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Improved gene delivery formulations and expression systems for enhanced transfection efficiency

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Abstract: Effective gene therapy requires efficient delivery of DNA to transfected cells followed by high levels of gene expression. Our laboratory has developed two new delivery formulations LPDI containing the DNA condensing agent protamine sulfate and reconstitued chylomicrons containing a hydrophobic lipid/DNA complex. LPDI can produce higher levels of gene expression than the corresponding liposome/DNA complexes alone both *in vitro* and *in vivo*. Reconstituted chylomicrons also induce high gene expression in the liver through intraportal injection. To increase overall gene expression of delivered DNA, an improved cytoplasmic expression system has been developed using a unique T7 RNA polymerase autogene pCMV/T7-T7pol. High levels of reporter gene expression were detected in the presence of this new autogene.

Introduction: Many advancements have been made over the past several years in the fields of non-viral gene therapy to enhance gene delivery and expression to cells both *in vitro* and *in vivo*. This had mainly been due to the development of formulations such as cationic liposomes for delivery of transgenes to cells. Cationic liposomes are good delivery formulations due to such charateristics as non-immunogenicity, minimal toxicity and simplicity of large scale production and use (ref. 1-3). However, these first generation cationic liposomes are serum sensitive, less efficient in cell transfections and are not targetable to become cell specific. To overcome these drawbacks, several new delivery formulations such as LPDI (ref. 4) and reconstituted chylomicrons have been developed in our laboratory. The current, improved version of LPDI consists of a cationic peptide, protamine sulfate, which condenses the DNA to form a DNA/polycation complex before the addition of cationic liposomes such as DOTAP to increase transfection efficiency both *in vitro* and *in vivo* (ref. 5-6). On the other hand, a hydrophobic lipid (TC-chol)/DNA complex can be incorporated into reconstituted chylomicrons by emulsifying with a mixture of trigylcerides, phospholipid and cholesterol to reduce serum sensitivity (ref. 7). The latter formulation also could be targeted to specific tissues for gene delivery.

Another major obstacle in gene therapy is inefficient expression of transfected DNA due to low nuclear transport where transcription machinery resides (ref. 8-9). Since the addition of a nuclear localization signal had limited success (ref. 10), a cytoplasmic expression system was developed to express DNA in the cytoplasm of transfected cells. Exogenous T7 RNA polymerase enzyme when delivered with a reporter gene driven by the T7 bacteriophage promoter can induce cytoplasmic reporter gene expression due to the lack of a nuclear localization signal in T7 RNA polymerase (ref. 11-13). This had led to the development of several T7 RNA polymerase autogenes made up of a T7 RNA polymerase gene driven by the T7 promoter to continuously supply transfected cells with high levels of endogenous T7 RNA polymerase (ref. 14-17). A novel T7 RNA polymerase autogene, pCMV/T7-T7pol, developed in our laboratory has been shown to induce higher, more sustained cytoplasmic reporter gene expression than previous autogenes or that found with a nuclear expression system driven by the cytomegalovirus promoter CMV and does not require exogenous T7 RNA polymerase enzyme.

LPDI particles containing protamine for enhanced *in vitro* and *in vivo* transfection efficiency

Polycations such as poly-L-lysine condense DNA into polycation/DNA complexes and when combined with cationic liposomes, can enhance gene expression over that seen with cationic lipid/DNA complexes alone (ref. 4). These LPDI particles were hypothesized to have a higher transfection efficiency for several reasons: 1) LPDI are highly compacted particles (50-75 nm in size) to assist in efficient cellular and nuclear uptake, 2) condensation of DNA protects against enzymatic digestion, 3) poly-L-lysine may mimic the nuclear localization signal to assist DNA delivery to the nucleus.

Our laboratory has investigated an alternative polycation to be used in LPDI. Protamine sulfate from salmon sperm is a small (MW~4000), highly postively charged peptide due to the presence of 21 arginine residues which contains a nuclear localization signal (ref. 18). Protamine sulfate can induce higher levels of gene expression than poly-L-lysine and other types of protamines when combined with DNA and DC-chol:DOPE cationic liposomes (ref. 5). As shown in Fig. 1, poly-L-lysine did enhance gene expression of a pCMV-Luciferase plasmid over that seen with DNA/DC-chol:DOPE liposomes alone by 60-fold in CHO cells. However, the protamine sulfates induced an even higher levels of gene expression which was 3-5 fold higher than expression with poly-L-lysine. Though minimal luciferase expression was observed with protamine phosphate and free base, protamine chloride produced high levels of gene expression but were still inferior to expression with protamine sulfate. This seems to be due to higher DNA condensation with protamine sulfate as seen in ethidium bromide displacement studies (ref. 4).

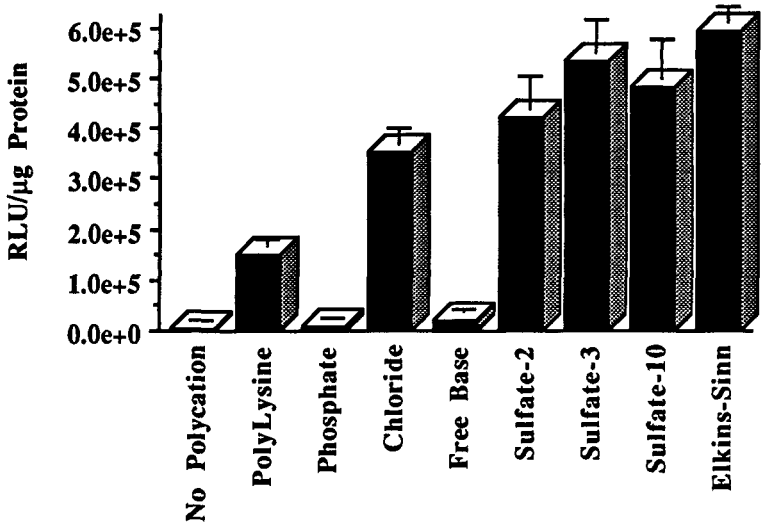

Fig. 1 Comparison of different polycations for enhancement of transfection efficiency in CHO cells. Various types of protamine (2 µg) or poly-L-lysine (1µg) was added to pCMV-Luciferase (1 µg) before complexing with 7.5 nmol DC-chol:DOPE liposomes.

Protamine sulfate was also compared with poly-L-lysine, protamine free base and protamine phosphate polycations *in vivo* by forming LPD particles with pCMV-Luciferase DNA and DOTAP liposomes at a protamine/DNA/DOTAP charge ratio of 1/1/11 (ref. 6, Fig. 2). Intravenous injection into mice showed that protamine sulfate induced the highest gene expression over all the polycations used and over that seen with DOTAP cationic liposome/DNA complexes alone. Gene expression was highest in the lung and spleen when injected intravenously as seen in Fig. 2. However, gene expression in the lung and spleen was dramatically reduced when LPD was injected intraportally (ref. 6). Therefore, LPDI particles containing protamine sulfate used both *in vitro* and *in vivo* can enhance gene expression over DNA/liposome complexes and other polycations such as poly-L-lysine. Protamine sulfate is also safe to use *in vivo* due the fact that it is an FDA approved compound with low immunogenicity.

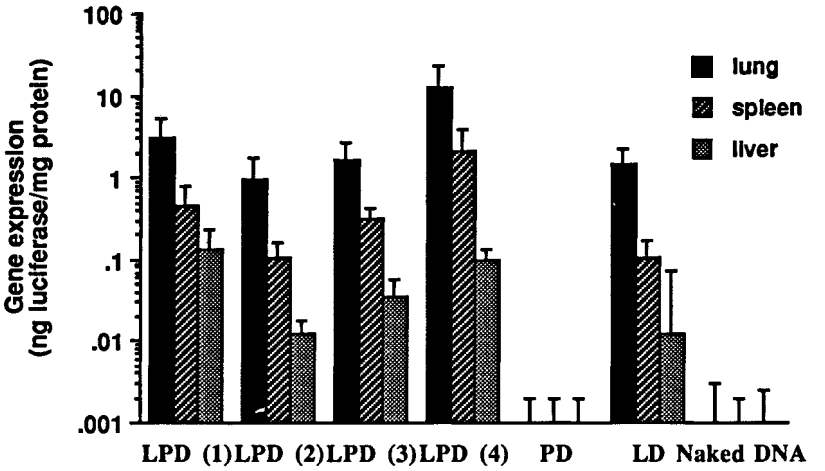

Fig. 2 Comparison of several different formulatios for their in vivo gene expression efficiency by i.v. administration to CD1 mice. LPD containing polylysine hydrobromide (1), protamine free base (2), protamine phosphate (3), or protamine sulfate-USP (4) was prepared at a protamine/DNA/DOTAP charge ratio of 1/1/11. PD: protamine sulfate/DNA complexes; LD: DOTAP cationic liposome/DNA complexes

Reconstituted chylomicrons *in vivo*

Though LPDI can increase transfection efficiency, this formulation still could not be targeted to specific cells. This is mainly due to the fact that LPDI is highly positively charged causing high interaction with negatively charged serum components. It would therefore be beneficial to use neutral or anionic formulations for targeting purposes. Our laboratory investigated the possibility of delivering a

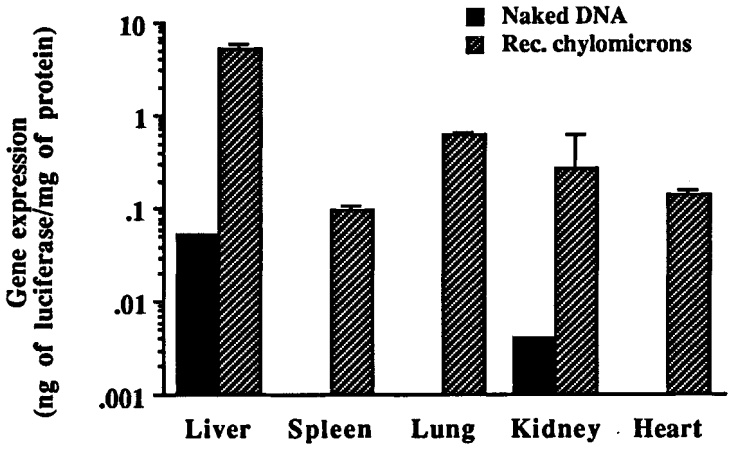

Fig. 3 In vivo gene expression following portal vein injection. CD1 mice were injected with 100 μg of naked DNA or DNA in the form of reconstituted chylomicrons.

hydrophobic lipid/DNA complex by incorporation into reconstitued chylomicrons. A cationic cholesterol derviative known as TC-Chol was added to pCMV-Luciferase DNA to form a hydrophobic lipid/DNA complex which increased the solubility of the DNA in chloroform (ref. 7). This complex was then incorporated into reconstituted chylomicrons by emulsifying with trigylceride, L-α-phosphatidylcholine (PC), lysophosphatidylcholine (lyso PC), cholesterol (Chol) and cholesteryl oleate in a 70: 22.7: 2.3: 3.0: 2.0 weight ratio. This resulted in 60% incorporation of the hydrophobic lipid/DNA complex with a mean diameter of 107 ± 16 nm (mean ± s.d.). The reconstitued chylomicrons were subjected to intraportal injection in mice and various organs were tested for luciferase expression (ref. 7, Fig. 3). High levels of luciferase expression were detected in liver, spleen, lung, kidney and heart with the highest levels seen in the liver. Expression levels in the liver with reconstituted chylomicrons were almost 100 times higher than injections with naked DNA.

Luciferase expression was only transient *in vivo*, lasting around 7 days in the liver; however, repeated injection could prolong expression (ref. 7). When injected intravenously, the reconstituted chylomicrons demonstrated low efficiency with minimal luciferase expression in the liver (ref. 7). However, this problem could possibly be overcome by the addition of targeting ligands such as apolipoprotein E for efficient receptor-mediated uptake by the liver.

Cytoplasmic expression by a novel T7 RNA polymerase autogene

New delivery formulations usually enhance the delivery of DNA to the cell, primarily to the cytoplasm; however, this does not solve the problem of limited nulcear transport of DNA where transcription machinery resides (ref. 8-9). Cytoplasmic expression systems utilizing T7 RNA polymerase were developed to avoid nuclear delivery and take advantage of the high levels of DNA in the cytoplasm. Many autogenes have been desinged to express the T7 RNA polymerase gene in the cytoplam via its own T7 promoter (ref. 14-17). Therefore, these autogenes work to continously produce T7 RNA polymerase in the cytoplasm to then drive expression of trangenes carrying the T7 promoter. These autogenes did improve expression levels, however, several problems still remained. These auotgenes were difficult to amplify and purify from bacteria. They still required the addition of exogenous T7 RNA polymerase to induce initial expression from the autogene which is expensive and possibly immunogenic. Finally, expression levels still remained low and only lasted a few days. One example of these autogenes is pT7 AUTO 2C$^-$ (Fig. 4A).

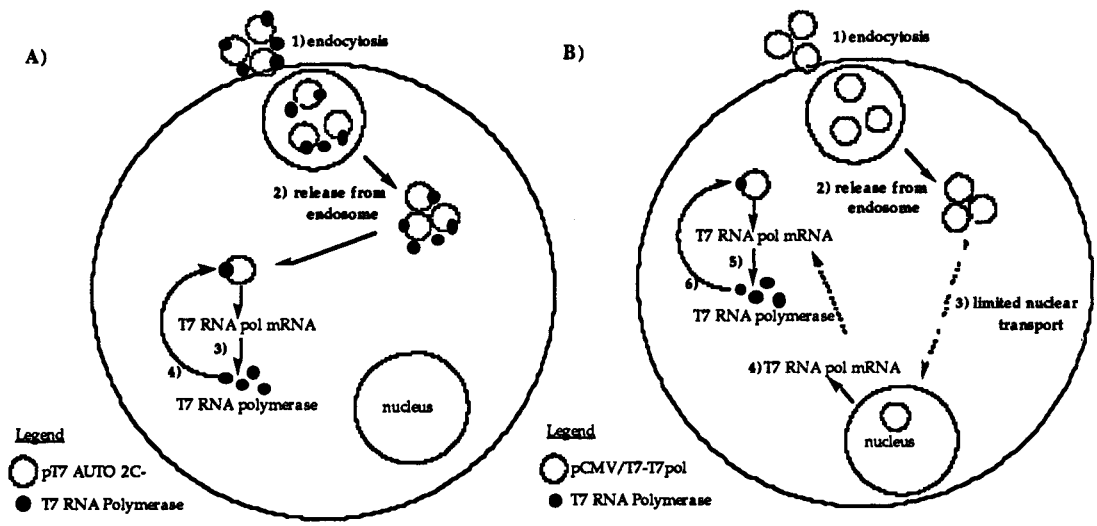

Fig. 4 Mechanisms for endogenous T7 RNA polymerase expression with pT7 AUTO 2C- (A) or pCMV/T7-T7pol (B)

When this autogene is co-delivered with exogenous T7 RNA polymerase enzyme to cells through endocytosis (1), they are released from endosomes into the cytoplasm (2). pT7 AUTO 2C$^-$ is then

transcribed by the exogenous T7 RNA polymerase enzyme and translated in the cytoplasm (3). The resulting endogenous T7 RNA polymerase then recognizes the T7 promoter on the autogene to induce further transcription/translation of T7 RNA polymerase.

Our laboratory has constructed a new T7 RNA polymerase autogene, pCMV/T7-T7pol, which avoids the problems associated with the previously used autogenes. It consists of a T7 RNA polymerase gene driven by a cytomegalovirus (CMV) promoter and a T7 bacteriophage promoter (Fig. 4B). When this autogene enters the cell through endocytosis (1), the pCMV/T7-T7pol is released from endosomes (2) to allow a small amount of DNA to enter the nucleus (3). This allows the first round of T7 RNA polymerase expression to begin in the nucleus driven by the CMV promoter (4) followed by translation in the cytoplasm (5). Only a small amount of the nuclear derived T7 RNA polymerase needs to be present to initiate expression of the autogene in the cytoplasm via the T7 promoter (6). Cytoplasmic expression will then continue to produce high, sustained levels of T7 RNA polymerase to then drive transgene expression in the cytoplasm.

pCMV/T7-T7pol was co-delivered with pT7-CAT to 293 cells in the absence or presence of exogenous T7 RNA polymerase enzyme. This was compared with activities produced with a previously used autogene pT7 AUTO 2C$^-$ driven only by the T7 promoter under the same conditions.

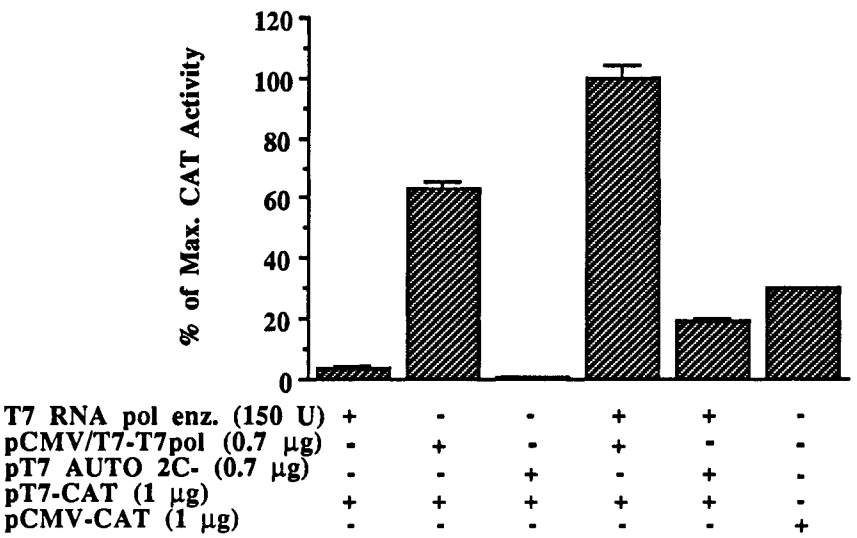

Fig. 5 Comparison of CAT activities produced by different sources of T7 RNA polymerase in 293 cells. pT7-CAT (1 μg) was co-transfected with the indicated amounts of autogene or exogenous enzyme via DC-chol:DOPE liposomes.

As shown in Fig. 5, pCMV/T7-T7pol produced higher levels of CAT activity than pT7 AUTO 2C$^-$ in the presence and absence of exogenous T7 RNA polymerase. pT7 AUTO 2C$^-$ only produced CAT activity in the presence of exogenous T7 RNA polymerase since it requires this enzyme to initiate expression at the autogene. pCMV/T7-T7pol can also produce higher CAT activity over longer periods of time (>7 days) than pT7 AUTO 2C$^-$ due to larger production of T7 RNA polymerase levels in the cytoplasm by pCMV/T7-T7pol detected by Western blot analysis (data not shown). pCMV/T7-T7pol can also induce CAT activities higher than that seen with a nuclear expression system pCMV-CAT (Fig. 5). Therefore, pCMV/T7-T7pol could replace exisiting autogenes for high, sustained cytoplasmic expression of transgenes.

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