

# Optimization of plasmids for gene delivery

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*The efficient and safe delivery of therapeutic genes to target cells upon direct in vivo administration of non-viral vectors is of great interest for the development of gene therapy. Successful non-viral gene transfer requires mastering of plasmid access to target cells, intracellular penetration and nuclear localisation; this review describes the optimisation of plasmid backbone with this respect. Several strategies have been developed for the optimum reduction of the prokaryotic sequences of expression vectors. Approaches such as minicircle and pCOR were developed to diminish the number of CpG motives, to avoid the presence of an antibiotic resistance gene for improved bio-safety, and to reduce the size of the plasmid for better bioavailability. Moreover, this paper outlines the field of plasmid nuclear targeting, either through the adjunction of targeting DNA sequences, or by covalent chemistry on plasmid DNA.*

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*Key words: Gene transfer — Gene therapy — Non-viral vector — Conditional replication — Site-specific recombination — Nuclear import — Nuclear localisation signal.*

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Considerable advances in molecular biology and genetics are extremely promising for gene therapy. Indeed, therapeutic gene transfer is an appealing strategy, not only to cure monogenic hereditary diseases, but also for life-saving treatment of diseases such as cancer or ischaemia. It is presently recognised that one of the factors limiting the development of gene therapy is the lack of sufficiently efficient or safe gene transfer vectors.

In particular, the efficient and safe delivery of therapeutic genes to target cells upon direct *in vivo* administration remains one of the most important challenges for the development of gene therapy. Non-viral systems use a plasmid as an expression vector in combination with chemical and/or biochemical delivery vectors such as cationic lipids or polymers. These vectors self-associate with plasmids to form particles that can transfect cells.

Non-viral vectors have many advantages over viral systems: a better safety profile, the absence of theoretical size limitation for the therapeutic expression cassette, the suitability of one single gene transfer delivery vector for any desired gene, and simpler development due to easier good manufacturing practices (GMP). On the other hand, concerns have been raised regarding the immunostimulatory prokaryotic CpG motives in plasmid DNA, and the efficiency of non-viral gene transfer remains to be enhanced, especially into non-dividing cells.

Successful non-viral gene transfer calls for several steps to be mastered: preparation, purification and formulation of the therapeutic plasmid and synthetic vector, administration, plasmid access to target cells followed by intracellular penetration and nuclear localisation. Although much work has been dedicated to the optimisation of the delivery vectors and to the expression cassette in the plasmid (promoter, etc.) over the last decade, relatively less research has been conducted into the plasmid prokaryotic backbone per se, i.e. the DNA moiety which does not include the expression cassette. Indeed, this backbone can still be optimised as regards the efficiency and safety of non-viral gene transfer.

In the present review, we describe the work that we and other investigators have carried out in order to obtain optimum reduction in the prokaryotic moiety of expression vectors. This allows the number of CpG motives to be reduced, the presence of an antibiotic resistance gene for improved bio-safety to be avoided, and the size of the plasmid to be decreased, thus leading to improved bioavailability. Moreover, we describe the growing field of plasmid targeting, which has been investigated in order to optimise plasmid cellular trafficking, either through the adjunction of suitable DNA sequences, or by covalent chemistry on plasmid DNA.

## I. OPTIMISATION OF PLASMID DNA BACKBONE FOR GENE THERAPY

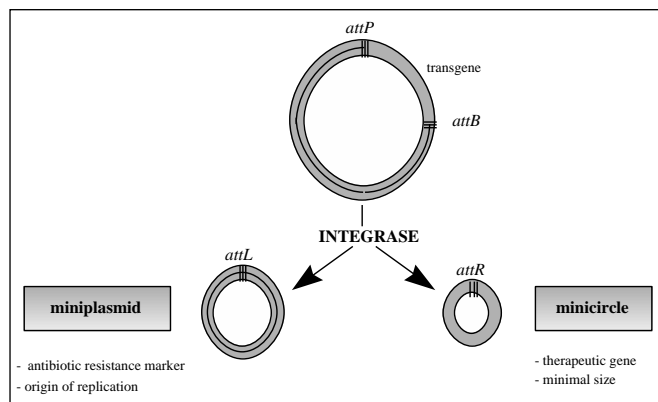
### 1. Minicircles

The first strategy to produce minimal backbone DNA molecules was developed by Darquet *et al.* [1]. They produced minicircles, supercoiled recombinant DNA molecules containing only the therapeutic gene expression cassette. Contrary to the plasmids currently used in preclinical and clinical trials of gene transfer, minicircles have neither an origin of replication nor an antibiotic selection marker. They cannot self replicate or confer antibiotic resistance to other micro-organisms. They carry only short bacterial sequences, limiting the production of antibodies against the bacterial proteins and the immune response due to CpG sequences present in the plasmid backbone [2].

The minicircles are the product of the *in vivo* excision of the expression cassette by site-specific recombination between the attP and attB recombination sequences, driven by *E. coli* bacteriophage  $\lambda$  integrase (*Figure 1* [3]). During the bacteriophage  $\lambda$  lytic cycle, the expressed integrase mediates the integration of the circular phage genome into the *E. coli* chromosome by site-specific recombination through the attP sequence of bacteriophage  $\lambda$  and the attB sequence of the *E. coli* chromosome. For minicircle production, the attP and

attB sequences are introduced in the same direction in the plasmid backbone just upstream and downstream of the expression cassette. This plasmid is introduced into the *E. coli* D1210HP strain. This strain harbours a  $\lambda$  thermosensitive lysogen ( $\lambda$  cI857 xis- kil-) phage defective for lethal and lytic functions of the prophage. Upon thermal shift to 42°C, the thermolabile cI857 integrase repressor becomes inactivated, thus inducing the integrase expression. The integrase is produced and its recombination activity on the att sites generates two recombination products, which are called minicircle (containing the therapeutic gene expression cassette and the recombinant attR site) and miniplasmid (containing the origin of replication, the antibiotic marker and the attL site) (Figure 1 [3]). In D1210HP, the yield of non-recombined plasmid is around 40% of the starting material. Only small quantities of minicircles are recovered due to the sub-optimal plasmid copy number, incomplete recombination and the presence of multimeric forms. The introduction of the parA gene (encoding a 24 kDa resolvase) into the D1210HP genome, together with the introduction of a multimer resolution site (MRS) between attP and attB allow the minicircle multimers to be resolved [4]. Up to 1.5mg DNA minicircle per litre of bacterial culture can be produced under optimised conditions [4].

After recombination, the minicircle is purified by digesting the miniplasmid with restriction enzymes. *In vitro* experiments were performed with a minicircle encoding the luciferase gene and the cationic lipid RPR120535 (2-(3-[4-(3-amino-propylamino)-butylamino]-propylamino)-N-dioctadecyl-carbamoylmethyl-acetamide [5]). The minicircle leads to a 2- to 10-fold increase in luciferase activity in various cell lines compared with the non-recombined plasmid with the same molarity of luc-cassette [3]. This transfection enhancement was confirmed with a modified firefly luciferase (luc<sup>+</sup>)-minicircle and a  $\beta$ -galactosidase-minicircle on NIH 3T3 cells [1]. *In vivo* injection of the minicircle into mouse skeletal muscle and experimental tumours gives strongly higher reporter gene expression compared with the non-recombined plasmid. Enhancement of mouse tibial cranial muscle gene transfer is up to 32 times higher with luc-minicircle, 50 times higher with



**Figure 1** - Main features of minicircle DNA. Minicircles are the product of site-specific recombination between the attB and attP sites driven by *E. coli* bacteriophage  $\lambda$  integrase. They thus lack an origin of replication and an antibiotic resistance gene (for detail see [2]). They cannot self-replicate nor confer antibiotic resistance to other micro-organisms or cells.

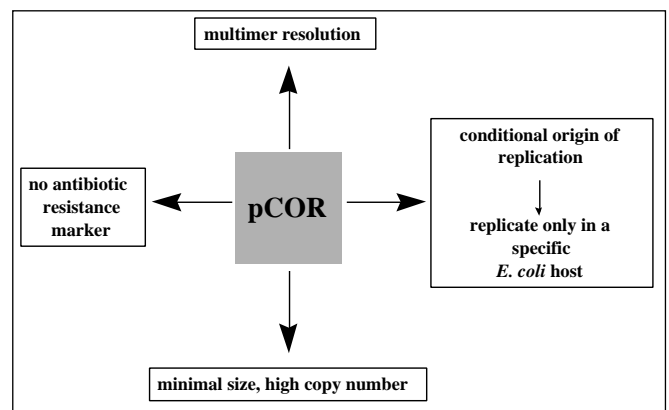
luc<sup>+</sup>-minicircle, and 13 times higher with  $\beta$ -gal minicircle [1]. The percentage of transfected myofibers per muscle is also higher when injecting luc<sup>+</sup>-minicircle than that observed following injection of the non-recombined plasmid, with minimal levels of muscle damage [1].

Minicircle purification is the major limitation of the achievement of this strategy: restriction enzymes cannot be used for the production of clinical DNA samples. Chromatographic purification methods have been tested and yield satisfying results (to be published). Triple helix-mediated affinity chromatography of plasmids [6] represents a promising technology for the efficient separation of a minicircle from a miniplasmid after successful recombination.

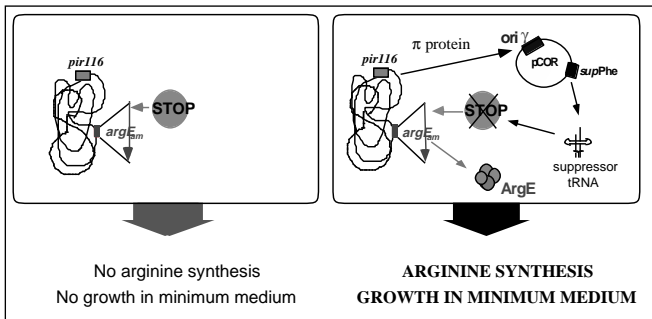
## 2. pCOR

More recently, an original host/vector system with a conditional origin of replication was designed [7]. The vector (pCOR for plasmid with conditional origin of replication) backbone consists of three bacterial elements: the R6K  $\gamma$  conditional origin of replication (0.4 kb ori  $\gamma$ ), a tRNA suppressor gene (0.2 kb sup Phe), and a cer (0.4 kb ColE1 resolution) fragment to resolve pCOR oligomers (see Figure 2). The size of the pCOR plasmid is thus minimal, which should limit the potential inflammatory response to the prokaryotic CpG sequences during clinical trials.

Propagation of pCOR plasmid is restricted to a specifically engineered bacterial strain expressing the R6K  $\pi$  initiator protein (product of the pir gene), which binds and exerts a trans-positive regulation on the conditional origin of replication (Figure 3 [8]). On the other hand, this bacterial strain carries an amber mutation in the argE gene, involved in arginine biosynthesis, and thus cannot grow on a minimal medium lacking arginine. This mutation can be corrected by the phenylalanine-specific suppressor tRNA encoded by the sup Phe gene on the pCOR plasmid, thus leading to a functionally active argE protein bearing a single Tyr $\rightarrow$ Phe mutation, allowing the strain to grow on minimal medium [8]. With this pCOR construction, no antibiotic resistance gene is required.



**Figure 2** - Main features of pCOR plasmid. pCOR plasmids can only replicate in  $\pi$ -producing (pir-116 gene product) bacteria considerably limiting their host range. pCOR selection does not require antibiotics but uses the expression of a synthetic amber suppressor tRNA gene, specific for phenylalanine (sup Phe). This suppressor corrects an amber mutation in argE gene allowing the recombinant host strain to grow on a minimal medium lacking arginine [1].



**Figure 3** - pCOR host vector system. The XAC-1pir116 strain requires arginine for growth on minimal medium whereas the arginine deficiency of the pCOR host is corrected by the tRNA sup Phe from the plasmid. The amber mutation is shown by a stop and its correction by a crossed-out stop.

The bacterial strain was optimised to enhance pCOR production. Firstly, the mutation of the endA gene avoids the production of endonuclease I which could trigger degradation of pCOR DNA at the pCOR production and purification stages. Then the insertional inactivation of the traD gene abolishes F' transfer and thus avoids low-frequency illegitimate co-transfer of pCOR by conjugation [8].

*In vitro* standard transfection assays and *in vivo* injection into OF1 murine tibial anterior muscles with luc and luc+ pCOR leads to higher levels of reporter gene expression compared with a conventional plasmid harbouring the same expression cassette [8]. The pCOR plasmid is presently being used in a phase I clinical trial using the FGF1 gene for peripheral artery occlusive disease.

## II. OPTIMISATION OF PLASMID DNA FOR NUCLEAR TARGETING

When a green fluorescent protein (GFP) plasmid was microinjected into the nucleus of CV1 cells, about 50% of the injected cells expressed GFP. On the other hand, when the same amount of plasmid was injected into the cytoplasm, no expression of the reporter gene was observed [9]. This may be due either to a lack of diffusion in the cell cytoplasm, or to the incapacity of the plasmid DNA to enter the cell nucleus.

The nucleus is the cellular compartment that encloses chromatin and the machinery necessary for gene transcription. Its composition has to remain intact in order to maintain the integrity of the nuclear structure. Although access to the nucleus is a highly restricted process, a multitude of macromolecules have to enter and exit the nucleus, for the control of the basic cellular metabolism and to respond to changing environmental conditions. Except during mitosis, when the nuclear envelope disappears, the only way macromolecules can enter the nucleus is through the nuclear pore complex (NPC). The NPC allows passive diffusion of small molecules, up to 9 nm, or active transport of larger molecules (up to 25 nm) [10]. The size of a plasmid (from 2 to 50 kbp) places it in the group of macromolecules which have to be imported into the nucleus through an active import process.

The nuclear import mechanism of macromolecules (proteins, RNAs and RNPs) is well documented [11-16]. This mechanism is energy-dependent and carrier-mediated. Karyophilic

macromolecules bear one or more peptide nuclear targeting signals called nuclear localisation sequences (NLSs) [17-20]. Alternatively, a macromolecule can form complexes with karyophilic proteins having NLSs for active importation into the nucleus ("piggyback" transport) [21, 22].

Depending on the imported protein, one or two free cytoplasmic transport factor(s) called karyopherin(s) associate(s) with the NLS sequence to form a pore-targeting complex [15, 23, 24]. These karyopherins are importin- $\alpha$  and importin- $\beta$  for proteins bearing the SV40 large T antigen NLS, snuportin and importin- $\beta$  for the m3G UsnRNPs, or transportin alone for the shuttling hnRNP protein A1 [22]. The complexes dock on the distal end of the fibrils protruding from the cytoplasmic ring of the nuclear pore complex. They are translocated through the pore by an energy-dependent mechanism, which has not been fully elucidated, consisting in a series of dissociation/re-association steps of the complex with the nucleoporins.

The nuclear import of proteins is also controlled by the cellular localisation of the protein. This is the case of transcription factors such as NF $\kappa$ B. To be active, these transcription factors have to enter the nucleus and bind to the DNA. Cytoplasmic retention of NF $\kappa$ B is the major regulatory mechanism for the inactivation of NF $\kappa$ B. When the cells are not stimulated, the NF $\kappa$ B are cytoplasmic, sequestered by the I $\kappa$ B family members. The  $\kappa$ B inhibitors mask the NF $\kappa$ B NLS, preventing them from nuclear import. When the NF $\kappa$ B/I $\kappa$ B complex dissociates, NF $\kappa$ B is targeted to the nuclear pore, nuclear translocation and DNA binding take place: the transcription factor is active [25].

### 1. A nucleotidic sequence can be a nuclear targeting signal

In a recent study, Dean microinjected plasmids containing the SV40 early promoter/enhancer sequence into the cytoplasm of TC7 cells and the plasmids were detected in the nucleus of the majority of the cells by *in situ* hybridisation 8 h after microinjection [26]. Plasmids lacking this sequence, or plasmids containing other parts of the SV40 promoter sequence, did not localise in the nucleus. In parallel experiments, the nuclear import mechanism was shown to be dependent upon energy and cytoplasmic factors. The nuclear import of SV40 bearing plasmids occurred through the nuclear pore complex, since import was inhibited by wheat germ agglutinin (WGA) and by antibodies raised against the nucleoporins. Moreover, the plasmids appeared localised in the areas of active transcription and message-processing inside the nucleus.

To confirm this study, the same plasmids were tested in the digitonin-permeabilised cell model [27]. The plasmids were hybridised with a fluorescein labelled PNA (peptide nucleic acid), in order to retain their transcriptional potential. This fluorescent plasmid, containing the nuclear targeting sequence of the SV40 early promoter/enhancer, was incubated with digitonin-permeabilised HeLa cells, in the presence of the import machinery and an energy-regenerating system. The nuclear import was effective after 90 mn, and maximal after 4 h.

The same group (Dean *et al.*) tested other promoter sequences but these sequences did not show similar nuclear targeting properties [27]. In order to identify another potential sequence

with these targeting properties, we have constructed a library of 7500 plasmids, each plasmid containing a 35 nucleotide-random sequence, and the lacZ reporter gene. After isolation of the plasmids we sequenced a representative number of random sequences to show that they were effectively degenerated. Pools of 96 plasmids were tested for nuclear import on digitonin-permeabilised HeLa cells. The pools were incubated for 30 min with permeabilised cells in the presence of the nuclear import machinery and an energy-regenerating system. The nuclear DNA was then extracted and purified, and DH5 $\alpha$  ultra-competent bacteria were transformed with this nuclear extract. None of the samples showed a representative increase in the number of colonies growing on ampicilline/X-Gal medium. Thus, this approach did not lead to the identification of a specific insert or a group of specific inserts conferring increased active nuclear uptake in the digitonin-permeabilised cells system used. We then transfected human aortic smooth muscle cells (hAoSMC) and HeLa cells with the 96 plasmid pools described above using the lipopolyamine RPR 120535. AoSMC are known to be poorly transfected cells, either because the plasmids cannot enter the cell, or because plasmids badly diffuse in the cytoplasm or cannot pass through the nuclear envelope. No increase in the reporter gene expression was observed 24 h after transfection. Transfection with a control fluorescent plasmid showed that the plasmid can pass the cellular membrane as fluorescent-spotted labelling of the entire cytoplasm but no nucleus labelling could be detected after 24 h transfection. In conclusion, none of the sequences of our library could be shown to significantly target a plasmid to the cell nucleus.

## 2. Covalent coupling of NLS peptides to the DNA plasmid

Sebestyen *et al.* [28] have developed a two-step method to link large SV40 T antigen NLS-peptides (PKKKRKV) to fluorescently labelled plasmid DNA. The NLS-peptide was first linked to a bromoacetamide derivative of cyclopropa-pyrroloindole (CPI) and then incubated with plasmid DNA. Between twenty-four and one hundred peptides were covalently linked per kilobase pair (kbp) DNA, resulting in a majority of nicked NLS-plasmids. This NLS-bearing DNA could be linearised and then ligated to a linear DNA containing a reporter gene expression cassette. These NLS-plasmids have been shown to be actively imported in the nucleus of digitonin-permeabilised cells when more than 40 NLS-peptides/kbp are linked to the plasmid. This resulted in a spotted staining of the nucleus of digitonin-permeabilised cells, and the spots did not co-localise with the nucleoli of the cells. The rate and the extent of nuclear import were related to the density of NLS peptides coupled to the plasmid. The modification of DNA by NLS peptides totally abolished reporter gene transcription. Moreover, no nuclear staining could be seen when such NLS-plasmids were microinjected into the cytoplasm of HeLa cells.

Zanta *et al.* [29] adopted a totally different approach. The authors developed a linear nucleic acid by digesting plasmid with restriction enzymes, and subsequently capping with hairpin oligonucleotides [30]. This molecule was coupled to a single NLS peptide on one of the hairpin caps. With this vector, the expression level was increased 10- to 1000-fold after transfection

of various cultured cells with a luciferase reporter gene. The time-course study of transgene expression showed that the presence of a single NLS peptide seemed to accelerate the rate-limiting step of transfection. The mechanism implicated here was hypothesised to involve a threading effect of the NLS. NLS would help docking DNA on NPC, and as soon as the DNA starts to enter the nucleus, the DNA would be condensed by histones into chromatin-like structures which would pull the filamentous molecule into the nucleus. According to the proposed model, the presence of multiple NLS on the linearised plasmid would then inhibit the nuclear import, since the NLS-importing complexes could dock on adjacent nuclear pores, and since the DNA could be pulled in opposite directions and blocked on the nuclear envelope.

Even if this approach shows an interesting enhancement of the expression level of the transgene, the molecule remains difficult to produce, and future pharmaceutical development and production of this chimeric DNA cannot be investigated at the present time. Moreover, linearised plasmid DNA seems to lose part of expression vector potency compared with the original supercoiled plasmid.

In a parallel effort, in order to develop a vector that would keep the structural integrity of plasmids, we covalently coupled large T SV40 antigen NLS to circular plasmid DNA [31]. This was achieved via the coupling of cysteine-NLS to p-azido-tetrafluoro-benzyl maleimide, and photoactivation of a controlled number of this compound in the presence of the plasmid. The structural integrity of the plasmid was preserved with this technique: the plasmid-NLS conjugates appeared on agarose gels as supercoiled DNA. This NLS-plasmid conjugate effectively bound to an importin- $\alpha$ -GST fusion protein in a NLS-dependent fashion, showing that NLS peptides bound to DNA could still interact with their receptor. Cytoplasmic microinjection of lissamine-rhodamine B labelled NLS-plasmids did not lead to any nuclear staining: the fluorescence remained cytoplasmic and progressively vanished to a residual spot, probably at the injection site, 6 h after injection. The reporter gene was expressed after cationic-lipid mediated-transfection of this NLS-plasmid in NIH-3T3 cells, showing that the functional integrity of the plasmid was maintained. However, expression enhancement was not significant when 3 or 8 NLS peptides were coupled to the plasmid, and a 60% expression decrease was observed when 43 NLS were coupled.

As indicated, the covalent association of numerous NLS on non-specific sites on the plasmids could inhibit the reporter gene expression. In a site-directed ligand-coupling strategy, we covalently coupled a single NLS peptide at a specific site on plasmid DNA by triple helix formation [32]. The NLS was chemically coupled to the 3' end of a GA<sub>19</sub> oligonucleotide (5'-AAGGAGAGGAGGGAGGGAA-3'), which relates to a specific GA<sub>19</sub> sequence via a triple helix. A psoralen residue was linked to the 5' end of the GA<sub>19</sub> oligonucleotide, allowing covalent attachment of the oligonucleotide to DNA after photoactivation. This GA19 sequence was cloned upstream of the LacZ expression cassette in a plasmid DNA. The plasmid was incubated overnight with an equimolar amount of the GA<sub>19</sub>-NLS oligonucleotide peptide conjugate, allowing the oligonucleotide to bind to the major groove of duplex DNA by

forming a triple helix. The plasmid was then photoactivated, in order to covalently link the oligonucleotide-peptide conjugate to the plasmid through the psoralen moiety at the specific site defined by the triple helix. With this approach, a single NLS was bound to the plasmid, and the plasmid remained supercoiled. By this technique, several of such sequences could be covalently linked in a site-specific fashion to a plasmid, by simply inserting the required number of target triple-helix sequences in the plasmid. Moreover, this approach can be used to produce the plasmid on a large scale. The expression of the transgene was maintained after transfection on NIH-3T3, but the increase of expression over non-modified plasmid was not significant, either *in vitro* or *in vivo* (unpublished data).

Subramanian *et al.* [33] studied another import system and used a non-covalent NLS coupling to plasmid DNA: they chemically conjugated the nuclear targeting M9 sequence of hnRNP A1 to a 13-peptide cationic peptide (scramble SV40 large T antigen NLS (ScT)), in order to increase the positive charge of the peptide, thus promoting ionic interaction with polyanionic DNA. They complexed M9-ScT with a lacZ plasmid and observed a 63-fold increase in  $\beta$ -Gal expression after bovine aortic endothelium cell (BAEC) lipofection. A classical nuclear import test on digitonin-permeabilised BAECs showed that the M9-ScT fluorescently labelled plasmid was located in the cell nucleus, whereas a fluorescent control plasmid was located in the remaining cytoplasm and on the nuclear envelope of the cells.

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In conclusion, significant progress has been made to reduce plasmid size and backbone, resulting in potentially less immunostimulatory plasmids for gene therapy. Such an improved expression vector is already in clinical use. Moreover, although an interesting enhancement in terms of the efficacy and specificity of gene transfer has been achieved by targeting the delivery vector associated to the plasmid, particularly in *in vitro* studies, further investigation should be geared towards conferring some intrinsic targeting properties on the plasmid molecule itself. This can be attempted either by inserting an appropriate DNA sequence, or by covalent or non-covalent grafting of a targeting moiety on the plasmid. Although such recent approaches have yielded results that are either still contradictory or unsuitable for pharmaceutical development, these new avenues of research undoubtedly show great promise for the future of gene therapy.

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## MANUSCRIPT

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