivery. Images show red fluorescent DNA signal (indicating labeled DNA) with respect to fluorescent cytoplasmic memberane in orange and nucleus in green. The first panel on top represents cytoplasmic diffusion and DNA internalization of parent pNN9, enhanced DNA minicircle, and enhanced DNA ministring vectors, respectively from left to right, at 3 h post-transfection. The second panel from the top represents cytoplasmic diffusion and DNA internalization of parent pNN9, enhanced DNA minicircle, and enhanced DNA ministring vectors, respectively from left to right, at 7 hours post-transfection. The third panel from the top represents GFP expression quality (green) in HEK cells transfected by parent pNN9, enhanced DNA minicircle, and enhanced DNA ministring vectors, respectively from left to right, in respect to the total cell population (orange) at 9 h post-transfection. Isogenic enhanced mini DNA vectors outperform parent plasmid with respect to time to reach the nucleus and initiate expression of GFP and DNA ministring vectors outperformed their isogenic minicircles in term of number of DNA vectors internalized and taken up by the nucleus.

4.3.3 Integration of LCC DNA into human genome results in chromosomal disruption and cell death

Next, we examined the safety profile of ministring DNA vectors and hypothesized that although, the integration of DNA vectors into the host genome is generally a rare event, the integration event of a LCC DNA vector into a human host chromosome would disrupt the chromosome at the site of integration, reduce the stability of the genome, and either kill the aberrant integrant cell or quickly target it for apoptosis. In contrast, we reasoned that the integration of a CCC vector, due to circular topology, would not result in such a disruption. To test this outcome, we forced site specific insertion of LCC or CCC DNA constructs into the human (HEK 293) genome (Figure 31) using the commercially available Flp-In™ System (Invitrogen) that exploits the Flp-*frt* high efficiency recombination system. Cells that integrated the CCC or LCC DNA vector were isolated after 3 to 7 weeks. The viable IF of LCC DNA was significantly (>500-fold) lower than that seen for the isogenic CCC counterpart (Table 9). Any surviving LCC integrant cell colonies were isolated and expanded for furture investigation. LCC integrants were found to be dramatically smaller in size and arrested in cell division compared to a completely normal morphology and growth rate seen with CCC integrants (Figure 32).

Table 9- Integration frequency of LCC pDNA vectors into human cells.

Plasmid Conformation	Integrant Viability
Circular (CCC::CAT)	1.0
Linear (LCC::CAT)	6.5×10^{-3}

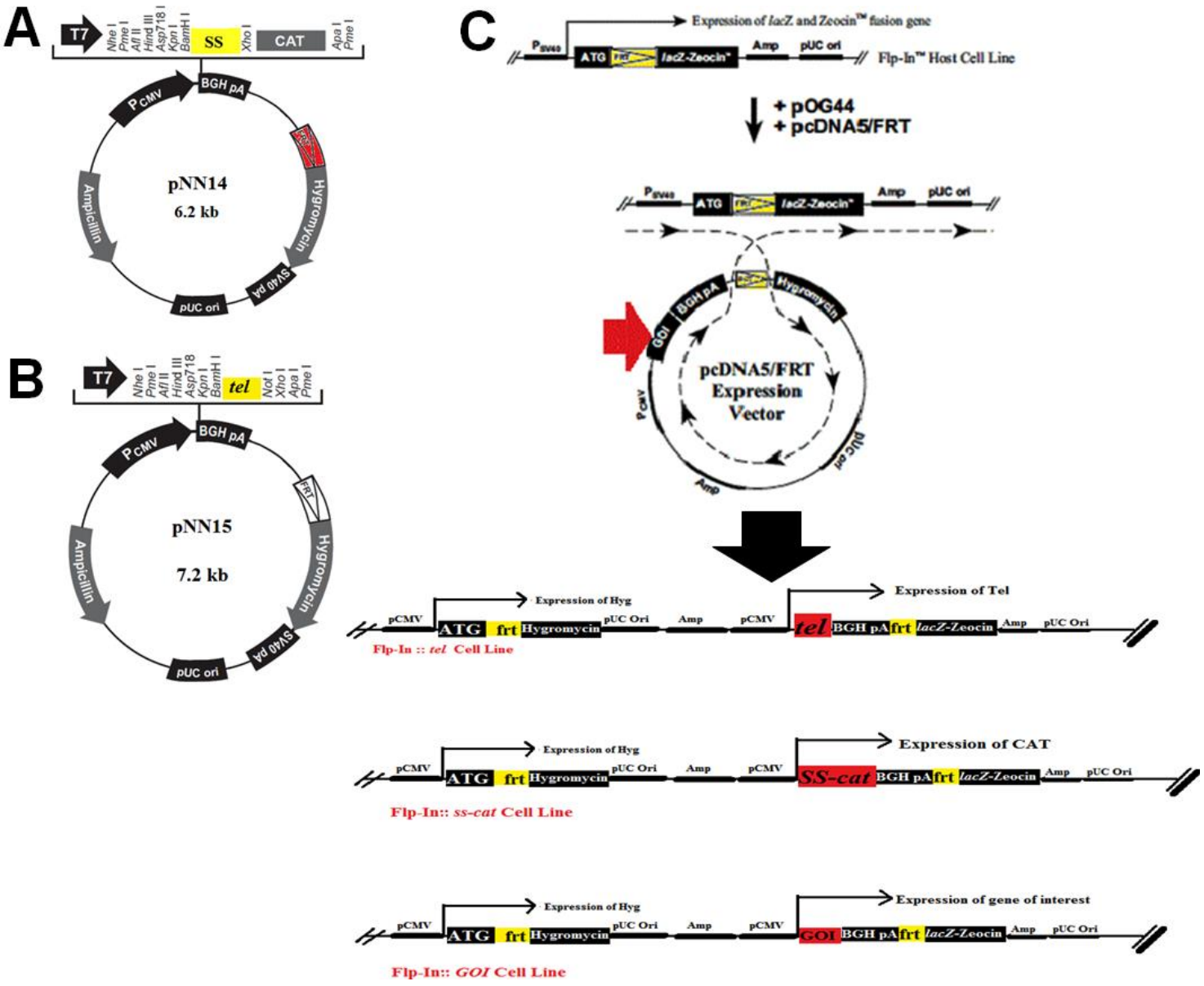


Figure 31- Schematic representation of site-spesific integration of pDNA vectors into human cells.

A) The SS multi-target site was moved from pNN12 by excising the fragment with BamHI and XhoI and moving it into the pcDNA5/FRT/CAT vector (Invitrogen) to generate the pNN14, integrating vector. **B)** The *tel* gene was amplified from PY54 lysate and cloned into the pcDNA5/FRT by BamHI and XhoI to generate the pNN15 integrating vector. **C)** Lipoplexed pOG44 (integrase expressing plasmid) and pDNA integrating vector carring the GOI were cotransfected at the molarity ratio of 9 : 1 into the Flp-In 293 cells (human embryonic kidney cells carring the *frt* target site for site-specific integration of the GOI into the genomic DNA). 3 (to max. 5) weeks later, Hyg^R, Zeo^S, B-Gal$^-$ cells were isolated and expanded to generate **Flp-In::*tel*** and **Flp-In::*SS-CAT*** cells lines.

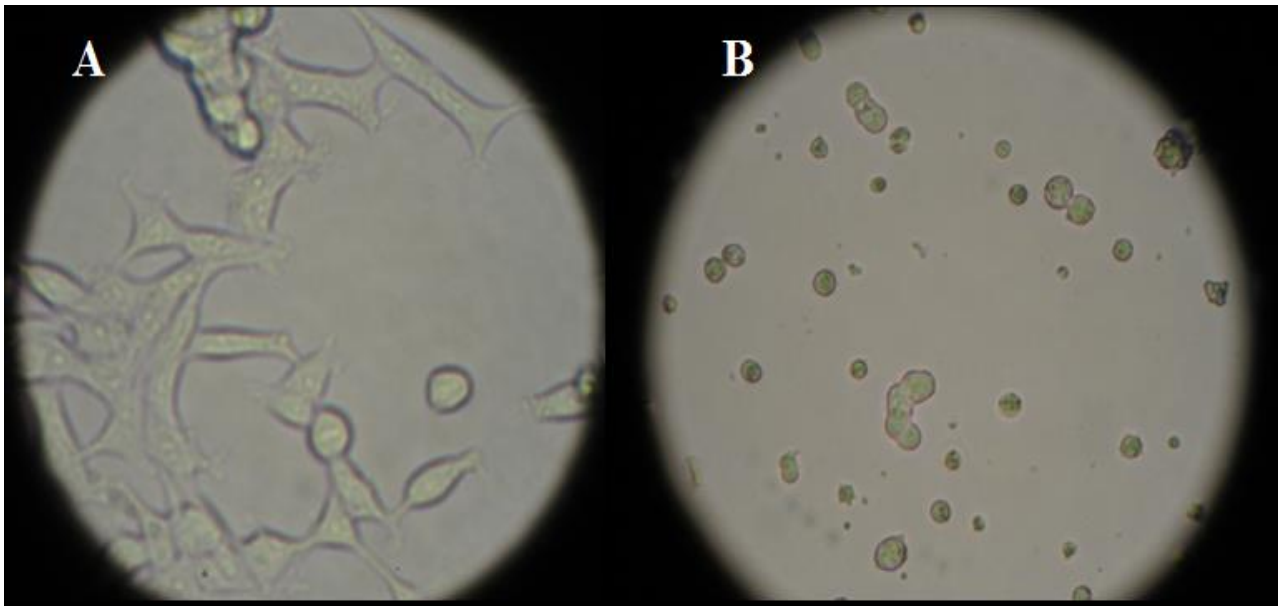


Figure 32- Morphology of integrated human cells.

Flp-In HEK 293 cells were integrated at site of *frt* by CCC and LCC DNA vectors. Three to 7 weeks post-antibiotic selection, surviving cells were observed by bright field microscopy. **A)** CCC integrants at 7 weeks post-selection, **B)** LCC integrants at 7 weeks post-selection.

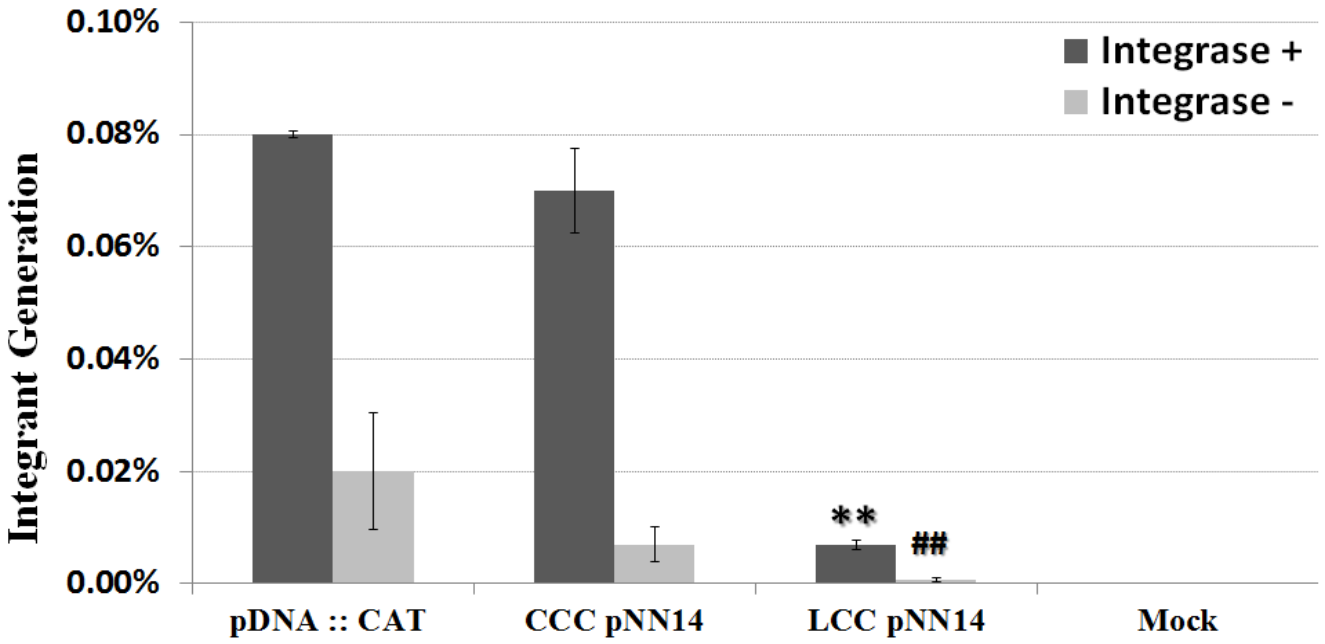


Figure 33- Integration of LCC DNA into the human genome targets integrant cells for death.

Flp-In 293 HEK cells (Invitrogen) carrying the attachment site (*att*), were transfected by the Flp-In kit plasmids pcDNA5/FRT and pcDNA5/FRT/CAT that possess the Flp target site *frt* and the indicator chloramphenicol acetyl tranferase (CAT). The SS was cloned into each plasmid and passaged through the Tel^+ R-cells to generate LCC topology of these plasmids. Transfection was performed in the presence or absence of integrase-expressing vector, pOG44, for site-specific single cross over or general integration, respectively. Integrated cells have been selected after 3 weeks in either Hygromycin or Zeocin. The Hyg^R, Zeo^S, B-Gal$^-$cells represent the result of site-specific integration. In contrast, Hyg^R, Zeo^R, B-Gal^+ cells represent low level illegitimate integration events. The mean was calculated from a minimum of three trials. Integration frequency (IF) is expressed as the fraction of integrant cells to total cells. Integration frequency (IF) of LCC was significantly lower than its CCC counterpart and control plasmid with no SS ($P < 0.001$).

To determine whether cells that had integrated the LCC vector now possessed, as hypothesized, a chromosomal disruption at the site of vector integration, we collected and pooled all LCC and CCC integrants and assayed chromosome integrity by PCR amplification over the site of vector integration (Figure 34-D). The LCC integrant DNA pool demonstrated chromosomal disruption as evidenced by the inability to amplify DNA over the integration site while CCC integrants demonstrated the expected presence and size of integrated DNA insert.

We next sought to determine whether these aberrant LCC integrant cells were viable, dead, or dying. To assess the cellular fates following LCC or CCC DNA vector integration, we employed Annexin and PI staining, and flow cytometry to distinguish early apoptotic cells (Annexin-V positive) from late apoptotic/necrotic cells (Annexin-V and PI positive) cells. Greater than 50% of LCC integrants were either in early or late stages of cell death compared to a significantly healthier population of CCC integrant cells (Figure 35).

4.3.4 Tel/*pal* cleaving-joining system is not functional in human cells

To determine whether phage PY54-derived Tel/*pal* protelomerase-mediated cleaving-joining system is functional in human cells, we used the Flp-In™ System (Invitrogen) to integrate and express the bacteriophage PY54-derived telomerase enzyme Tel in HEK 293 cell line. The Flp-In™ System involves introducing an Flp Recombination *frt* target site into the genome of the HEK 293 cells. The expression vector pNN15 that contains bacteriophage PY54 *tel* was then integrated into the genome *via* Flp recombinase-mediated DNA recombination at the *frt* site as previously described (O'Gorman et al., 1991). We also inserted the SS multi-target site into Flp-In 293 cells at the *frt* site using the pNN14 vector to generate Flp-In::*tel* and Flp-In::*SS-CAT* cell lines, respectively (Figure 36). New Flp-In::*tel* human cells were transfected by pNN17 (pcDNA $3.1^{(+)}$/SS-CAT) providing stable expression of Tel and episomal expression of CAT and Flp-In::*SS-CAT* cells were transfected by pNN16 (pcDNA$3.1^{(+)}$/*tel*) providing episomal expression of Tel and genomic CAT expression. We reasoned that if Tel was functional in eukaryotic cells, it would target the SS site available on the pNN17 or integrated into the chromosome and would disrupt the CAT expression cassette and abrogate CAT expression. We collected transfected cells and assayed Tel activity by CAT ELISA and found positive CAT expression in all cases. This finding implies that Tel, in its wild type pristine sequence, is not able to process target *pal* sequences in the nucleus.

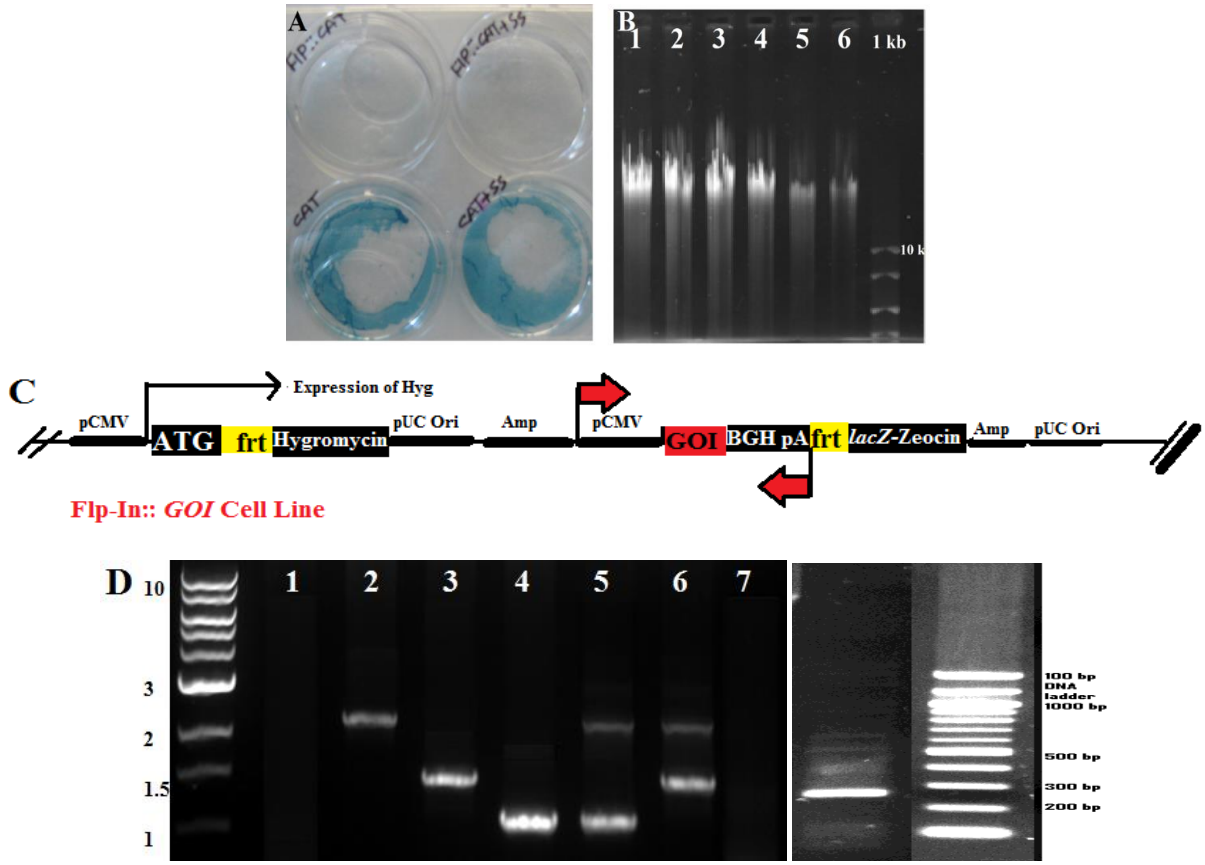


Figure 34- LCC integration results in chromosomal disruption at the site of vector insertion.

A) B-Gal staining test confirms site-specific integration of *cat* and *ss-cat* into the *frt* target site results in interruption of the *lacZ* gene and negative B-Gal staining compared to random integration. **B)** The Hyg^R, Zeo^S, $B\text{-}Gal^-$ integrants were collected and genomic DNA (gDNA) was extracted by Omega DNA Extraction kit. Purity and integrity of gDNA was confirmed by 260/280 nm spectrophotometer analysis and AGE and EtBr staining, respectively. NanoDrop results showed pure gDNA (1.8-1.9) ratio, and AGE analysis demonstrated a single sharp amplified band. From left to right, **L1**. Flp-In 293 (Negative control); **L2**. Flp-In::*tel* integrants; **L3**. Flp-In::*SS-CAT* integrants; **L4**. Flp-In::*CAT* (positive control); **L5** Flp-In::*SS-CAT/tel*; and **L6**. LCC Flp-In::*CAT* (linearized plasmid control for disrupted sequence). **C)** Schematic representation of the primer used to amplify the site of integration on Flp-In 293 genomic DNA. These primers are specific for the Flp-In kit that amplifies over the region of vector insertion. **D)** LCC integration event were tested by PCR at site of integration. CMV and BGH primers were used to amplify genomic DNA extracted from modified/integrated cells. From left to right: 1 kb ladder; **L1:** Flp-In 293 (Negative control) (240 bp); **L2:** Flp-In::*tel* (240 bp + 1900 bp); **L3:** Flp-In::*SS-CAT* (342 bp + 1058 bp); **L4:** Flp-In::*CAT* (positive control) (1058 bp); **L5:** Flp-In::*CAT*/tel; **L6:** Flp-In::*SS-CAT*/*tel*; **L7:** Flp-In::LCC CAT (linearized plasmid control for disrupted sequence yielding no bands). This analysis was repeated with identical findings.

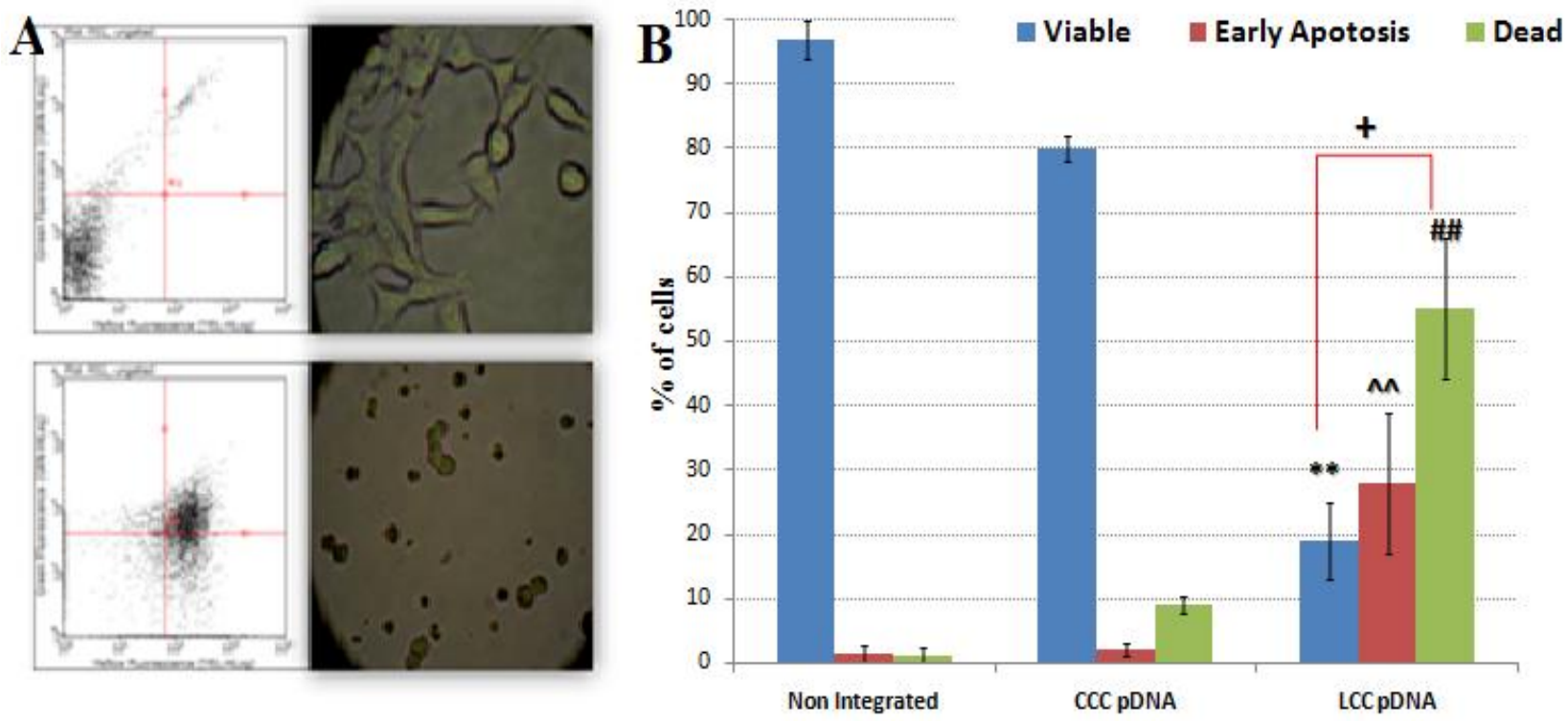


Figure 35- LCC single cross over integration into human cell induce apoptotic cell death.

LCC and CCC integrant cells (10^6) were stained by Annexin V and PI and results were analyzed by FACS. Measurements were normalized against controls: 1) unstained non-integrated cells 2) non-integrated cells stained with only FITC Annexin V, and 3) non-integrated cells stained with only PI. **A)** Two channel reading of FACS results aligned with cell morphology of CCC (upper) and LCC (bottom) integrants. **B)** Graphs represent percentage of healthy, apoptotic, or necrotic cells. Mean of a minimum of three trials. LCC integrants show significantly lower viability and higher apoptotic and necrotic index compared to the CCC integrant ($P < 0.001$). The number of dead cells within the LCC integrant group is significantly higher than healthy cells ($P < 0.05$).

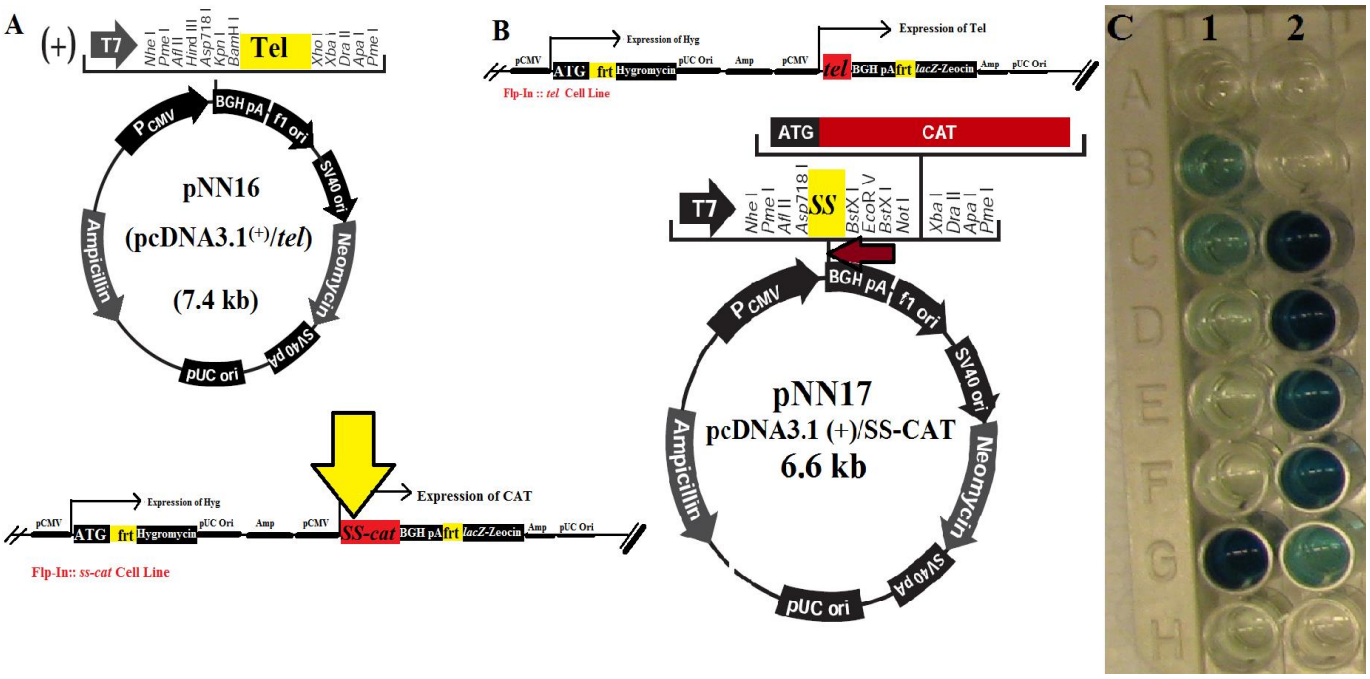


Figure 36- Schematic representation of assessment of Tel/*pal* activity in human cells.

Two different strategies have been tested to assess the functionality of the Tel protelomerase cleaving-joining activity over its target site in human cells. **A)** Flp-In::*SS-CAT* cells were transfected by pNN16 (pcDNA3.1$^{(+)}$/*tel*), providing episomal expression of Tel and genomic CAT expression. **B)** Flp-In::*tel* human cells were transfected by pNN17 (pcDNA 3.1$^{(+)}$/SS-CAT) providing stable genomic expression of Tel and episomal expression of CAT. The *pal* target site that was incorporated into the multi-target site (SS) was inserted into the CAT expression cargo between the CMV promoter and ATG start codon. In both settings, the functionality of the Tel/*pal* protelomerase system would result in cut-join activity of the Tel enzyme at the *SS* site that would abrogate CAT expression. **C)** CAT expression level was measured by ELISA test on whole cell lysate, 1A: blank, 1B to:1F: CAT enzyme standard curve 1, 0.5, 0.25, 0.125, 0.06 ng/ml, respectively; 1G: Flp-In::*CAT* cell lysate (integration positive control) , 1H: blank, 2A: Flp-In 293 cell lysate (integration negative control), 2B: Flp-In::*tel* cell lysate, 2C: Flp-In::*tel* cells and episomal expression of CAT (transfection control), 2D: Flp-In::*SS-CAT* cell lysate, 2E: Flp-In:: *SS-CAT* cells and episomal expression of Tel, 2F: Flp-In::*tel* cells and episomal expression of SS-CAT (5 µg pNN17), and 2G: Flp-In::*tel* cells and episomal expression of SS-CAT (1 µg pNN17), 2H) PBS.

4.4 Discussion

Gene therapy and modern vaccination require highly safe and efficient gene expression vectors. Plasmid DNA-based (nonviral) gene delivery offers attractive opportunities for molecular medicine. However, the injection of naked pDNA imparts poor transfer efficiency, which is likely due to three factors: 1) inefficient DNA uptake by cells; 2) inefficient cytoplasmic diffusion and nuclear uptake; and 3) low pDNA expression levels. Complexing DNA vectors with highly efficient synthetic carriers and generation of minimally sized and bacterial sequence-devoid DNA vectors can dramatically circumvent these limitations. In this study, we combined a superior mini DNA vector, the DNA ministring vector, with an efficient vector delivery method, the cationic lipid/ polymer. Ministring DNA represents the next generation of plasmid-derived DNA vectors that possesses LCC ends, minimal transgene expression cassette elements devoid of prokaryotic sequences, and DTS SV40 enhancer sequences. Such vector processing certainly results in a significant reduction in the size of the construct obtained. We previously described the exploitation of recombination systems encoded by bacteriophages PY54 and N15, to generate enhanced isogenic DNA ministring or minicircle vectors through a simple, one-step *in vivo* production platform (*10*). In this study, we compared the TE and cytoplasmic kinetics of ministring to minicircle DNA vectors and further investigated the safety of LCC and CCC DNA vectors by determining the integration efficiency and the fate of cells following the integration of these vectors into the human host genome.

Standard pDNA vectors suffer from immunogenicity of their bacterial sequences and antibiotic resistant genes, large size, and the imparted potential for insertional mutagenesis and genotoxicity. More recently, DNA vectors have been optimized to carry minimal immunogenic components while improving TE and the absence of bacterial DNA sequences resulting in more robust and persistent transgene expression *in vivo* (*90*). Mini DNA vectors can be CCC or LCC in conformation and their production can be streamlined by exploiting phage recombination systems in their generation (*10, 36*). Mini DNA vectors represent a promising alternative to conventional plasmids in terms of biosafety, bio- and immune-compatibility, improved gene transfer, potential bioavailability and cytoplasmic diffusion due to its smaller size, and low immunogenicity due to the lack of bacterial sequences and immunogenic motifs (*65, 144*).

Commonly used nonviral DNA transfection procedures include: 1) chemical methods, including lipid- and polymer-mediated DNA transfer; and 2) physical methods, including electroporation and microinjection. While the majority of previous investigations have focused on assessing the efficiency of transgene expression, little is known about the kinetics of transgenic DNA intracellular diffusion and localization to the nuclear membrane of the target cell. This is largely due to the fact that most conventional methods for monitoring gene transfer assess protein expression levels of reporter genes, such as *luc* and *gfp*, for 24 to 48 h

post-transfection, which are not suitable times to investigate early or intermediate transfection events. We have described here a method to monitor the intracellular diffusion of modified or unmodified DNA vectors post-transfection.

Recent results have shown that DNA vector/cell membrane interaction is necessary but not sufficient for transgene expression suggesting that a minimum of DNA copies interacting with the permeabilized cell membrane is needed to promote vector internalization and transgene expression. Therefore, if the number of copies interacting with the membrane increases with the ministring DNA vectors, it could explain why ministrings enhance TE. In addition, translocation of DNA across the nuclear membrane is a crucial step for successful gene expression and the intercellular kinetics of ministring DNA vector is inversely proportional to its lower molecular weight and linear topology, thereby promoting gene expression. We noted that transgene delivery *via* DNA ministring vectors demonstrated superior cellular uptake, transfection efficiency, and GOI expression compared to both minicircles and the parental CCC parental plasmid precursor in non-dividing cells. We attributed this finding to both the minimal size and LCC conformation that has been previously noted to improve TE and gene expression compared to a CCC counterpart by up to 17-fold in a tissue specific manner, in addition to imparting improved endosomal escape and intracellular trafficking (*92*). While both bacterial-sequence-free mini DNA vectors demonstrated improved cytoplasmic diffusion due to their smaller size, ministring DNA exhibited preferable kinetics compared to isogenic enhanced minicircles, which can only be attributed to DNA conformation due to vector isogenicity. Furthermore, the incorporation of SV40 enhancer DTSs was shown to facilitate vector nuclear localization and TE in slowly dividing cells. The presence of four sequences in CCC and LCC, thus likely contributes to the enhanced TE exhibited by both mini vector conformations. These findings agree with previous reports on the impact of DTS on transgene expression level (*70, 74*). We showed that not only the size of DNA vectors, but also the DNA topology and conformation considerably influence cytoplasmic distribution. Bacterial sequence-free ministring DNA showed higher TE in both cationic lipid- and polymer-mediated manner which, like minicircles, can be attributed to the smaller size, better cytoplasmic diffusion, and DTS-mediated nuclear uptake. Interestingly, we noted that TE continued to improve as additional DTSs were present on the vector. We are currently investigating whether further addition of SV40 E sequences will continue to improve TE in non-dividing cells.

While bacterial sequence-free mini DNA vectors offer improved efficacy, LCC vectors such as ministrings were shown to offer a superior safety profile in terms of insertional mutagenesis. Circular DNA vectors can compromise safety as they permit unpoliced vector integration into the host DNA while LCC DNA vectors target integrant cells for cell death. As expected, we found that the integration of an LCC DNA vector into host chromosomal DNA yields a chromosome disruption event (Figure 23) that should separate the

centromere from the telomere in a mammalian chromosome. We noted that such genomically unstable integrant cells are arrested for growth and are targeted for apoptosis. The natural elimination of such cells prevents the propagation of potentially genotoxic integrants in the transfected cell population, thereby providing a safer option for DNA vector-mediated transgene delivery by prohibiting the manifestation of potentially oncogenic insertional mutations.

We also tested whether the wild type *tel* protelomerase gene, derived from a *Yersinia* phage, is able to be appropriately expressed and function efficiently in human cells. We noted that the enzyme was functional in *E. coli (10)* and thus lacked any requirement for *Yersinia*-specific cofactors. On this basis, we then asked whether the enzyme might have the ability to function in human cells. To do so, we expressed the enzyme in human cells (HEK) and investigated Tel/*pal* functionality in human embryonic kidney by assaying whether Tel could catalyze efficient site-specific recombination at its target site (*pal*), incorporated into the multi-target region (SS), available on human chromosomes. In all tested cases, no Tel/*pal* activity was noted. However, the Flp-In::*SS-CAT* cells generated in this study are good models for this test due to its ease of modification and screening of the CAT expression.

One of the greatest reasons that the wild-type Tel lacks a function in human cells can be attributed primarily to its non-mammalian origin. Achieving steady-state expression levels and functionality of non-endogenous genes in eukaryotic systems, under the simplest of circumstances, is a sophisticated endeavor, due to codon bias, unexpected cryptic splice acceptor/donor sites in the introduced transgene and other factors associated with the eukaryotic gene transcription and translation mechanisms. To accelerate the compatibility of the protelomerase mRNA translation in human cells, Tel was engineered according to the native gene sequence but with some modifications compatible to the eukaryotic transcription and translation mechanism, such as placement under the control of a CMV promoter, introduction of a Kozak consensus translational enhancement sequence, and the terminal polyA sequence. Although these modifications would be adequate to achieve Tel expression, they do not ensure efficient nuclear translocation of translated Tel, and hence, functionality over the *pal* site incorporated into the genomic DNA. We are currently re-engineering the telomerase coding sequence to avoid internal TATA-boxes, ribosomal entry sites, unfavorable mRNA secondary structures and repetitive sequences, or extreme AT- and GC-rich sequences to prolong mRNA half-life and achieve a high level of expression and activity (*147*). Additionally, as it was shown previously that by reducing the number of CpG dinucleotides, we would avoid gene silencing associated with DNA methylation in human cells (*148*) or by adding SV40 NLS to the C-terminal (*105*) and/or to the N-terminal (*149*), we can improve the nuclear uptake rate of the recombinant protelomerase to achieve functional phage PY54-derived Tel/*pal* cleaving-joining processing in human cells. In addition to conferring Tel/*pal* functionality, it is even

more important to fuse a C-terminal/N-terminal NLS to the *tel* gene to promote NLS- mediated importation of the NLS-Tel protein into the nucleus so that it is free to act on resident *pal* sequences.

4.5 Conclusion

Enhanced DNA ministring vectors at least preliminarily represent a strong standard in gene transfer vectors in molecular medicine. In addition to expedited cytoplasmic diffusion and improved TE, DNA ministring vectors offer a superior safety profile due to its lower immunogenicity and the natural elimination of the unwanted and potentially oncogenic vector insertional genotoxicity [Nafissi *et al.*-in progress].

Chapter 5

Summary

5.1 General discussion

"Innovation is often a combination of serendipity, accidents, and spin offs from other research. So it might be a mistake not to think that something is going to happen in one or two years that may pave the way for gene therapy in humans within a relatively short period of time" Aposhian. Quoted in Morrow, 1976)

It was suggested that what is currently called "gene therapy" is principally a "model intended to aid in developing solutions to a problem set". Ultimately, the gene therapy paradigm is typically modified by scientists who work on this model. " This is a dynamic process which occurs each time a paradigm is used to address problems, encounter obstacles, resolve some components of the original problem set, and define new problems" (*150*).

DNA vectors have been widely used to prevent or treat various of diseases and showed promising results in nonviral gene delivery with different applications from gene therapy of several types of diseases such as inherited and metabolic disorders and cancer, to the development of prophylactic DNA vaccines, and more recently, in reprogramming stem cells, genome editing, and cell therapy. With the discovery and widespread use of pDNA vectors, certain advantages of DNA relative to other biological products can be appreciated—characteristics such as relative ease of manufacturing, characterization, storage, and delivery. From the viewpoints of both vaccines and therapeutics, DNA may be favored as an approach in order to generate the desired gene product(s) *in situ*. Administration of the gene rather than the protein provides multiple advantages: 1) proteins produced *in situ* from DNA may potentially persist locally or systematically longer without the toxic effects associated with the administration of the proteins; 2) gene transfer allows administration of DNA to the desired site, and 3) any necessary posttranslational modifications would be imparted, thus avoiding many of the challenges for production of recombinant proteins in mammalian cells.

Highly effective and safe DNA vectors can confer a cell-specific and more convenient gene delivery approach with higher and sustained expression of the GOI, improved bioavailability, enhanced immunocompatibility, and reduced/eliminated genotoxicity. The optimization of DNA vectors with improved efficiency and safety, is essential toward the growing the number of applications of nonviral vectors in clinical trials. Recent improvements in design, synthesis, production, and purification of advanced, safe, and

effective gene delivery methods such as biological and synthetic carriers, facilitate the development of novel technologies for mass production of pharmaceutical-grade mini DNA vectors. As such, gene and cell therapy may replace many traditional treatments in near future, particularly with continued movement toward personalized medicine.

Conventional pDNA vectors are comprised of the bacterial backbone and the GOI transcription unit. The cistron component usually carries the transgene or target sequence along with necessary regulatory elements. The bacterial backbone includes the necessary elements for production and maintenance of plasmid in bacterial cells like an antibiotic resistance gene, an origin of replication, and unmethylated CpG motifs. The bacterial backbone of pDNA vectors, however, can yield serious biological safety problems. It was previously shown that the antibiotic resistance gene in the plasmid backbone which are used for selection of plasmid vector during bacterial growth is undesirable for administration to human body due to the adverse effects such as: 1) the potential transfer of antibiotic resistance genes to normal microbial flora by endogenous horizontal gene transfer; and 2) the adverse effect of antibiotic resistant genes on the expression of the gene of interest under control of mammalian promoters (*111*). It was also shown that the immune response through toll-like receptors against unmethylated CpG dinucleotides present in the bacterial backbone of plasmid vectors cause decrease or in some cases loss of transgene expression (*111*). This property makes bacterial sequence-free DNA an excellent vector for nonviral gene therapy (*87*).

Minimized DNA vectors as therapeutic or marker gene expression cassettes circumvent the unwanted effects imparted by the prokaryotic plasmid backbone. Mini DNA vectors offer a number of advantages as a nonviral transgene delivery component, most remarkably, improved diffusion due to relatively smaller size, a lower insertional mutagenesis rate owing to their smaller size and topology, and reduced carriage of immunostimulatory CpG motifs. A number of strategies have been developed to produce mini DNA vectors, but large scale production of pure and pharmaceutical-grade vectors for clinical application poses a challenge. In this study, we used bacteriophage recombination systems to design and construct an *in vivo* robust technology to generate highly effective and safe mini DNA vectors in large quantities at lower cost. We introduced phages P1-derived Cre recombinase, N15-derived TelN telomerase, and for the first time, PY54-derived Tel protelomerase enzymes into the *E. coli* chromosome by homologous recombination. The recombinant recombinases were governed by a temperature-controllable expression system toward construction of a cost effective *in vivo* platform, and we showed that Cre/*loxp*, TelN/*telRL*, and Tel/*pal* are active in a new heterologous prokaryotic environment (R-cells: W1NN, W2NN, and W3NN *E. coli* cells). Expression of the three enzymes, as inducible expression systems integrated into the genomic DNA of R-cells conferred the processing of parent pDNA vectors carrying the specialized SS target site. Derived from conventional plasmid DNA vectors, mini DNA vectors in both forms of CCC and LCC provide a promising

improvement over traditional vectors and are currently being tested in clinical trials (*90*). However, to date, production of mini LCC DNA vectors that were conducted *in vitro*, added expense and complexity to the developmental process, especially in the industrial scale. We demonstrated that the potential for large scale production of mini LCC (ministring) DNA vectors includes applying phage PY54 derived telomerase-mediated recombination in R-cells. However, like any other process, after constructing the novel engineered *E. coli* cells (WNN series) to chromosomally encode specific recombinases and the pDNA vectors carrying the SS sites we successfully optimized the heat-regulated recombinant telomerase expression by applying the *pL* and *pR* promoters from the λ phage. Below a temperature of 37°C, association with the CI repressor reppresses the promoter. Above 37°C, the repressor is inactivated and the promoter recovers its activity to the maximum level (*10*). Furthermore, we examined whether certain variables such as the heat induction strategies, duration of induction, and the culture media formula would affect bacterial growth rate, expression level of recombinant telomerase, and the mini DNA vector production rate.

Recently, a powerful *in vivo* platform has been described for the mass production of minicircle DNA vectors (*36*). The mass production of mini LCC DNA vectors has not been broached elsewhere, as such, the *in vivo* mini LCC (ministring) production technology described in this work, is the first on its kind. This innovation has been provisionally patented and confers scalable production of enhanced isogenic ministring or minicircle DNA vectors form a single isogenic parent plasmid precursor. This technology represents the second generation of plasmid-derived DNA vectors in two forms of LCC and CCC that in addition to possessing minimal transgene expression cassette elements devoid of prokaryotic sequences is also equipped with multiple DTS SV40 enhancer sequences to boost DNA translocation efficiency. The work here represents the technological proof of principle comparing the transfection efficiency and cytoplasmic kinetics of our ministring to its isogenic minicircle and precursor counterparts and further comparing the safety profiles of LCC and CCC DNA vectors by investigating LCC and CCC DNA integration and the fate of cells following a rare integration of these vectors into the bacterial and human host genome. Still an important safety concern associated with gene delivery strategies, whether by viral or nonviral means, is the potential for genotoxicity. While bacterial sequence-free DNA mini vectors offer improved efficacy, LCC vectors such as ministrings were also shown to offer a superior safety profile in terms of preventing insertional mutagenesis. Circular DNA vectors can compromise their safety profile when they permit chromosomal stability and integrity following an undesirable vector integration event, while the integration of an LCC DNA derivative yields a chromosomal disruption event at the site of integration. We found that genomically unstable integrant cells (both bacterial and human) are arrested for growth and are targeted for cell death. The natural elimination of such cells prevents the propagation of potentially genotoxic integrants, which provides a safer option for DNA vector-mediated transgene delivery by prohibiting the manifestation of cells that have undergone a potentially oncogenic insertional mutagenic event. Moreover, we found that transgene delivery

via ministring DNA vectors conferred superior cellular uptake and TE compared to both minicircle and the parental CCC parental plasmid precursor counterparts in slowly-dividing cells.

Unlike previous investigations that have only focused on assessing the efficiency of transgene expression by measuring the gene and protein expression levels of reporter genes more than 24 h post-delivery, we further investigated the kinetics of transgenic DNA intracellular diffusion and localization to the nuclear membrane of the target cell within few hours post DNA delivery, showing that LCC mini DNA vectors outperform CCC derivatives with respect to time to reach the nucleus.

5.2 Future directions

5.2.1 DNA ministring application to the generation of induced pluripotent stem cells (iPSCs)

Nonviral and enhanced ministring DNA vectors offer the ideal attributes toward the generation of iPSCs. Minicircle vectors containing a single cassette expressing four reprogramming factors and a *gfp* reporter gene have already been successfully applied to this end to induce pluripotency in human adipose stem cells (hASCs) (*90*). As ministring DNA vectors benefit from higher transfection efficiencies and natural elimination of integrants in case of undesired integration event owing to their precise LCC shape, they represent a logical evolution of this application. Owing to ease of construction and manipulation, basic molecular principles, and straightforward manufacturing protocols, ministring could dramatically facilitate the translation of iPSC research.

5.2.2 DNA ministring application to gene therapy of monogenetic hereditary disorders

Hemophilia and sickle-cell anemia are caused by the malfunctioning of a particular gene(s) in the blood clotting cascade. Replacement of the defective allele with the wild type should solve the problem; this is the underlying principle of gene replacement therapy. In the case of hemophilia, *in vivo* GT or *ex vivo* cell therapy through gene replacement by a DNA vector offers a promising life long treatment option. However, in contrast to *in vivo* gene therapies, during *ex vivo* therapies there is no exposure of the patient to the gene transfer vector, and the target cell of gene editing can be selected, expanded and/or differentiated before gene transfer in order to improve efficacy and safety (*151*). Although it is currently in its infancy as a treatment for disorders caused by a monogenic defective gene, patients have received genetically engineered cells as an experimental treatment for missing genes (*152*). In this concept, ministring DNA vectors would play a key role in a tightly controlled gene replacement into the desired chromosomal site without jeopardizing the host cell genome stability. While it is currently an effective treatment for nonhereditary disorders, researchers are

attempting to engineer bone marrow cells to enhance the abilities of immune cells to treat cancer or resist HIV infection (*4*).

When compared to traditional DNA vectors, enhanced ministring DNA vectors could play a crucial role in ensuring controllable gene replacement and genome editing strategies for the *in vivo* gene therapy or the *ex vivo* cell therapy of various hereditary and nonhereditary disorders.

5.2.3 DNA ministring application to develop DNA vaccines

It has been more than 16 years since DNA vaccines made their scientific debut, and since their advent, DNA vaccine technologies have generated both excitement and disappointment. Currently, pDNA vectors comprise about 27% (354 out of 1,311 trials) of all gene delivery systems studied in Phase I to Phase III trials since 2007 (data obtained from Gene Therapy Trials Worldwide provided by the *Journal of Gene Medicine*). However, low efficiency and safety issues remain the primary concerns. Previous clinical disappointments highlight the possibility that complexity in DNA vaccine design, including better and improved formulations, better delivery technologies, enhanced DNA and delivery approaches, and more thoughtful clinical implementation will drive continued enhancement of this therapeutic platform, both of which are significantly addressed by ministring DNA products. The further advancements in one-step production and purification of ministring DNA in large-scaled *via* our *in vivo* platform will likely continue to drive the new global therapeutic standard for nonviral DNA vectors. Our lab is currently applying ministring technology in combination with dendritic cell targeting toward the development of a novel DNA-Virus like particle DNA (DNA-VLP) vaccine. This work is being conducted in collaboration with Dr. Chil-Yong Kang at the University of Western Ontario, using HIV self-assembling VLP sequences designed by Dr. Kang.

5.2.4 Purification of enhanced mini DNA vectors

While the production and purification technology introduced here is simple and straightforward in theory, the theory was only transformed into a workable platform through a barrage of delicate and gradual optimization improvements. In this regard it is also evident that further optimization will be required along the commercialization pathway. Since the processing of the parent plasmid, even if 100% efficient results in the production of the desired and undesired products, an efficient purification strategy is still required.

Various methods have been tested in the purification of mini DNA vectors, particularly density gradient, cesium chloride/EtBr ultracentrifugation. While this technique is excellent for large scale production, cesium toxicity is not conducive for translation to large-scaled pharmaceutical-grade production. In this study very simple techniques such as AGE and gel purification were employed to prepare adequate amounts of mini DNA to carry out the studies described. Our one-step *in vivo* recombination process in *E. coli* generates three plasmid species: 1) DNA mini vectors (minicircle and ministring), 2) miniplasmids, and 3) residual parent

plasmids. Thus, the DNA mini vector was separated from the other residual bands due to its smaller size and it was purified from agarose gel. However, this approach is considered to be labor-intensive due to the multiple steps involved that makes this approach not conducive for large-scale preparation of mini DNA vectors. To improve scalability of the system, our lab is currently attempting to incorporate and bicistronically co express a restriction enzyme with the recombinant Tel/TelN/Cre enzymes that possesses a unique target site present within the bacterial backbone of a parent plasmid vector. Cutting at this site should serve to degrade any LCC and CCC DNA that carries the backbone bacterial DNA, leaving the LCC ministring intact as the sole episomal "survivor", thereby simplifying purification. A similar system was reported by Chen *et. al.,* who applied this system successfully to co express SceI and ΦC31 recombinase as a bicistronic messenger RNA (mRNA). Chen *et. al*. showed that after 240 min of induction of the recombinase and the endonuclease, only 3% of mini plasmids and parent plasmids were detectable (*36, 88*). In contrast to their approach, our lab will employ a different enzyme and target site and instead of two different induction system, our lab is developing the CI857/*pL*/*pR* systems to co-express the telomerase and restriction enzyme in the same operon. Another approach being examined is based on affinity chromatography purification of the mini DNA vectors where a short recognition sequence is integrated into the plasmid eukaryotic backbone after recombination is located on the mini DNA vector. This technique is a one-step approach but cannot separate mini DNA vector from its parental plasmid and requires a highly efficient complementary recombination process.

5.2.5 Application of phage PY54 derived protelomerase system in human cells

We used site specific recombination to insert the PY54 derived *tel* and its target site *pal* into human embryonic kidney (HEK 293) chromosomal DNA to assess the functionality of Tel/*pal* system in human cells. In contrast to prokaryotic cells, the wild type Tel/*pal* system did not show activity in human cells during the first trial. We are currently modifying the wild type *tel* sequence for testing Tel/*pal* activity in human cells due to the complexity of these cells compared to bacterial cells.

5.3 Conclusion

Enhanced mini LCC DNA vectors (DNA ministrings) outperform conventional and even isogenic minicircle counterparts with respect to transfection efficiency and insertional genotoxicity. This nonviral transgene delivery system offers applications to gene and cell therapy, DNA vaccination, knock-in and gene replacement strategies in regenerative medicine and generation of transgenic plant/animal models, conferring superior efficiency and safety. In addition to sustained and high expression level of the GOI, ministring DNA vectors show superior cytoplasmic diffusion, improved transfection efficiency, and a superior safety profile with lower immunogenicity. The lower immunogenicity is a result of a lack of undesired bacterial sequences

and the natural elimination of the unwanted and potentially oncogenic vector insertional mutagenesis. To achieve a high yield of DNA ministring vectors production, a robust technology by applying bacteriophage PY54-derived cleaving-joining system was developed and characterized to provide a time/cost effective, *in vivo E. coli* platform for the production of DNA ministring vectors. It is anticipated that this novel technology will set the standard to drive the design and high-yield production of virtually limitless innovative DNA vectors with applications to health, agriculture and industry.

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