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A. I. Aronsohn & J. A. Hughes

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Nuclear Localization Signal Peptides Enhance Cationic Liposome-Mediated Gene Therapy

A. I. ARONSOHN and J. A. HUGHES*

Department of Pharmaceutics, University of Florida, Gainesville, FL 32610

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The use of genes as therapeutic drugs will likely involve non-viral delivery systems. While traditionally less effective for gene expression, the advantages of a non-viral delivery system include ease of production, lower toxicity, and no risk of infection. However, most non-viral systems do not incorporate a mechanism for gene transport into the nucleus. Nuclear localization signal peptides can combine the increased expression of viral delivery systems with the safety and ease of preparation of non-viral delivery systems. A novel non-viral delivery vehicle consisting of a conglomerate of a synthetic nuclear localization signal peptide derived from the SV40 virus, a luciferase encoding PGL3 plasmid, and a cationic lipid DOTAP-:DOPE (1:1 w/w) liposome was transfected into SKnSH mammalian neuroblastoma cells. A three-fold increase in luciferase expression was seen with the delivery system containing a NLS peptide over cationic liposome controls. Examination of the factors that limit the rate of transgene expression can potentially lead to the discovery of new ways to improve the efficiency and efficacy of nonviral methods of gene therapy.

Keywords: Cationic liposomes, gene therapy, signal peptides

Gene therapy is an exciting new avenue in the treatment of diseases arising from single gene defects such as cystic fibrosis (Caplen et al. 1994) and for the production of therapeutic peptides to treat a variety of disorders (Pappas 1996). In viral-based delivery systems, a replication-deficient virus particle such as a retrovirus, adenovirus, or an adeno-associated virus is used to introduce DNA into the host cell either permanently or transiently, depending on the virus. Viral delivery systems are efficient but typically suffer from problems including immunogenicity and the potential for host infection with the wild type virus.

Non-viral gene therapy attempts to mimic viral methods of DNA delivery such as DNA compaction and endosomal escape, while eliminating the risks. A commonly employed method uses cationic liposomes (Felgner et al. 1987) complexed with nucleic acids. During complexation, the anionic DNA and cationic liposomes form a new entity which is

*All correspondence: Jeffrey Hughes, Ph.D., University of Florida, Department of Pharmaceutics, PO BOX 100494, Gainesville, FL 32610. e-mail: hughes@cop.health.ufl.edu

resistant to nuclease digestion and can transfect cells *in vitro* (Geschwind et al. 1994) and *in vivo* (Osaka et al. 1996).

While substantial effort has been spent on the development of cationic liposomes for gene therapy, little is known concerning the factors which influence the expression of the transgene after delivery of the cationic liposome/DNA complex to the cell. One factor limiting the rate of transgene expression may be the cell's intrinsic ability to produce protein from extracellularly administered DNA. This will be governed by factors such as the cell's transcription factors and stability of mRNA. However, this study focuses on factors involved in delivery of the DNA/liposome complex to the cell, and any of several steps in this process could limit the rate of transgene expression.

After administration, the DNA/liposome complex must reach the cell surface where it will most likely enter the cell through endocytosis (Xu and Szoka 1996). After the complex is internalized, the DNA must escape from the endosome and reach the cytoplasm. This process most likely will occur via membrane leakage, rupture, or fusion with other cellular compartments. A major barrier to efficient transgene expression is the final transport step, when the DNA must reach the nucleus for transcription.

Nuclear pore structures of approximately 70 nm in diameter are formed by points of fusion in the double layered nuclear membrane. The pores are spanned by a macromolecular assembly known as the nuclear pore complex (Goldberg and Allen 1995). The central aqueous channel of the pore complex allows for the free exchange of small molecules and regulates the vertical transport of macromolecules in a sieve-like fashion. Measurements with exogenous tracer molecules suggest an effective pore size of 9-10 nm in diameter, making it unlikely that large molecular complexes such as DNA can freely enter the nucleus.

However, addition of nuclear localization signal (NLS) peptides have been demonstrated to enhance nuclear localization of several macromolecules (Michaud and Goldfarb 1993, Stochag et al. 1993, Allbritton et al. 1994, and Xiao and Wilson 1994) including histone-bound DNA. The exact location of NLS receptors has yet to be firmly established, but candidates, collectively referred to as signal-binding proteins, are found on the nuclear envelope and in the cytosol. This observation supports the possibility that signal recognition by a cytoplasm protein is followed by transfer of the signal-protein complex to specific receptors on the nuclear envelope and/or the pore complex.

We hypothesized that the addition of NLS peptides to a cationic liposome/DNA complex delivery system would enhance transgene expression. Theoretically, an NLS peptide bound to a DNA/liposome complex could be targeted to the nucleus similar to other macromolecules. Also, most NLS peptides are characterized by a short stretch of positively charged lysine and/or arginine residues which are excellent for electrostatic interactions with pDNA. The location of the signal within the protein does not appear to affect its function since it is active at the amino or carboxyl terminal ends of proteins and also active when located internally. Based on these characteristics, we selected one of the most studied of the NLS peptides (KKKRK) to address its effects on non-viral gene therapy. Additional basic amino-acids were added to the sequence of this base peptide to enhance the electrostatic interaction with nucleic acids. In addition, this sequence was specifically designed to include the possibility of cyclelizing the peptide via the glutamic acid and the second lysine from the amino terminal. The studies with the cyclic peptide are still on-going and will be presented in a later manuscript.

All peptides used in this study were synthesized at the University of Florida Protein Core Facility, identified by mass spectrometry, and purified by reverse-phase HPLC. The core nuclear localization sequence (KKKRK) was derived from the Simian virus SV40 T antigen. The sequence used in all experiments was KKPNKKKRKE. A pGL3-control plasmid that codes for a cytoplasmically localized luciferase (Promega) was propagated in *E. coli* bacteria. Liposomes were prepared from a rehydration of a dried lipid film (Hughes et al. 1994), consisting of 5 mg dioleoyl phosphatidylethanolamine (DOPE)

(Avanti Polar Lipids, Alabaster, AL) and 5 mg of dioleoyl trimethylammonium propane (DOTAP) (Avanti) reconstituted in 5 ml of sterile water and sonicated until clear.

Plasmid DNA incubated with either NLS peptide or DOTAP:DOPE liposomes exhibited a decreased fluorescence after being treated with the DNA fluorescent stain SYBR green (Figure 1). The decrease in fluorescence is indicative of an interaction between the two species. Three μg of pDNA showed a 90% decrease in fluorescence when complexed with 10 μg of liposomes. The fluorescence of the pDNA remained at this level with increasing amounts of the liposome. A similar effect was seen with the pDNA-NLS complexes. A maximum of 42% decrease in arbitrary fluorescence units was measured with a complex consisting of 3 μg of plasmid and 10 μg of NLS peptide. The addition of 3 μg pDNA with 2.5 μg cationic liposome and 5 μg NLS peptide showed an additive effect in the decrease of arbitrary fluorescence units (Figure 2). The pDNA, liposome, and NLS complex showed a two fold decrease in fluorescence when compared to the pDNA/NLS or the pDNA/liposome complexes.

These results indicate that when the various components interact, there is at least an additive effect and perhaps a synergistic effect on the interaction between the three components. A second interpretation is that two types of particles are formed: one between the peptide and nucleic acid and the second one between the lipid and DNA. The resulting particle structure is difficult to predict since the hydrophobic interaction would push the hydrophilic peptide from the final particle. This effect may be

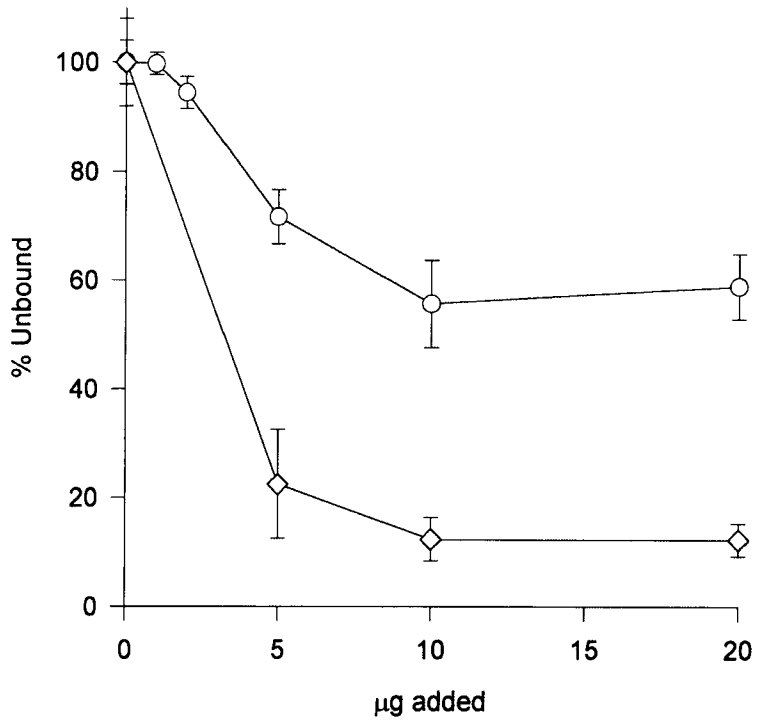

FIGURE 1 *Interaction of Either NLS or Liposome with pDNA.* Three μg of plasmid DNA (pGL3) was incubated with nuclear localization signal peptide (○) and/or cationic liposome (◇). The samples were incubated at room temperature for thirty minutes in SHE buffer (8 mM NaCl, 2 mM HEPES, 0.05 mM EDTA [pH = 7]). After incubation, the DNA was stained with the fluorescent stain SYBR (Molecular Probes, Eugene, OR) and quantified by fluorescence spectroscopy λ_{ex} = 497 nm and λ_{em} = 520 nm. Each data point is the mean of 3 independent experiments ± the standard deviation.

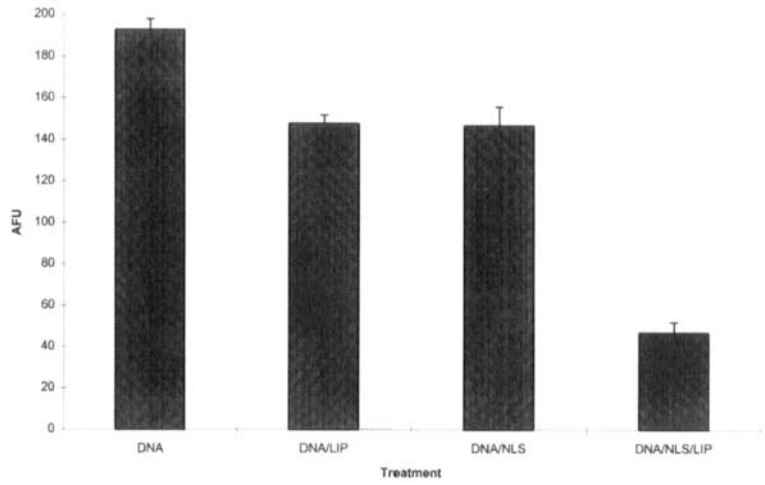

FIGURE 2 *Interaction of NLS, Liposomes and pDNA.* The additive binding effect of 2.5 μg cationic liposome (LIP) and 5 μg NLS peptide to plasmid DNA (DNA) can be seen by an additional decrease in SYBR green fluorescence of the pDNA/NLS/LIP complex when compared to the pDNA (3 μg) alone, pDNA/NLS (5 μg) complex, and the pDNA/LIP (2.5 μg) complex. Each bar represents the mean of 4 independent experiments ± the standard deviation.

balanced between the electrostatic interaction between the components and additional hydrogen bonds.

In addition to measurements of interaction between the interacting species, the final complex was evaluated by electron microscopy. Figure 3 illustrates the differences between the complexes formed between plasmid DNA and cationic liposomes and the addition of the peptide to the same complex. Morphologically, there are distinct differences in the two types of particles formed. In the cationic liposome preparation, typical cationic liposome DNA structures are observed including toroids and lipid coated DNA. With the addition of the

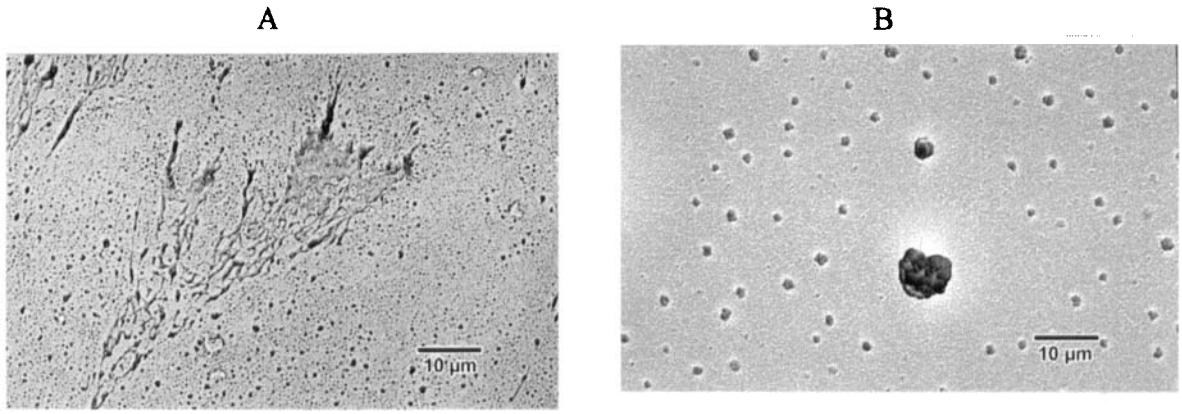

FIGURE 3 *Electron Micrographs.* Samples were prepared for visualization by first mixing pDNA and liposomes in 1:4 (w/w) ratio (A) or the addition of the NLS peptide (B). The complex was allowed to incubate for 15 minutes at room temperature. Carbon films, made by evaporation onto freshly cleaved mica, were placed on 400 mesh nickel grids. Suspensions of the particles were adsorbed to the surface of the carbon films. The grids were exposed to 1% OsO_4 for one hour, washed with water, and dried. The specimens were rotary shadowed with carbon/platinum by electron bean evaporation at a 10 degree angle and observed at 75 kV on a Hitachi H-7000 transmission electron microscope. Photomicrographs were taken at 25 K magnification.

NLS peptide the shape of the final particle becomes more spherical. There also appears to be a variation of particle size.

To address the question of whether DNA uptake is increased by the addition of NLS peptides, pDNA was covalently labeled with ethidium monoazide. Covalently attaching a fluorophore directly to the plasmid molecule allowed for quantification of delivery. As Figure 4 illustrates, with increasing concentrations of the peptides, greater amounts of pDNA were associated with the cell. This implies that the addition of the peptide to the pDNA-cationic liposome complex creates a particle with greater affinity for cellular endocytotic events or the cell membrane surface. Only cellular associated plasmid DNA was measured and no subcellular compartments were quantified.

SKnSH cells were transfected in serum-free medium with complexes consisting of pDNA and either DOTAP:DOPE liposomes, NLS peptide, or both. The NLS-pDNA complex and the pDNA transfected alone resulted in negligible luciferase expression, measured in relative light units (RLU). The pDNA-liposome complex resulted in luciferase expression on the order of 1500 RLU/μg protein. The pDNA-NLS-liposome complex resulted in higher luciferase expression of 4700 RLU/μg protein (Figure 5). These results, in combination with the cellular uptake studies, indicate that the NLS peptide interacts with the plasmid-liposome complex and that the inclusion of NLS peptides into the delivery system increases the total amount of pDNA internalized. We draw these conclusions due to the fact that NLS alone was unable to facilitate transgene expression.

The inclusion of NLS peptides could facilitate transgene expression by two routes: 1) the peptide could enhance transport of the complex to the nuclear pore complex or 2) the peptide might increase pDNA uptake or escape from the endosome. We did not attempt to delineate between the two possibilities, but rather we verified that the inclusion of the peptide increases the total amount of pDNA entering the cell. This finding correlates well with a recent report (Fritz et al. 1996) in which the use of SV40 NLS-tagged histones were used to increase cationic mediated gene therapy. These investigators report that the inclusion of the signal peptide on Histone H1 could enhance liposome mediated gene therapy in tissue culture models but did not enhance the amount of transgene produced upon microinjection into the cytoplasm or increase

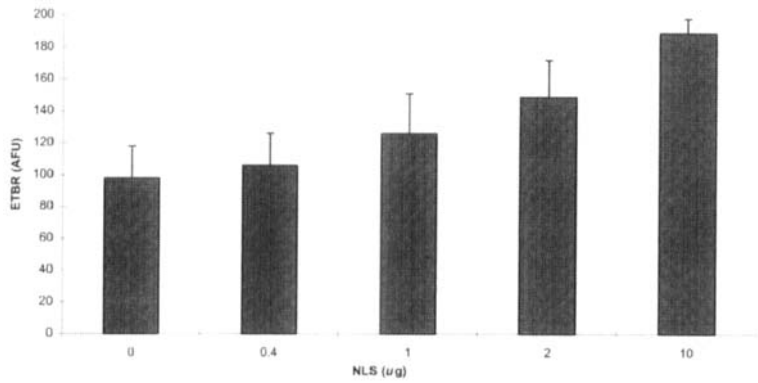

FIGURE 4 *DNA-Liposome-NLS Cellular Uptake Studies.* Plasmid DNA (1 mg) was reacted with (1 mg) ethidium monoazide (Molecular Probes) in TE buffer (pH = 7.5) for 10 minutes on ice. Ethidium was covalently linked to the pDNA by exposure to light for 10 minutes. Ethidium linked plasmid DNA was purified by ethanol precipitation (2 ×). Cellular uptake studies were conducted by incubating 3 μg of the DNA, 5 μg of the cationic liposome, and various amounts of the NLS peptide at room temperature for 15 minutes. The complexed DNA was used to transfect SKnSH cells (2×10^5) in serum-free medium (500 μL) for 4 hours. Medium was replaced with serum-containing medium and the cells grown for an additional 24 hours. The cells were lysed in luciferase lysis buffer and the amount of ethidium linked DNA was determined by fluorescence spectroscopy with λ_{ex} = 260 nm and λ_{em} = 600 nm. Each bar represents the average of 3 independent experiments ± the standard deviation.

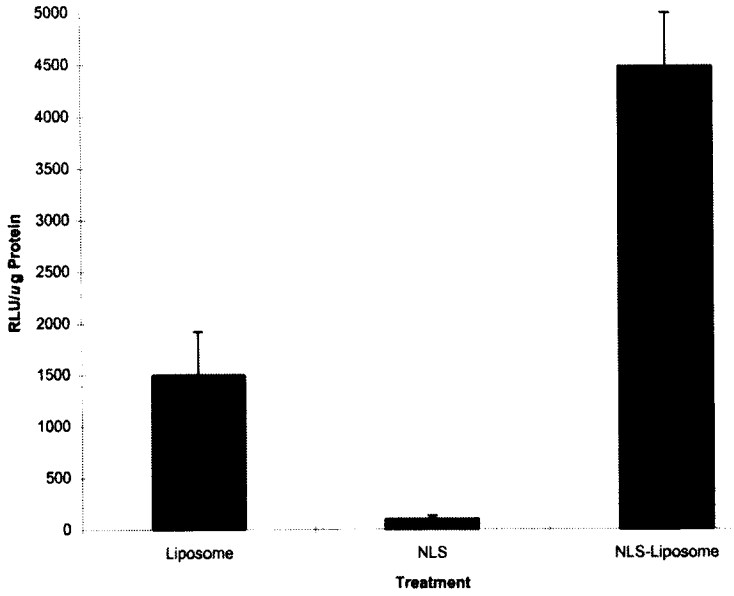

FIGURE 5 *DNA-Liposome-NLS Conglomerate Expression Study.* SKnSH cells were plated on 24 well plates in RPMI 1640 medium containing 10% FBS with 100 U/mL penicillin and 100 μg/mL streptomycin. When the cells reached 75% confluency, they were treated with DNA conjugates. The conjugates consisted of a 3 μg of plasmid DNA control treatment [DNA] with either liposome (15 μg) [DNA/LIP], NLS peptide (15 μg) [DNA/NLS], or liposome and NLS peptide (7.5 μg each) [DNA/NLS/LIP]. In these studies the peptide was always added prior to the liposomes. After the conjugates were prepared, they were incubated at room temperature for 30 minutes in serum free medium. The cells were transfected with the various DNA conjugates in serum free RPMI 1640 medium and were incubated for 4 hours. After this period, the medium was changed back to the serum-containing medium. 24 hours after the transfection, the cells were lysed in a cell lysis buffer (0.1 M K_2HPO_4, 1% Triton X-100, 1 mM DTT, 2 mM EDTA [pH = 7.8]) and the luciferase expression of each sample was quantified and corrected by using total cellular protein. Each bar represents the mean of 4 separate experiments ± the standard deviation.

the content of plasmid DNA in the nucleus. These last two findings indicate that the NLS protein may not be functioning via enhanced nuclear transport. The inclusion of basic peptides (poly-lysine) has been shown to alter the conformation of pDNA-cationic liposome complexes (Gershon et al. 1993). It is reasonable to assume that the NLS peptide might also alter the conformation of the final particle. This new type of particle that comes from the interaction of the three components might have superior transport characteristics. A second limitation of the current approach to target the nucleus is that the complex is held together via electrostatic interactions. During the endocytotic process, escape from the endosome or transport in the cytoplasm, the complex may disassociate. This dissociation event has been proposed as a possible mechanism of pDNA escape from the endosome via cationic mediated gene transfer (Xu and Szoka 1996). In future studies, we will include the covalent attachment of NLS peptides to pDNA to assist in understanding the rate limiting steps in non-viral gene therapy.

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