The Role of Proteoglycans in the Delivery of Cationic-DNA Complexes and Enhanced Delivery by Folate Receptor-Mediated Endocytosis

Thesis by

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Acknowledgments

Graduate school has been the greatest, most exhilarating challenge of my life. I have learned some hard lessons about research, about life and about growing up, but looking back, I would not trade these experiences for anything. I have developed the ability to conduct independent research, which in my opinion, is the most privileged skill anyone can have. Science will challenge me for the rest of my life, and I am thrilled! Through research, I can live my life in a constant state of bewilderment and fascination, and I consider this to be a gift. The positive experiences that I have had at Caltech are the result of many people who have helped me along the way.

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Finally, I'd like to dedicate this thesis to my family who has always encouraged me to go for the gusto! I love you!

Abstract

Experiments were conducted to elucidate the mechanisms of polylysine-mediated transfection into mammalian cells *in vitro*. In the first chapter, the role of membrane-associated proteoglycans in transfection was investigated by testing transfection efficiency under a number of assay conditions. Cells were treated with sodium chlorate in order to desulfate glycosaminoglycan chains. Chlorate treatment inhibited expression of luciferase, the intracellular uptake of DNA, and binding of DNA to the cell surface. Expression, uptake, and binding of DNA was also inhibited by exogenous glycosaminoglycans and by glycosaminoglycan lyases. Similarly, each of the transfection steps was severely inhibited in CHO cell mutants, incapable of synthesizing proteoglycans. Transfection by certain cationic liposomes was also inhibited in the mutant cell line. The possible implications of these results for gene therapy of diseases affecting hematopoietic cells is described. In the second chapter, polylysine-mediated gene delivery via the folate receptor was developed. Gene delivery by folate receptor-mediated endocytosis was approximately 18 times higher than by nonspecific endocytosis of polylysine-DNA in the presence of chloroquine. A number of controls confirmed that the folate receptor was critical in the gene delivery mechanism. When chloroquine was removed from the media, transfection efficiency dropped approximately 30 fold, suggesting that gene delivery occurred via a lysosomal pathway. In the final chapter, transfection by folate receptor-mediated endocytosis was characterized by 2-photon laser scanning microscopy. KB cells were transfected with folate-polylysine-DNA complexes labeled with YOYO, a dimeric

cyanine intercalator, and a single cell was examined over a two hour period. Although additional controls are required, preliminary evidence suggests that mitosis is not a strict requirement for delivery of DNA into the nucleus of cells.

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Chapter 1: Introduction

Advances in molecular biology and genetics have stimulated a worldwide effort to map the human genome. As a result, thousands of genes have been located and sequenced. This powerful information has spawned research in genetic screening technology and in gene therapy (1).

Gene therapy is broadly defined as the treatment of disease states through the genetic modification or augmentation of cells. Because treatment occurs at the level of DNA transcription, gene therapy has the potential to eradicate disease. However, gene therapy technology is not fully developed or characterized, and most gene therapy protocols are still carried out in animal models and *in vitro*. The purpose of this introduction is to acquaint the reader with the general principles and goals of gene therapy and with the properties and limitations of the viral and non-viral vectors currently employed to deliver foreign DNA into eukaryotic cells.

Gene Therapy Modalities

Gene therapy may be accomplished by two modes. Individual cells may be removed, transfected *ex vivo* and implanted back into the patient. Alternatively, whole tissues or solid tumors may be genetically modified *in vivo* by delivering the gene intravenously, intranasally, intraperitoneally, or in situ (2).

Many diseases such as severe combined immunodeficiency disease (3), familial hypercholesterolemia (4), Gaucher's disease (5), and cystic fibrosis are encoded on a single locus. For these diseases, a single replacement gene is transfected into the defective cells or tissues and the required gene product is transcribed.

Other diseases are encoded by a set of multiple, discontinuous genes. In these cases, the replacement of a single gene is unlikely to lead to elimination of the disease but, a single "suicide" gene may be transfected to induce the self-destruction of diseased cells and tissues. This approach is currently being developed to treat a number of cancers (6). In one strategy, target tissues are transfected with the Herpes Simplex Virus-thymidine kinase (HSV-tk) gene and then treated with gancyclovir, a nucleotide analog. Phosphorylated gancyclovir produced by transfected cells is incorporated into genomic DNA and further elongation is prevented (7). This mode of treatment is enhanced by the "bystander effect" i.e. the passage of phosphorylated gancyclovir through gap junctions into neighboring non-transduced cells (8). Thus, the growth of the tumor is inhibited by transfecting only a fraction of the tumor mass.

Cancer cells may also be transfected with a gene to upregulate or activate an immune response against them. Cancer cells typically avoid destruction by T cells because, the display of tumor antigens by class I HLA antigens on the tumor membrane and the tumor cell production of B7 co-stimulatory molecules are defective. However, if the cDNA for class I HLA antigens or B7 antigens is transfected into the tumor cells, a T-cell dependent immune response is mounted against the tumor and tumor proliferation is inhibited (9) .

Despite the potential impact of gene therapy, the technological barriers to transferring a gene from outside of the body into the nucleus of a cell are enormous. The human body is a complex system designed to resist the invasion of foreign matter. The circulatory system and digestive systems are proficient at eliminating matter based

merely on size and chemical composition. Foreign DNA surviving this process is challenged by the adaptive and humoral immune response. Upon reaching a target cell successfully, a gene faces an entirely new set of resistances to entering the nucleus. The plasma membrane permits the passage of select or very small molecules only, and a gene ingested by pinocytosis is rapidly degraded by lysosomes and cellular deoxyribonucleases. To survive the protective mechanisms of the body, a gene would have to be disguised by a rationally designed vehicle. The remainder of this introduction discusses the vehicles currently available for gene therapy and the advantages and disadvantages associated with them.

Recombinant Viral Vectors

Viruses can circumvent many of the obstacles presented by the body. Each class of virus is endowed with characteristics that promote the successful transfer of nucleic acids into host cells. Viral nucleic acid is surounded by a protein capsid to protect the nucleic acids from serum nucleases (10). In some viruses, an envelope synthesized from lipids and glycoproteins from the host helps to disguise the virus from immediate elimination by the adaptive immune response (11). Specific proteins on the envelope or capsid enable viruses to bind to cell surface molecules and to enter cells via membrane fusion or endocytosis. In the acidic environment of the lysosome, viral proteins may change conformation as a way to facilitate the virus's escape into the cytoplasm (12). Retroviral enzymes catalyze the integration of viral nucleic acids into the host's genome (10). Viruses are natural transfection agents designed over

billions of years of evolution. It is not surprising that recombinant viruses are the most common gene therapy vectors in clinical trials.

To produce a recombinant virus, a portion of the pathogenic viral DNA or RNA is excised and replaced with the gene of interest. The viral DNA is transfected into a packaging cell line that clones the recombinant vector (Figure 1). Although unable to replicate, the recombinant virus maintains the ability to infect cells with the therapeutic gene (13). Currently used in gene therapy research are three classes of viruses: retroviruses, adenoviruses, and adenoassociated viruses (Table 1).

Recombinant retroviruses, such as the LASN virus (14) were employed in some of the first human gene therapy protocols (15). After entering the cell by receptor mediated endocytosis, the retroviral reverse transcriptase transcribes viral RNA into DNA. The DNA is circularized and then integrated into the genome of the host (11). In theory, repeated administration of recombinant retroviruses is unnecessary since daughter cells carry the gene. However, retroviral vectors are useful for gene delivery into dividing cells only and can not be produced to high titers. Moreover, retroviruses can induce cells to undergo neoplastic transformation by insertional mutagenesis (16).

Adenoviruses are also used for gene therapy. These viruses have very high titers, are trophic for quiescent respiratory and gastrointestinal cells, and can carry large pieces of DNA (17). However, expression of the gene is only short-term and repeated administration is necessary, and since adenoviruses are particularly susceptible to destruction by killer T cells, the number of possible treatment's an

individual could receive would be limited by the ensuing immune response (18). Adenoassociated viruses can yield longer term expression, eliminating the need for repeated treatments, but the research in this field is too immature to ascertain whether adenoassociated viruses will be a safe, viable alternative to retroviruses and adenoviruses (16).

In summary, viruses are promising gene therapy vehicles, but the potential dangers that could result by using them for gene therapy and the technological limitations associated with them has stimulated research into the design of alternative gene delivery techniques and synthetic virus-like vehicles.

Non-Viral Vehicles for Gene Therapy

Physical Techniques

Physical techniques use forces such as electric field gradients to compromise the integrity of the plasma membrane and enable plasmids or genes to enter cells (19). In the "particle bombardment" technique, DNA is coated onto the surface of gold or tungsten beads, accelerated by the use of an electric discharge device and fired at the tissue. Because this method imparts a large force on cellular membranes, the coated beads are able to penetrate directly into the cytoplasm (20). Particle bombardment is most efficient in cells of the epidermis, although successful transfection has been accomplished in both muscle and liver tissues *in vivo*. Expression of transfected genes persists for only 3-5 days, and plasmid DNA is not incorporated but rather exists as an unstable episome (21). These limitations and the requirement for invasive procedures

are the major reason why these methods are not being considered seriously for widespread use in human subjects.

Cationic Gene Delivery Vehicles

Dozens of laboratories are developing positively charged macromolecules that combine with DNA to form self-assembling gene delivery vehicles. The goal of this research is to develop vehicles that exploit the advantageous features of viruses but avoid their inherent limitations. In the following paragraphs, cationic gene delivery agents and the factors that influence their efficiency are described in order to set the motivation for the research discussed in this thesis.

Dendrimers

Dendrimers, or polyamidoamine cascade polymers, are a class of spherical polycations. The dendrimer core is synthesized from ammonia, ethylenediamine and methyl acrylate and grows radially (22). By controlling the number of generations, a dendrimer with a specific diameter and number of amines can be synthesized (Figure 2). The spherical polymers associate with DNA via electrostatic interactions between positively charged amines and the negatively charged phosphate backbone. Dendrimer/DNA complexes are thought to bind to anionic cell membrane components and to enter the cell via adsorptive endocytosis (Figure 3) (23).

The low pKa of the terminal amines confers a significant advantage in dendrimer mediated gene delivery. As dendrimers are transported into lysosomes, the terminal amines buffer the pH change to neutrality and inhibit the activity of

acidophilic proteases. Thus, the DNA complex is protected from lysosomal degradation. In accordance with this feature, the transfection efficiency of dendrimer-DNA complexes is unaffected by chloroquine, an agent shown to raise the pH of the lysosomal compartment (24).

To enhance gene delivery out of the endosome, a membrane destabilizing peptide, GALA (Trp-Glu-Ala-Ala-Leu-Ala-Glu-Ala-Leu-Ala-Glu-Ala-Leu-Ala-Glu-His-Leu-Ala-Glu-Ala-Leu-Ala-Glu-Ala-Leu-Glu-Ala-Leu-Ala-Ala), can be covalently linked to the terminal amines. Dendrimer-GALA-DNA complexes have transfected cells to the highest levels ever reported by a synthetic vector *in vitro* (23). Despite their low toxicity and high efficiency, dendrimers are being studied by only a handful of laboratories. As these compounds are developed and improved, they will undoubtedly gain popularity for gene therapy. Common properties of dendrimers are summarized in Table 2.

Cationic Amphiphiles

Cationic amphiphiles are the most widely used non-viral gene therapy constructs. They are composed of a quaternary ammonium group (25) or a polyamine backbone (26) linked to a lipid tail (Figure 4). Cationic amphiphiles bind cooperatively to DNA and form a positively charged bilayer around it enabling the DNA complex to associate with anionic residues on the cell membrane (27). For highest efficiencies, the charge ratio between the amphiphile and DNA must be optimized. At low lipid:DNA charge ratios, association with the anionic cell membrane is limited, and thus transfection efficiency is small. At very high

lipid:DNA, unbound lipid can competitively bind to anionic molecules on the cell membrane and can be cytotoxic. Most amphiphiles yield optimal transfection efficiencies at lipid:DNA charge ratios between 2 and 6 (26, 28). Agents that lack a carbon spacer between the quaternary amine and lipid lead to the highest transfection efficiencies (29). Possibly, the spacer deficient amphiphiles form DNA complexes that consist of a higher concentration of localized cations which can interact more strongly with anionic membrane residues. Common properties of cationic amphiphiles are listed in Table 2.

Until recently, it was believed that amphiphiles entered cells by fusing directly with the plasma membrane (27). However, recent electron microscopy data support a nonspecific endocytic uptake mechanism (Figure 3). Cationic-lipid DNA complexes are found in endocytic vesicles and at later time points, in large vacuoles in the perinuclear region (30-31). Transfection is only moderately sensitive to chloroquine treatment although the mechanism whereby complexes escape lysosomal degradation is still unknown (Table 2). Zabner *et al.* were unable to localize any transfected DNA in the nucleus by electron microscopy even though expression of the transfected gene was observed (30). Thus, the mechanism of amphiphile mediated transfection is still largely uncharacterized.

The presence of serum in the media also influences transfection. Felgner demonstrated that transfection could be eliminated by adding purified, sulfated proteoglycans to the transfection media *in vitro*. He suggested that the presence of similar polyanions in serum could competitively bind to cationic complexes and thus

inhibit transfection (32). Some amphiphilic vehicles are more sensitive to serum than others, but the reasons for this are currently unknown. This serum sensitivity could seriously impede efforts to use these vehicles *in vivo*.

One of the most important variables in cation mediated transfection is the cell type. Suspension cells and primary cells are notoriously difficult to transfect (33-34). When transfected with Lipofectin (GIBCO-BRL), K562 suspension cells, derived from a human leukemia cell line, express approximately 4 orders of magnitude less reporter gene than NIH 3T3 cells (35). Primary hemotopoietic cells generally yield 1-2 orders of magnitude less expressed protein than cell lines. This low efficiency prohibits the use of cationic amphiphiles for gene replacement therapy of hematopoietic stem cells *ex vivo*. However, Harrison *et al.* discovered that if primary human mononuclear cells and hematopoietic stem cells differentiated in culture for two days, transfection increased by an order of magnitude. Cells artificially differentiated by treatment with tetradecanoylphorbol 12,13-acetate (TPA), a protein kinase C activator, or sodium butyrate yielded higher expression levels as well (36). Methods to increase transfection efficiency without inducing differentiation need to be developed.

Cationic amphiphiles are the leader for *in vitro* gene delivery. Because of their lipid content, amphiphiles are expected to be non-immunogenic. In most cell lines, they yield high levels of transfection even without chloroquine. Indeed, they possess some of the properties of viruses. The major disadvantages of cationic amphiphiles are their inability to target specific tissues and their relative inefficiency in primary

cultures. In contrast, gene delivery to specific receptors in both established cell lines and primary cultures can be accomplished using derivitized polylysine.

Polylysine

Polylysine binds cooperatively to DNA. Upon addition of a stoichiometric amount of positive charge, polylysine-DNA complexes undergo a comformational change and condense into toroidal or rod like structures of varying size, depending on the ionic strength of the buffering solution and other factors (37-38).

Compared to cationic amphiphiles and dendrimers, native polylysine is an inefficient gene delivery vehicle (39). Because binding of the polylysine to the DNA utilizes most of the available lysines, the resulting complex lacks a uniform, cationic surface. However, DNA titrated with a slight excess of polylysine yields moderate reporter gene expression in some adherent cell lines (33).

The efficiency of polylysine can be enhanced dramatically when a fraction of the ε -amine groups of polylysine are covalently linked to a receptor ligand (Figure 5) (40). Gene delivery complexes are formed by titrating a plasmid to neutrality with the polylysine conjugate (Figure 6). When the composition of the complex is optimized with respect to charge and the number of covalently bound ligands, the DNA is efficiently transported by receptor mediated endocytosis (Figure 7) (41). This technique is very appealing, because it may enable one to accomplish gene delivery to specific cells and tissues *in vivo* that overexpress a particular receptor. To date, no cationic amphiphile or dendrimer has been developed with this feature. In contrast, derivitized polylysine has demonstrated promise *in vitro* and has been used to deliver

DNA by endocytosis of a number of receptors (Table 3). Because transfection by native polylysine-DNA is usually very low, high levels of receptor specific expression are obtained. Modified polylysine is one of the best methods to transfect suspension and primary cultures, since specific receptors on the cell surface can be targeted and non-specific methods of transfection by cationic amphiphiles are inefficient (41).

The greatest limitation of receptor-mediated gene delivery is the degradation of complexes by lysosomes. Degradation is minimized *in vitro* by incubating cultures with chloroquine (Figure 7). Alternatively, Wagner *et al.* covalently linked the influenza virus hemagluttinin fusion peptide (GALA) directly to the transferrin polylysine conjugate. In the endosomal compartment the fusion peptide undergoes a conformational change and destabilizes the membrane allowing the DNA complexes to enter the cytoplasm. Along this same line, Cristiano *et al.* incubated cells with whole, attenuated adenoviruses and polylysine-DNA complexes (44). Since both adenoviruses and polylysine-DNA complexes are delivered into common endosomes, DNA complexes can escape into the cytoplasm following adenoviral destabilization of the endosomal membrane. Using these techniques, transfection efficiency can be enhanced a thousand fold over chloroquine augmented transfection (45).

The preliminary research conducted in this field suggests that the use of polylysine DNA may be useful for *in vivo* gene delivery as long as lysosomal degradation is minimized. The characterization and development of polylysine-based gene delivery is the major focus of this thesis

Thesis Organization

Chapter 2

The nature and identity of the anionic molecules on the cell membrane that mediate transfection by cationic molecules has never been studied. In Chapter 2, the interaction of polylysine-DNA coacervates with cells is investigated. Evidence that membrane associated sulfated proteoglycans mediate transfection of polylysine and cationic amphiphile DNA complexes is presented. A possible explanation for why some cell types are more "transfectable" than others is discussed.

Chapter 3

Polylysine is useful for transfecting cells by receptor-mediated endocytosis. In Chapter 3, the development of a polylysine-based method for transfecting cells via folate-receptor mediated endocytosis is described. The implications for this research on gene therapy and on the understanding of the intracellular pathway of folate conjugates are discussed.

Chapter 4

In Chapter 4, the intracellular uptake of folate-polylysine DNA complexes is followed in real time by 2-photon laser scanning microscopy. Physical barriers to gene delivery including the formation of insoluble aggregates and limited nuclear transport is shown. Evidence for transport of complexes into the nucleus of a non-mitotic cell is demonstrated.

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Table 1. Viral vectors used for gene therapy.

	retrovirus	adenovirus	adeno-associated virus
nucleic acid	ss-RNA	ds-DNA	ds-DNA
envelope	yes	no	?
titer	low	high	high
mitosis	required	not required	not required
oncogenic	possible	no	?
other	stable expression	short-term expression	stable expression

Table 2. Properties of cationic amphiphiles.

amphiphile	serum sensitivity	cell types	chloroquine	+/-(charge) ratio
DOTMA/DOPE	yes	wide range	yes	>2
lipopolylysine	slight	only adherent	?	6
DOGS	yes	adherent, primary	slight	4
dendrimers	slight	adherent	no	6

Table 3. Receptor mediated endocytosis of polylysine-DNA complexes: receptors used and common properties.

ligand	cell type	conj. ratio	charge ratio	estimated size (nm)	reference
transferrin	K562, HeLa	2.5:1	1:1	80-150	(34)
asialoglyco-protein	HepG2	5:1	1:1	?	(40)
lactose	HepG2	68:1	1.1:1	20-35	(46)
mannose	HepG2, macrophage	50:1 1:1	1:1	20-35 10-20	(46), (42)
folic acid	KB	0.5:1	1:1	?	(43)

Figure 1. Construction of a recombinant adenoviral (AD5) vector for gene therapy. *Abbreviations: ITR (inverted terminal repeat), Amp (ampicillin resistance gene), Ori (origin), AD5 mu 9.4-18 (adenovirus sequence from map unit 9.4-18).* A new plasmid from the AD5 virus is produced by removing E1 sequences and replacing them with the gene of interest. This virus is then linearized and transfected into 293 cells, containing the E1 region. The two DNA sequences combine homologously to form a recombinant genome which is propagated in the 293 cell line. The resulting recombinant virus is replication deficient but contains the gene of interest which can then be introduced into different cells. *Figure adapted from (13).*

Figure 1. Construction of a recombinant adenoviral (AD5) vector for gene therapy.

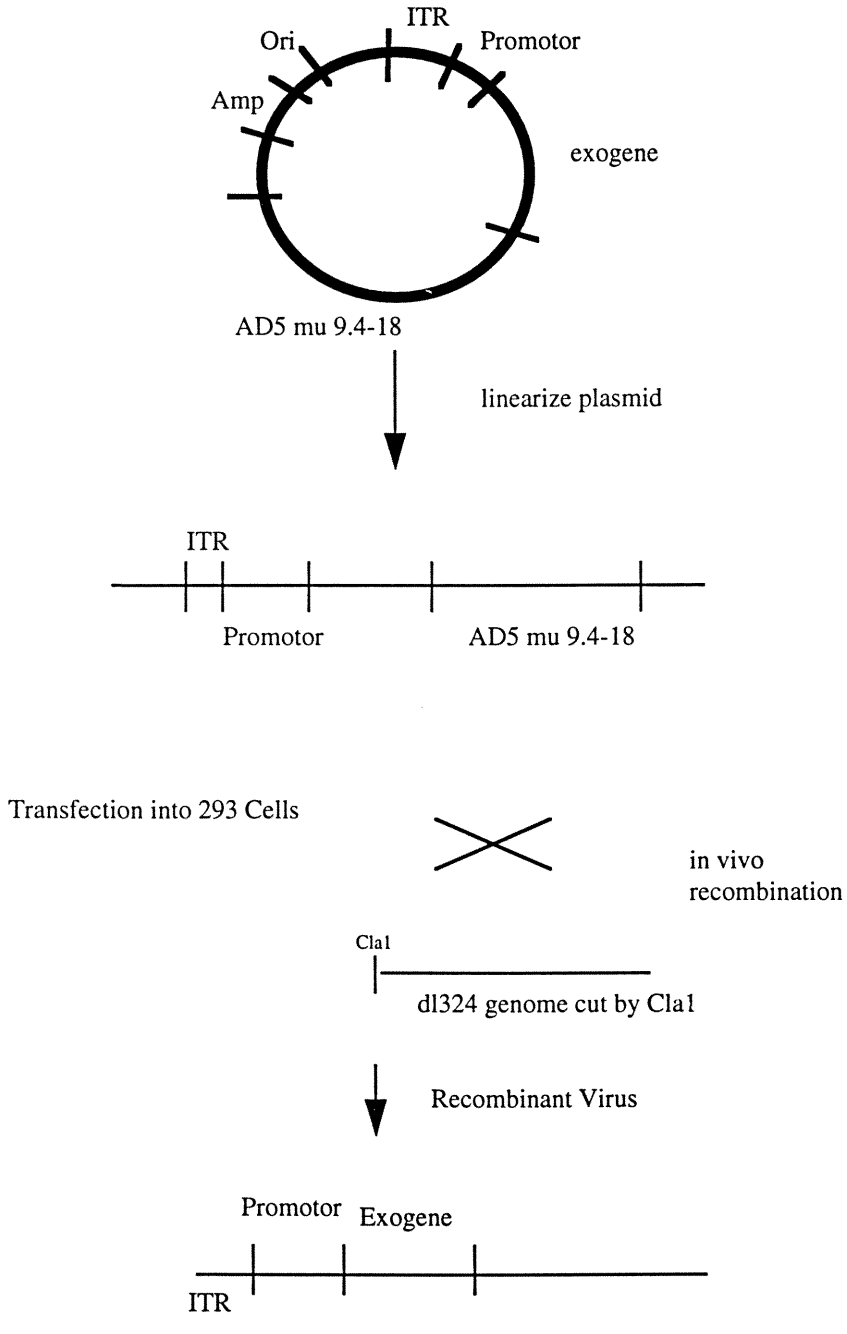


Figure 2. Structure of a dendrimer. The dendrimer core is synthesized from methyl acrylate and ethylene diamine. Synthesis proceeds radially to produce a spherical polymer of a controlled number of generations. The terminal amines are roughly 50% protonated at physiological pH allowing the dendrimer to associate with the phosphate backbone of a plasmid. The dendrimers associate with the plasmid to form a complex that is coated with a positive charge, which facilitates binding of the complex to anionic residues on the cell surface. Dendrimers are believed to enter the cell by adsorptive endocytosis. *Figure adapted from (39).*

Figure 2. Structure of a dendrimer.

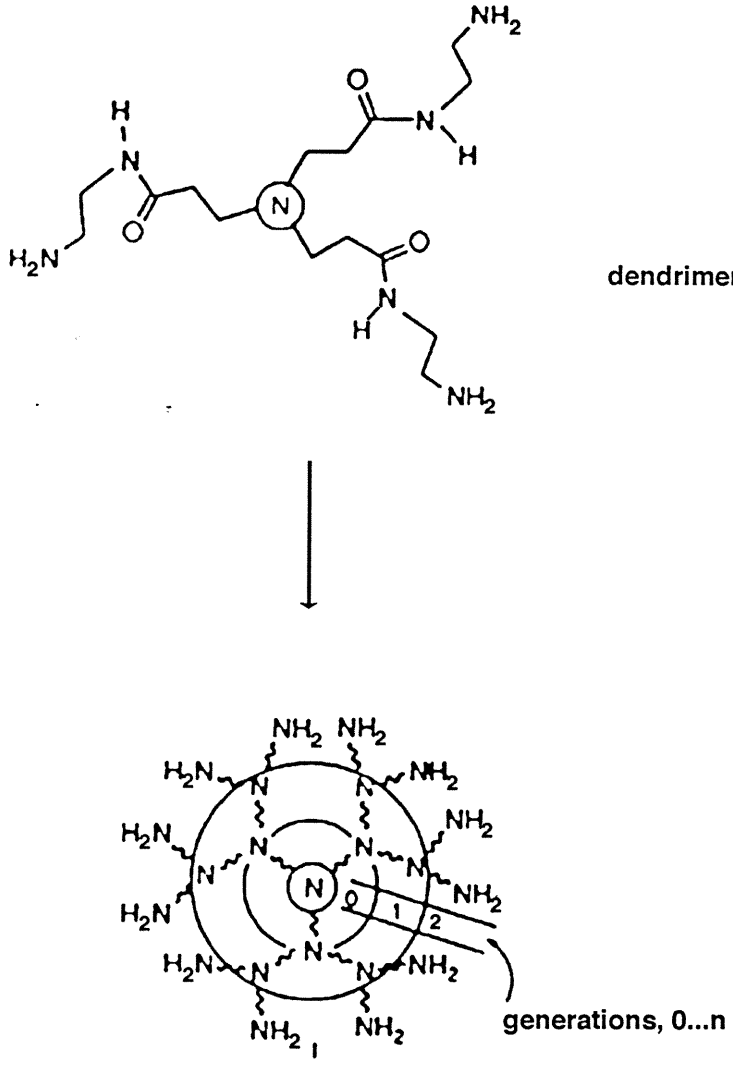
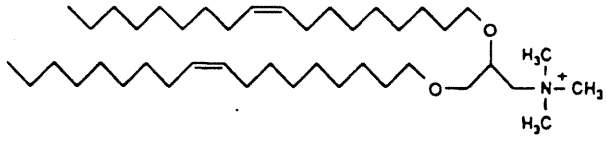
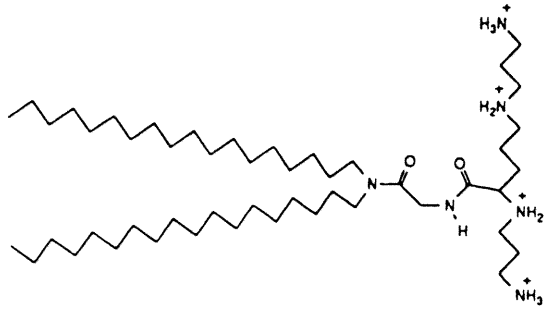


Figure 3. Structures of cationic amphiphiles. Cationic amphiphiles contain a quaternary amine (DOTMA) or a polyamine (DOGS, LPLL) backbone linked to a lipid tail. In the presence of plasmid DNA, these cationic macromolecules bind cooperatively to DNA to form a DNA complex with a positively charged layer surrounding it. The positive charges associate with anionic residues on the cell surface. The complexes are ingested by adsorptive endocytosis. *Figure adapted from (34).*

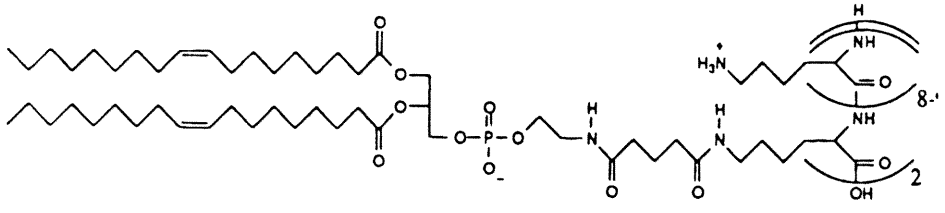
Figure 3. Structures of cationic amphiphiles.



DOTMA



DOGS



Lipopolylysine

Figure 4. Non-specific endocytosis of cationic-DNA complexes. Cationic-DNA complexes bind to negatively charged membrane residues, and endocytosis brings the complexes into endosomes and lysosomes. By a number of mechanisms, a fraction of the DNA enters the cytoplasm in nondegraded form. The DNA enters the nucleus by an unknown mechanism.

Figure 4. Non-specific endocytosis of cationic-DNA complexes.

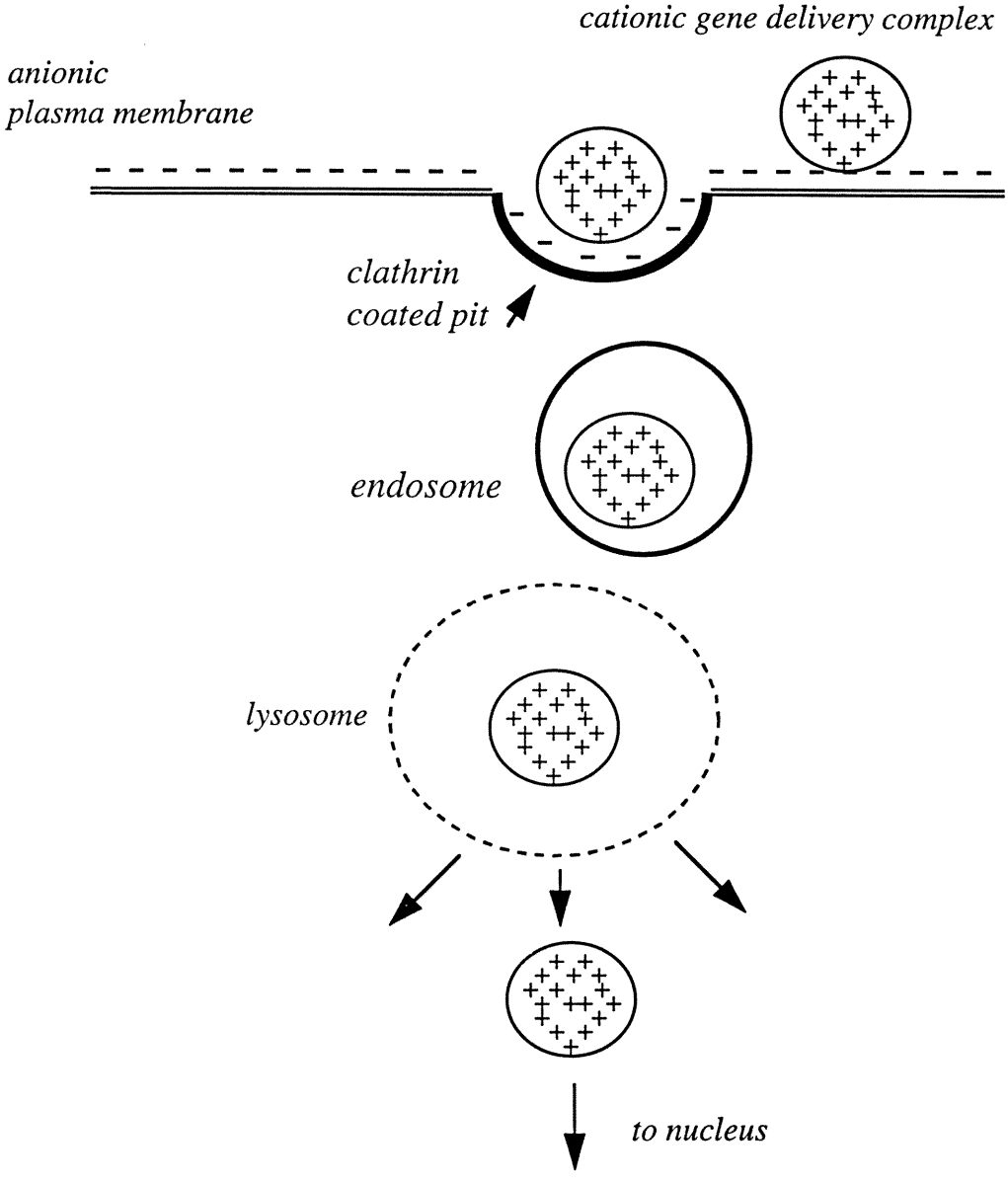


Figure 5. Structure of derivitized polylysine. Through the covalent modification of ε -amine groups, poly-L-lysine is covalently linked to a receptor ligand (X) to form the polylysine conjugate, XPLL.

Figure 5. Structure of derivitized polylysine.

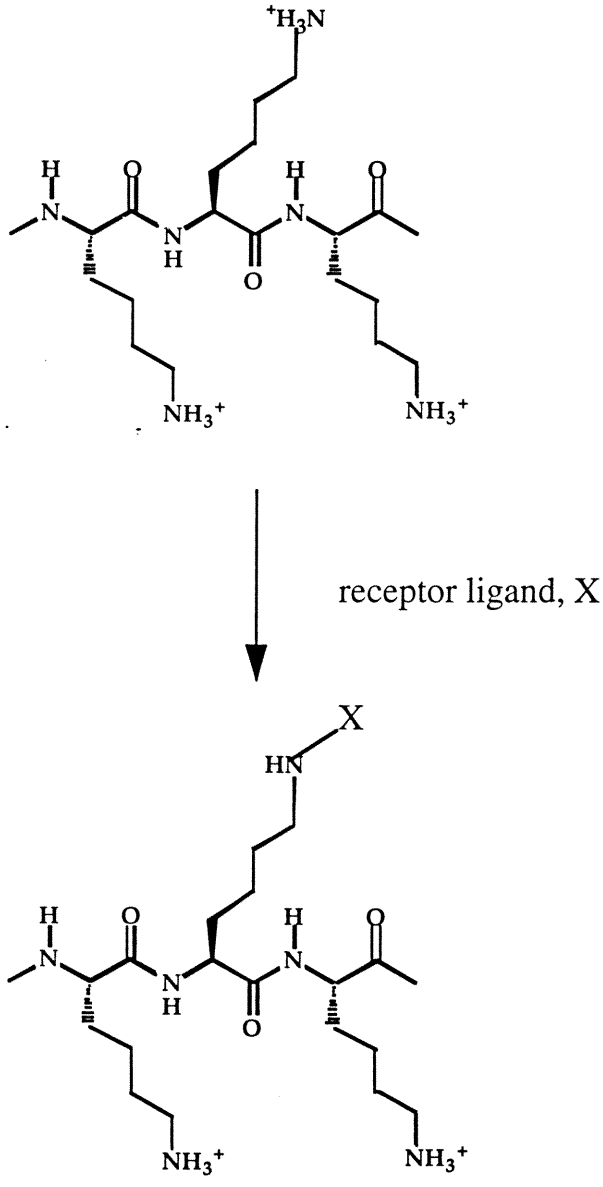


Figure 6. Formation of receptor targeted polylysine-DNA complexes. DNA, encoding the gene of interest, is titrated with XPLL in a dilute buffered solution. Upon the addition of an aliquot of XPLL that charge neutralizes the phosphate backbone, the plasmid undergoes a conformational change and condenses into a gene delivery complex approximately 10-150 nm in diameter.

Figure 6. Formation of receptor targeted polylysine-DNA complexes.

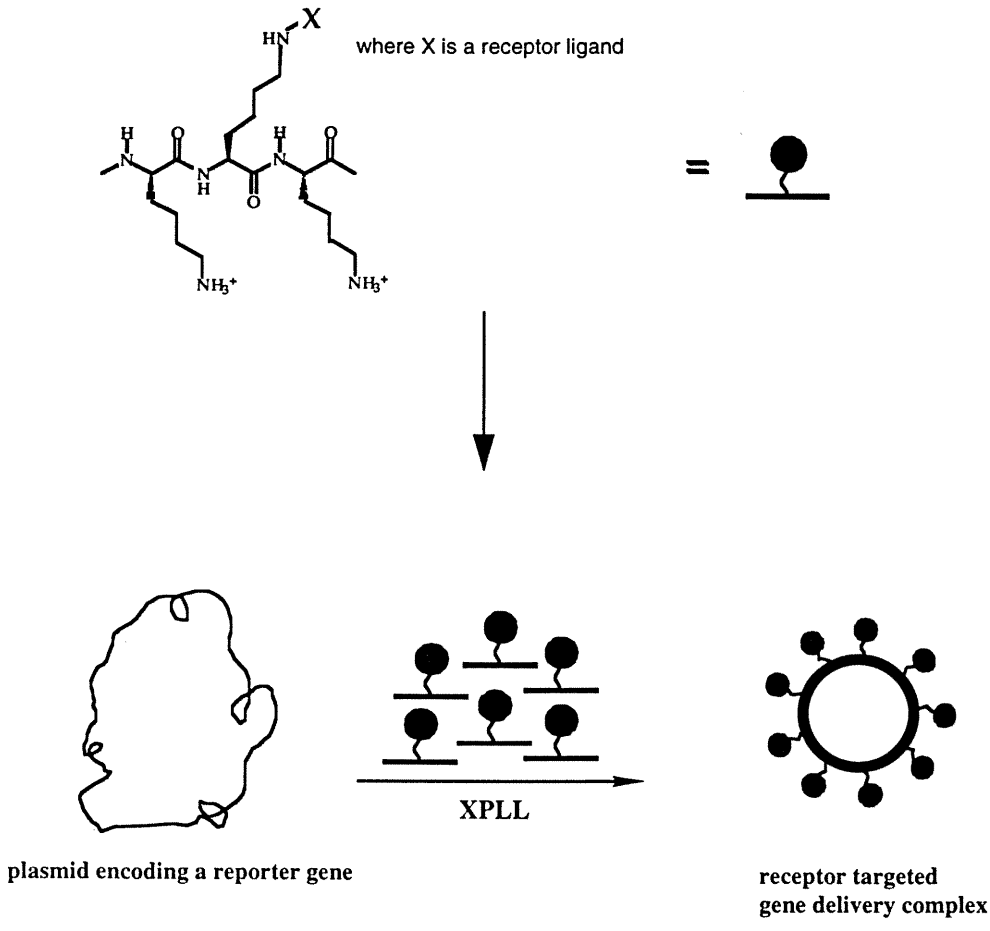
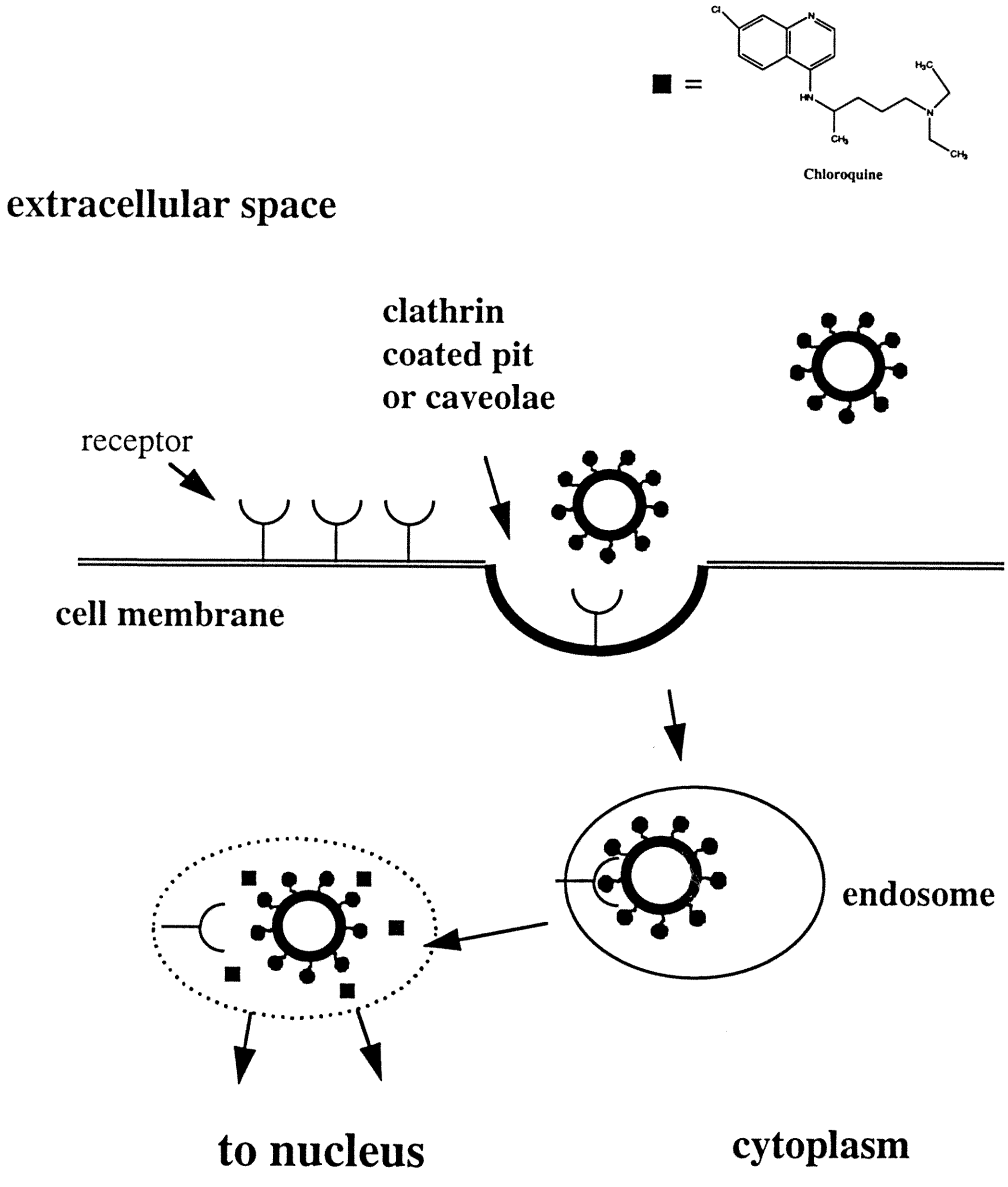


Figure 7. Uptake of DNA complexes by receptor-mediated endocytosis. Chloroquine is added to the culture media of target cells. This compound accumulates in endosomes and lysosomes, buffers their pH to neutrality, and inhibits endosome/lysosome fusion. Approximately 30-60 minutes later, XPLL-DNA complexes are added to the cells. Complexes bind to receptors localized on clathrin coated pits or caveolae in the cellular membrane. Endocytosis is triggered and the complex is delivered into an endosome. Due to the presence of chloroquine in the endosome, few complexes are delivered into lysosomes. Those that are deposited in the lysosomal compartment are protected due to inactivation of proteases by neutral pH. Complexes enter the cytoplasm and nucleus by an unknown mechanism. Maximum expression of the transfected gene typically peaks after 24-48 hours.

Figure 7. Uptake of DNA complexes by receptor-mediated endocytosis



Chapter 2: The Role of Proteoglycans in Cation-Mediated Gene Delivery

Abstract

We report evidence that gene complexes, consisting of polylysine and plasmid DNA, exploit membrane-associated proteoglycans to transfect cells. Treatment of HeLa cells with sodium chlorate, a potent inhibitor of glycosaminoglycan sulfation, resulted in a 69% reduction in reporter gene expression. Cellular treatment with heparinase and chondroitinase ABC inhibited expression to 22% and 80%, respectively, of expression in control cells. Transfection in the presence of exogenous glycosaminoglycans was inhibited most drastically by heparin and heparan sulfate and to a smaller extent, by chondroitan sulfate B. Transfection in mutant, proteoglycan deficient CHO cells was 53 times lower than in wild-type cells. In each of these experimental systems, the intracellular uptake of DNA at 37 °C and the binding of DNA to the cell membrane at 4 °C was impaired. Preliminary transfection experiments conducted in mutant and wild-type CHO cells suggest that gene delivery by some cationic lipids may also depend on cellular proteoglycan expression. The variable distribution of proteoglycans among tissues may in part, explain why some cell types are more susceptible to transfection than others.

Introduction

Because of the current interest in gene therapy, a number of non-viral transfection agents have been developed. Among them are polylysine and lipid based amphiphiles, which belong to a diverse class of macromolecules that form stable complexes with DNA. At optimum formulations, these agents surround DNA with a net positive charge, that in turn, enable the DNA complex to bind to anionic residues on the cell surface. Following entry into the cell by endocytosis a fraction of the DNA enters the nucleus and the gene of interest is transcribed (1-2).

To date, the identity of the anionic molecules on the cell membrane that are responsible for the transfection of cationic DNA complexes has not been investigated. Here, we report evidence that sulfated, membrane-associated proteoglycans mediate transfection by polylysine and cationic amphiphiles.

Proteoglycans: Synthesis and Function

Sulfated proteoglycans are among the most negatively charged components of the cell. They consist of a core protein covalently linked to one or more sulfated glycosaminoglycans: heparin, heparan sulfate, dermatan sulfate, chondroitan sulfate, and keratan sulfate (Figure 1) (3-4). Proteoglycan synthesis begins in the endoplasmic reticulum (ER) with the formation of the protein core (5-6). As the protein core is transported from the rough ER to the Golgi, serine residues are linked to d-xylose by xylosyltransferase (7). The protein core is further modified with two galactosyl residues and a glucuronosyl residue. Starting from this sugar, the glycosaminoglycan backbone is synthesized. Sulfates are transferred from adenosine 3'-phosphate 5'-phosphosulfate

(PAPS) by sulfotransferase onto the glycosaminoglycans (8). Finally, the glycosaminoglycan residues are epimerized before the proteoglycans are exported to their final destination (9-10).

Proteoglycans are a fundamental component of basement membranes (11) and the extracellular matrix and play a pivotal role in cellular proliferation, migration and differentiation (12-13). As integral and glycosylphosphatidyl inositol-linked membrane proteins, heparan sulfate and chondroitan sulfate proteoglycans sequester protease inhibitors, growth factors, and proteases and serve as a receptor for infection by the Herpes Simplex Virus (for reviews see (14-15)).

Because glycosaminoglycans are highly anionic, the interactions between proteoglycans and their ligands occur largely through ionic forces. Based on this information, we proposed that membrane-associated proteoglycans mediate the binding and delivery of cationic DNA complexes into cells. To test this hypothesis, a number of assays were employed. Since proteoglycan charge is derived mostly from the sulfation of the glycosaminoglycan chains (3), transfection was assayed in HeLa cells cultured in the presence of sodium chlorate, an inhibitor of glycosaminoglycan sulfation (16). Tranfection was also tested in cells pre-treated with glycosaminoglycan lyases to remove extracellular glycosaminoglycans (17) and in the presence of purifed glycosaminoglycans. In addition, the transfection efficiency of polylysine-and cationic lipid:DNA was tested in wild-type Chinese Hamster Ovary (CHO) cells and in mutant CHO cells unable to synthesize proteoglycans (18).

Materials and Methods

Cell Culture

HeLa cells were obtained from the American Type Culture Collection and grown in Dulbecco's Modified Eagles Media (DMEM, Gibco) containing Basal Medium Eagle Amino Acids (Gibco), Non-essential Amino Acids (Gibco), 10% fetal bovine serum (Hyclone), and 40 mg/ml gentamicin (Gibco).

Mutant (CHO-*pgs745*) and wild-type (CHO-K1) cells were generously donated by Dr. J.D. Esko (University of Alabama, Birmingham, School of Medicine). The mutant cell line lacks xylosyltransferase, the enzyme that initiates glycosaminoglycan synthesis. Both cell lines were allowed to proliferate in Ham's F-12 media, supplemented with 7.5% FBS and subcultured every four days.

For all cell types, polylysine transfection media consisted of regular growth media (DMEM, 10% FBS) supplemented with 100 μM chloroquine (Sigma). Lipid-DNA transfections were carried out in wild type and mutant CHO cells in serum free Ham's F-12 media. Experiments were conducted in cultures that had reached approximately 70% confluency.

Preparation of Fluorescent and Nick-Translated Plasmids

The PGL2 plasmid (Promega) encoding the firefly luciferase reporter gene was amplified in competent JM109 *Escherichia coli* (Promega) and purified by chromatographic methods (Qiagen). Plasmid DNA was diluted to 100 μg/ml in sterile, distilled water.

DNA (1.5 μg) was labelled with α-^{32}PdCTP (3000 Ci/mmol) using a nick translation kit (Boehringer Mannheim) and purified by repeated chloroform: phenol extractions and ethanol precipitation (19). A stock solution of DNA was prepared by mixing nick translated plasmid with approximately 30 μg unlabelled plasmid. The concentration of DNA was determined by absorbance at λ=260 nm.

To prepare fluorescent DNA, plasmid (100 μg /ml) was incubated with a fifty - fold molar excess of YOYO-1 (Molecular Probes), a fluorescent DNA intercalator (20-21), and incubated for two hours at 4 °C. The solution was loaded onto a Centricon-30 desalting unit and spun for 3 hours at 6000 rpm and 4 °C to remove unbound YOYO. The retentate was diluted to its original concentration in sterile water. The concentration of YOYO per mole plasmid was determined by spectrophotometric analysis. The fluorescence of YOYO-DNA (YYDNA) was monitored through excitation fluorimetry (λ_{ex} = 488 nm, λ_{em}= 512) in a Hitachi model F-4500 fluorescence spectrophotometer.

Desulfation of Cells

HeLa cells (20 K cells/ml) were seeded into 12 well plates (Falcon). Twelve hours later, media was supplemented with sodium chlorate (Aldrich, 35 mM) or a combination of sodium sulfate (Baker, 80 mM) and sodium chlorate (35 mM). Following an additional 48 hour incubation period, cells were rinsed twice with 1 ml PBS and placed into 1 ml transfection media. Transfection, fluorescence uptake, or binding experiments immediately followed.

Treatment of Cells with Glycosaminoglycan Lyases and Purified Glycosaminoglycans

HeLa cells were seeded at a density of 50 K/ml into 12 well plates (Falcon). Eighteen hours later, cells were rinsed twice with 1 ml PBS and placed into a bovine serum albumin/PBS solution containing either 10 units/ml chondroitinase ABC (Sigma) or heparinase II (Sigma) (17). Cells were incubated with lyases for one hour at 37 °C. Following the enzymatic digestion of surface glycosaminoglycans, cells were rinsed twice with PBS and transfected according to the normal protocol.

Glycosaminoglycans (chondroitan sulfate A, bovine trachea; chondroitan sulfate B, bovine mucosa; chondroitan sulfate C, shark cartilage; heparan sulfate, bovine trachea; heparin, bovine trachea; hyaluronic acid, bovine mucosa) were purchased from Sigma. To evaluate the effect of free glycosaminoglycans on transfection, DNA complexes and glycosaminoglycans (40 μg/ml heparan sulfate, heparin, chondroitan sulfate A, chondroitan sulfate B, chondroitan sulfate C, or hyaluronic acid) were added together at the time of transfection. Transfected cells were treated according to the usual protocol.

Preparation of Complexes and Transfection

An aliquot of poly-L-lysine (100 μg/ml, Sigma) was added to DNA (1.5 μg, 100 μg/ml) diluted in 150 μl HBS (Hepes Buffered Saline, 50 mM Hepes, 150 mM NaCl, pH 7.4). Polylysine-DNA (PLL-DNA) samples were mixed gently, incubated for 30 minutes at room temperature, and added to cells in transfection media. Four hours later, cells were rinsed twice in 1 ml phosphate buffered saline (PBS, 150 mM NaCl, 150 mM NaP_i, pH 7.4) and placed into fresh culture media at 37 °C. Following an additional 21 hour

incubation at 37 °C, the media was removed and cells were rinsed twice in 2 ml PBS. Luciferase expression in cellular lysates was determined using an Enhanced Luciferase Assay Kit (Analytical Luminescence Laboratories) according to the manufacturer's instructions. Expression was quantitated in terms of Relative Light Units (RLU) on an Analytical Luminescence Laboratories Model 2010 Luminometer.

Fluorescence Uptake Experiments and Confocal Microscopy

HeLa, CHO-pgs745, and CHO-K1 cells were incubated with PLL-YYDNA for four hours and then rinsed three times in PBS. To remove surface bound DNA, cells were treated with DNAse (Sigma, 1 mg/ml) for 15 minutes and then detached by trypsin. Cell suspensions were pelleted, washed twice in PBS, and fixed in 4% paraformaldehyde/PBS for 10 minutes at room temperature. After a final rinse in PBS, cell pellets were resuspended in Biomeda/PBS solution (90:10) and mounted between a slide and coverslip to dry. Fluorescence images were obtained using a BioRad Confocal Microscope.

Results

Polylysine Mediated Transfection Requires Positively Charged Complexes

The net charge on a DNA complex can significantly influence transfection efficiency. Optimum cationic amphiphile:DNA complexes require between a 2 and 6 fold molar excess of positive charge (2). To determine the optimum charge ratio in the polylysine system, a series of complexes prepared with 1.5 μg DNA and varying amounts of polylysine were transfected into HeLa cells.

Complexes composed of a small amine/nucleotide charge ratio yielded very little expression (Figure 3). Expression increased nonlinearly with polylysine concentration and reached a peak (5 x 10^5 RLU/10^6 cells) at an amine/nucleotide ratio equal to 1.5. Complexes with ratios larger than 1.5 yielded lower expression values (22). These results are consistent with the proposed ionic model to describe the interactions between the cell membrane and cationic DNA complexes. Complexes formulated with the optimum ratio were used for all subsequent experiments.

Sulfates Mediate PLL-DNA Transfection

The role of proteoglycans in cellular events has been demonstrated by inhibiting glycosaminoglycan sulfation with sodium chlorate (23-24). Chlorate is a non-toxic inhibitor of ATP-sulfurylase, an essential enzyme in the synthesis of 3'-phosphoadenosyl-5'-phosphosulfate, a glycosaminoglycan sulfate donor (16, 25).

To test the role of sulfated proteoglycans in transfection, cells were grown in the presence of sodium chlorate for 48 hours and transfected with PLL-DNA. As demonstrated by Figure 4, expression was inhibited by chlorate in a concentration dependent manner. At 35 mM chlorate, luciferase expression was 30% relative to untreated cells. The inhibition of gene expression was probably not caused by chlorate toxicity, because cellular morphology and proliferation remained normal at all chlorate concentrations.

However, these results could possibly be due to intracellular artifacts unrelated to the delivery mechanism of PLL-DNA. To elucidate the effects of chlorate more clearly, we tested whether chlorate treatment inhibited the uptake of DNA into the cell. DNA was

labeled with YOYO, a fluorescent intercalator, complexed with polylysine and incubated with chlorate treated (35 mM) and untreated HeLa cells. After 4 hours, cells were washed, fixed, and imaged by confocal microscopy to localize intracellular fluorescence. As shown in Figure 5 uptake of YYDNA into chlorate treated cells was severely impaired suggesting that the chlorate induced decrease in expression was due to an inhibition of YYDNA entering the cell.

In order to demonstrate a direct correlation between the uptake of PLL-DNA and sulfated cell membrane residues, we tested whether chlorate treatment inhibited binding of DNA to the cell surface. Complexes of polylysine and nick translated ^{32}P-labeled plasmid were prepared and incubated with chlorate treated (35 mM) and untreated HeLa cells for four hours at 4 °C to inhibit endocytosis. Cells were washed extensively with ice cold PBS, dissolved, and assayed for cell-associated radioactivity. As shown in Figure 6, binding of PLL-^{32}P-DNA to chlorate cells was inhibited 69 (+/- 14)% with respect to untreated cells. HeLa cells co-treated with chlorate and excess sulfate or treated with chlorate for only four hours bound as much PLL-^{32}P-DNA as untreated cells. We conclude that the effects of chlorate on expression are due to desulfation of membrane molecules that mediate transfection. Although these results clearly suggest that sulfated proteoglycans participate in gene delivery, additional evidence was sought to implicate them directly.

Effect of Exogenous Glycosaminoglycans and Glycosaminoglycan Lyases on Transfection of PLL-DNA

To test if glycosaminoglycans could competitively inhibit gene delivery, HeLa cells were co-transfected with exogenous glycosaminoglycans (40 μg/ml). Following

exposure to DNA complexes and glycosaminoglycans, cells were washed, incubated in fresh culture media and assayed for luciferase expression 22 hours later (Figure 7). The most anionic glycosaminoglycans, heparin and heparan sulfate, nearly eliminated luciferase expression while chondroitans A and C and hyaluronic acid had no effect. Chondroitan sulfate B , the third most anionic glycosaminoglycan, reduced expression by approximately 40%.

Since excess glycosaminoglycans in the media could affect DNA transcription (26), we tested whether the intracellular uptake of YYDNA was affected by exogenous glycosaminoglycans. PLL-YYDNA complexes and glycosaminoglycans were incubated with HeLa cells for four hours at 37 °C. Cells were rinsed, fixed, and mounted onto glass slides. Confocal microscopy images of intracellular fluorescence (Figure 8) revealed a correlation between expression and YYDNA uptake in the presence of competing glycosaminoglycans. Heparin and heparan sulfate nearly eliminated the uptake of YYDNA into the cells, but chondroitan sulfates A and C did not have any obvious effect. Chondroitan sulfate B moderately reduced the uptake of YYDNA into the cell. The binding of ^{32}P-DNA to the cell surface was tested in the presence of glycosaminoglycans and a similar pattern of inhibition was observed (Figure 9). The effects of soluble glycosaminoglycans on luciferase expression and binding of ^{32}P-DNA are summarized in Table 1.

It could be argued that any highly sulfated, anionic polymer in the media could inhibit transfection by destabilizing the interactions between polylysine and DNA. Indeed, when dextran sulfate, a synthetic glycosaminoglycan analog was added to the media,

reductions in uptake and expression comparable to heparin were observed (not shown). Therefore, as an additional control, HeLa cells were transfected following treatment with glycosaminoglycan lyases in order to remove glycosaminoglycans from the cell surface. By removing heparin/heparan sulfate and chondroitan sulfates with heparinase II and chondroitinase ABC, expression was reduced to 22% and 80%, respectively of untreated cells (Table 1). Moreover, treatment of cells with hyaluronase to remove non-sulfated hyaluronic acid glycosaminoglycans found in the extracellular matrix did not inhibit luciferase expression (not shown). These results suggest that heparin/heparan sulfate proteoglycans and possibly chondroitan sulfate proteoglycans are mediators of the transfection of PLL-DNA.

Cells Lacking Proteoglycans Are Transfection Deficient

To demonstrate that the role of proteoglycans in gene delivery is not specific to HeLa cells, transfection efficiency was tested in wild-type CHO-K1 cells and in mutant CHO-*pgs745* cells deficient in xylosyltransferase, an essential enzyme necessary in the first step of proteoglycan synthesis. Despite their inability to produce proteoglycans, CHO-*pgs745* cells are highly proliferative and are morphologically indistinct from their wild-type counterparts (18).

Both CHO-K1 and CHO-*pgs745* cells were transfected with the same amount of DNA and with the same amine/nucleotide charge ratio. However, luciferase expression in proteoglycan deficient cells was 53 times lower than in wild-type cultures. Consistent with previous observations, the uptake of PLL-YYDNA into CHO-*pgs745* cells was

dramatically lower than into CHO-K1 cells (Figure 10). Moreover, the binding of ^{32}P-DNA to CHO-*pgs745* cells was inhibited 80% with respect to wild-type, CHO-K1 cells.

Role of Proteoglycans in Cationic Lipid Mediated Transfection

The experiments described above demonstrate that transfection by PLL-DNA is mediated by membrane-associated proteoglycans. In practice, however, polylysine-DNA complexes are not widely used for transfection experiments, because the transfection efficiency is low compared to transfection by other methods such as cationic lipids. Therefore, to evaluate the role of proteoglycans in a more relevant transfection system, the transfection efficiency of a series of cationic lipids (obtained from a PerFect Transfection Kit, Invitrogen) were tested in wild-type and mutant CHO cells. The formulations tested were either a mixture of two cationic lipids (pfx-1), a 1:1 mixture of DOPE and a cationic lipid (pfx-6 and pfx-7), or a single cationic lipid (pfx-8) (summarized in Table 3; the exact structures of these lipids are proprietary information).

Each formulation was transfected into CHO-K1 and CHO-*pgs745* cells (~2 x 10^5 cells/well) using 4 μg DNA and the optimum amount of lipid (as determined by the manufacturer). Cells were exposed to lipid:DNA complexes for four hours in serum free Ham's F-12 media, rinsed twice with PBS, and incubated in regular growth media. Twenty hours later, a signifcant number of dead cells and cellular debris were observed especially in cultures transfected with pfx-7 and pfx-8. Cationic liposome induced toxicity has been observed previously, although the reasons for this toxicity are unclear.

To eliminate the possible artifacts that might arise from toxicity, cells were transfected again with a 20 fold lower concentration of lipid:DNA. At these

concentrations, very few dead cells were observed in any of the cultures. Following the transfection, all cultures were rinsed and assayed for luciferase activity.

A correlation quotient, *X*, defined as (RLU in wild-type cells/RLU in mutant cells) was calculated for each type of lipid. Raw luciferase expression (RLU) as well as *X* values were plotted for the various lipid formulations (Figures 11A and 11B). For pfx-1 formulations, *X* was equal to 81 indicating that wild-type cells expressed 81 times more luciferase than proteoglycan deficient cells. The correlation quotient determined for the other lipid formulations was 10.7, 1.7, 4.8, and 1.69 for pfx-4, pfx-6, pfx-7, and pfx-8, respectively. In addition, the highest levels of raw luciferase expression in both cell lines were obtained by pfx-1:DNA and pfx-4:DNA and the lowest levels from pfx-6, pfx-7, and pfx-8:DNA. Therefore, the highest expression was obtained by transfections with lipids composed of a mixture of two cationic lipids and the lowest expression from a 1:1 mixture of cationic lipid and DOPE or a single cationic lipid.

Discussion

In this paper we investigated the identity of the anionic membrane sites that mediate transfection by polylysine. Because proteoglycans bind and internalize a wide range of cationic substrates in the body, we hypothesized that gene delivery is mediated by proteoglycans bound to the cell surface. Transfection was tested in cells treated with chlorate in order to evaluate the possible role of sulfated proteoglycans in the transfection mechanism. Chlorate diminished binding of ^{32}PDNA to the cell surface at 4 °C suggesting that the first step in the transfection process involved binding to sulfated proteoglycans. Chlorate diminished the uptake of DNA as well the expression of luciferase. Taken

together, these results suggested that DNA complexes are effectively bound and internalized by membrane-associated proteoglycans. Accordingly, exogenous heparin and heparan sulfate and to a smaller extent chondroitan sulfate B added to the transfection media competitively inhibited binding and uptake of the DNA and expression of the reporter gene. The least anionic anionic glycosaminoglycans had very little effect on transfection, indicating that binding to glycosaminoglycans is dependent on charge effects rather than on structure. Along the same line of argument, pre-treatment of cells with heparinase was a more effective transfection inhibitor than chondroitinase ABC. Thus, PLL-DNA complexes are preferentially transfected via heparin/heparan sulfate proteoglycans. By showing that binding, uptake and expression of PLL-DNA was lower in mutant proteoglycan deficient cells relative to wild-type cells, we confirmed that proteoglycans participate in polylysine mediated gene delivery into other cell lines as well.

However, it is not clear whether the transfection inefficiency of CHO-*pgs745* cells is due solely to inefficient binding and transport of PLL-DNA. In CHO-*pgs745* cells, cellular binding of ^{32}PDNA at 4 °C was five fold lower than binding to wild-type cells, but expression was inhibited 50 fold. To account for this difference, it is possible that CHO-*pgs745* cells possess other intracellular deficiencies that can affect the expression of the reporter gene. Alternatively, in the absence of proteoglycans PLL-DNA may still bind to other anionic sites but may not enter the cell efficiently. The lack of YYDNA uptake into CHO-*pgs745* cells is consistent with this hypothesis. Regardless of whether proteoglycans are the sole membrane binding sites for transfection, we conclude that a

correlation exists between membrane-associated proteoglycans the efficiency of polylysine-mediated transfection.

Since polylysine is not a widely used transfection agent, preliminary experiments were conducted to evaluate the possible role of proteoglycans in the transfection of cationic lipid:DNA complexes. Luciferase expression in wild-type cultures relative to proteoglycan deficient cells varied depending on the lipid. The strongest correlation between transfection and proteoglycans and the highest expression levels were obtained by pfx-1 and pfx-4 lipids which consist of a mixture of two cationic lipids. The weakest correlation and the lowest expression levels were obtained with lipid formulations consisting of a 1:1 mixture of a cationic lipid and DOPE or a single cationic lipid. Experiments were not conducted to verify that the inhibition of expression was directly caused by decreased binding and uptake of lipid:DNA, but they are currently in progress. However, these preliminary results are consistent with the hypothesis that proteoglycans play a role in the transfection of certain cationic lipids. It is possible that the role of proteoglycans in lipid mediated transfection is dependent on whether DOPE is included in the formulations. Future studies will be conducted to test the role of proteoglycans in the transfection of cationic lipids of known structure so that a correlation between proteoglycan-mediated transfection and precise structural features can be elucidated.

In conclusion, we suggest that the variable expression of proteoglycans among tissues may explain why some cell types are more susceptible to transfection than others. Although all tissues express proteoglycans to some level (27), proteoglycan expression is regulated by both the state of differentiation and growth of the cell. Immature myeloid

and lymphoid cells express the hematopoietic proteoglycan core protein (HpPG) at low levels only but upregulate the HpPG gene 10 fold upon differentiation (28). Recently, the undifferentiated erythroleukemia cell line, K562, was shown to express 2-3 times more of the basement membrane proteoglycan, perlecan, if the cells were artificially stimulated to differentiate with 12-O-tetradecanoylphorbol-13-acetate, a phorbol diester (29). Incidentally, K562 cells are one of the most difficult cell lines to transfect by cationic methods but yield high levels of expression by transferrin-polylysine transfection (30). This implies that the susceptibility of a cell type to transfection may partially depend on the mechanism of entry and not entirely on intracellular factors. The development of methods to increase expression in these hematopoietic cell lines is important for *ex vivo* applications of gene replacement therapy. Experiments that attempt to modulate proteoglycan expression as a means to increase transfection by cationic methods are currently in progress.

Finally, the elucidation of the role of proteoglycans in gene delivery brings to light the possible barriers that might be encountered by gene delivery *in vivo*. The inhibition of transfection by heparin and other charged components in serum has been established (31). This problem is avoided *in vitro* by conducting transfection experiments in serum free media. To avoid this problem *in vivo* will be more difficult, and the administration of drugs that bind selectively to heparin might be required. Gene delivery might also be inhibited by wasteful binding of DNA complexes to the extracellular matrix and basement membranes of tissues in the body. If these problems prove to be significant *in vivo*, then

alternative gene delivery vectors that are are optimized at more neutral charge ratios may have to be developed.

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Table 1. Summary of the effects of exogenous glycosaminoglycans and glycosaminoglycan lyases on expression and binding of PLL-^{32}PDNA. Results of the effects of exogenous glycosaminoglycans on luciferase expression and on binding of PLL-^{32}PDNA are summarized. Experimental methods are provided in Figures 7 and 9. To test the effect of lyases on transfection, cells were incubated in the presence of either chondroitinase ABC or heparinase for 1.5 hours and transfected according to methods described in Figure 3.

Table 1. Summary of the effects of exogenous glycosaminoglycans and glycosaminoglycan lyases on luciferase expression and binding of PLL-^{32}PDNA.

glycosaminglycan or glycosaminoglycan lyase	Expression (% of Control)	Binding (% of Control)
heparin	0.185	2
heparan sulfate	0.269	3
chondroitan sulfate A	110	109
chondroitan sulfate B	58	38.5
chondroitan sulfate C	104	63.5
hyaluronic acid	95	85
heparinase	22%	**
chondroitinase ABC	80%	**

Table 2. Luciferase expression and binding of PLL-^{32}PDNA in wild-type (CHO-K1) and mutant (CHO-*pgs*745) cells. Wild-type and mutant cell lines were transfected with PLL-DNA (lysine:nucleotide=1.5) and assayed for luciferase expression as described (Figure 3). Results are presented in terms of relative light units (RLU). PLL-^{32}PDNA complexes were prepared and incubated with wild-type and mutant CHO cells for 4 hours at 4 °C. Cells were rinsed extensively, dissolved, and quantitated. Results are given in terms of counts per minute. (+/-) gives the range of the observed values for duplicate samples.

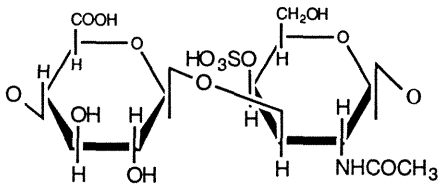
Table 2. Luciferase expression and binding of PLL-^{32}PDNA in wild-type (CHO-K1) and mutant (CHO-*pgs*745) cells.

	CHO-K1	**CHO-*pgs745***
expression (RLU)	3.1×10^5 (+/-) 7.5×10^4	6×10^3 (+/-) 3×10^3
		% of CHO-K1=1.9
binding (counts per minute)	7.0×10^4 (+/-) 1.3×10^4	1.4×10^4 (+/-) 2×10^3
		% of CHO-K1=20

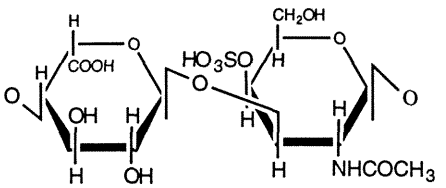
Table 3. Cationic lipid formulations used for transfections into wild-type and mutant CHO cells.

lipid	*formulation*
pfx-1	1:1 mixture of two cationic lipids
pfx-4	1:1 mixture of two cationic lipids
pfx-6	1:1 mixture of a cationic lipid and DOPE
pfx-7	1:1 mixture of a cationic lipid and DOPE
pfx-8	single cationic lipid

Figure 1. Structure of disaccharide and polysaccharide units that comprise the glycosaminoglycan backbone.



Chondroitan Sulfate A



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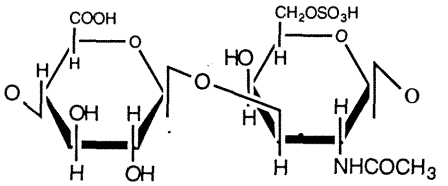
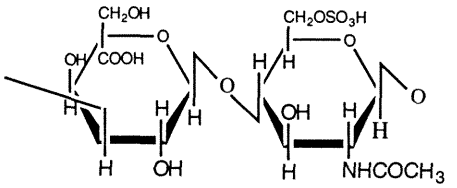
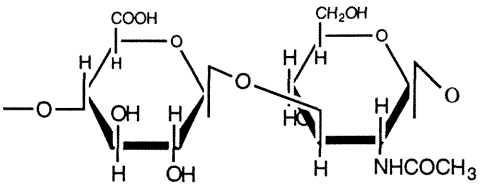


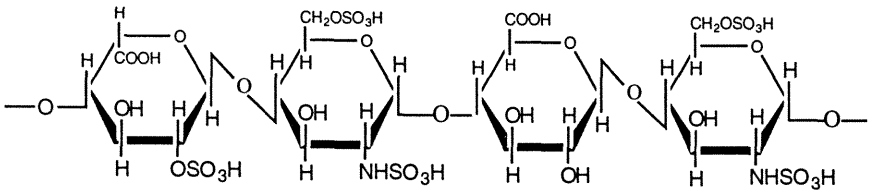
Chondroitan Sulfate C



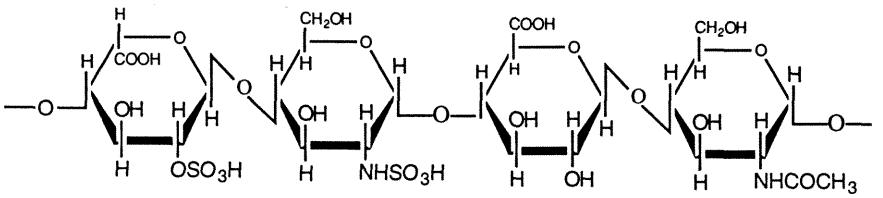
Keratan Sulfate



Hyaluronic Acid



Heparin



Heparan Sulfate

Figure 2. Proposed model for the interaction of cationic DNA complexes with membrane-associated proteoglycans. DNA is titrated with polylysine or a cationic lipid to formulate a complex with a net positive charge. The cationic complexes interact with anionic, membrane bound proteoglycans and enter the cell by endocytosis.

Figure 2. Proposed model for the interaction of cationic DNA complexes with membrane-associated proteoglycans.

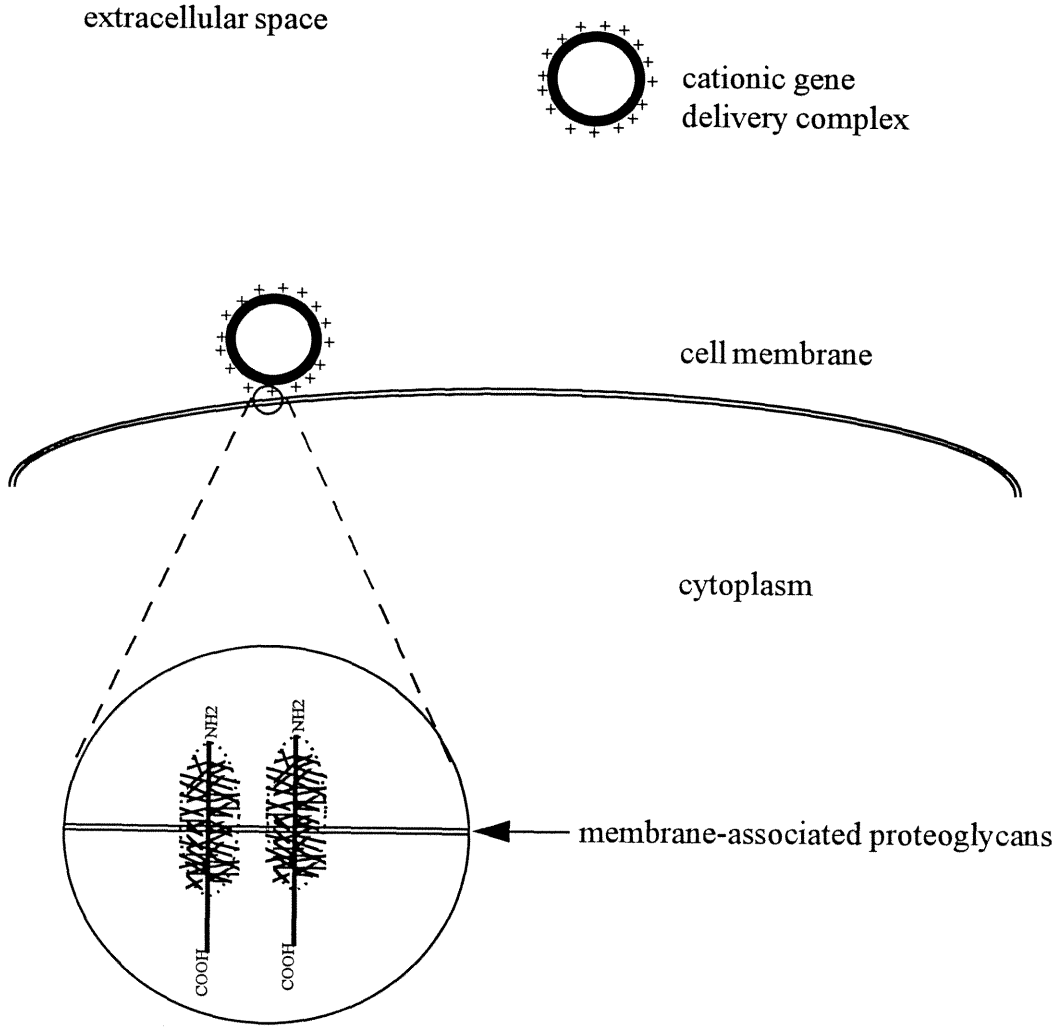


Figure 3. Determination of the optimum charge ratio (lysine:nucleotide) for the transfection of PLL-DNA. Varying amounts of polylysine (100 μg/ml) were added to 1.5 μg plasmid DNA diluted in 150 μl HBS. Samples were gently agitated, equilibrated for 30 minutes, and added to HeLa cells in transfection media. Following a five hour incubation at 37 ^{o}C, transfection media was removed and cells were washed twice in 2 ml PBS. Cells were grown for an additional 21 hours in fresh growth media and then washed in PBS. Luciferase expression in cellular lysates was determined using an Enhanced Luciferase Assay Kit and a Monolight Luminometer (Analytical Luminescence Laboratories) according to the manufacturer's instructions. Results obtained from different charge ratios are presented as a percent of the maximum observed expression.

Figure 3. Determination of the optimum charge ratio (lysine:nucleotide) for the transfection of PLL-DNA.

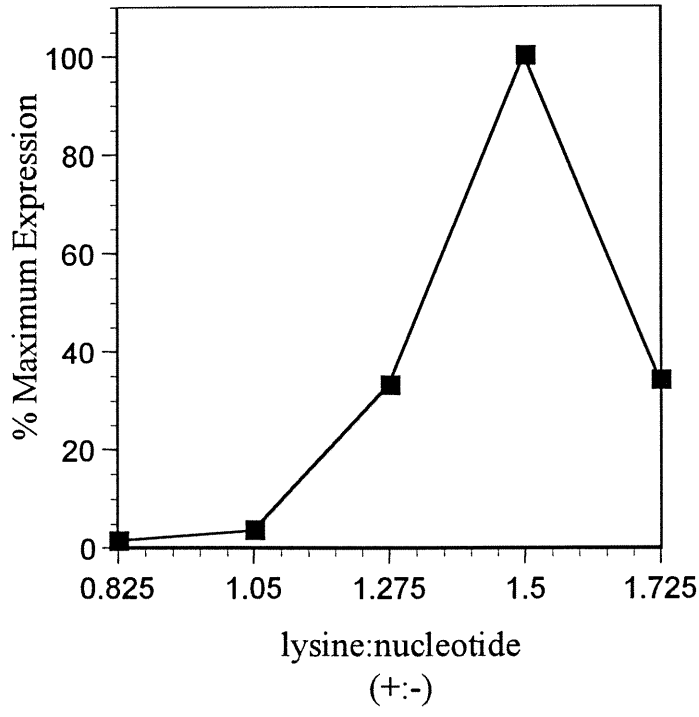


Figure 4. Effect of sodium chlorate concentration on luciferase expression. HeLa cells were grown for 48 hours in the presence of varying amounts of sodium chlorate or were left untreated as a control. Chlorate treated and untreated HeLa cells were transfected as described (Figure 3) using PLL-DNA complexes made with the optimum charge ratio.

Figure 4. Effect of sodium chlorate concentration on luciferase expression.

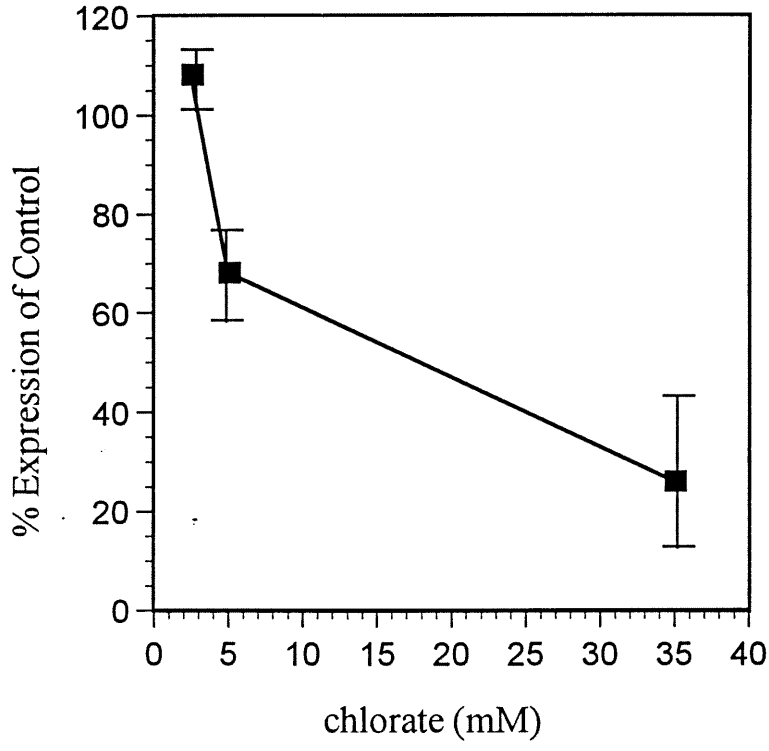


Figure 5. Effect of 35 mM chlorate on the uptake of PLL-YYDNA. HeLa cells were grown for 48 hours in 35 mM chlorate or left untreated as a control. Complexes composed of YOYO labeled DNA (YYDNA) and polylysine were prepared and incubated with chlorate treated and untreated HeLa cells at 37 °C. After 4 hours, cells were rinsed extensively and incubated in 1 ml PBS/deoxyribonuclease (Sigma, 50 μg/ml) for 15 minutes. Cells were trypsinized, fixed in 4% paraformaldehyde, and suspended in Biomeda/PBS (90:10) solution. Cells were mounted onto a glass slide and cover slip and imaged twelve hours later using a Biorad Confocal Microscope. Each panel contains approximately the same number of cells.

Figure 5. Effect of 35 mM chlorate on the uptake of PLL-YYDNA.

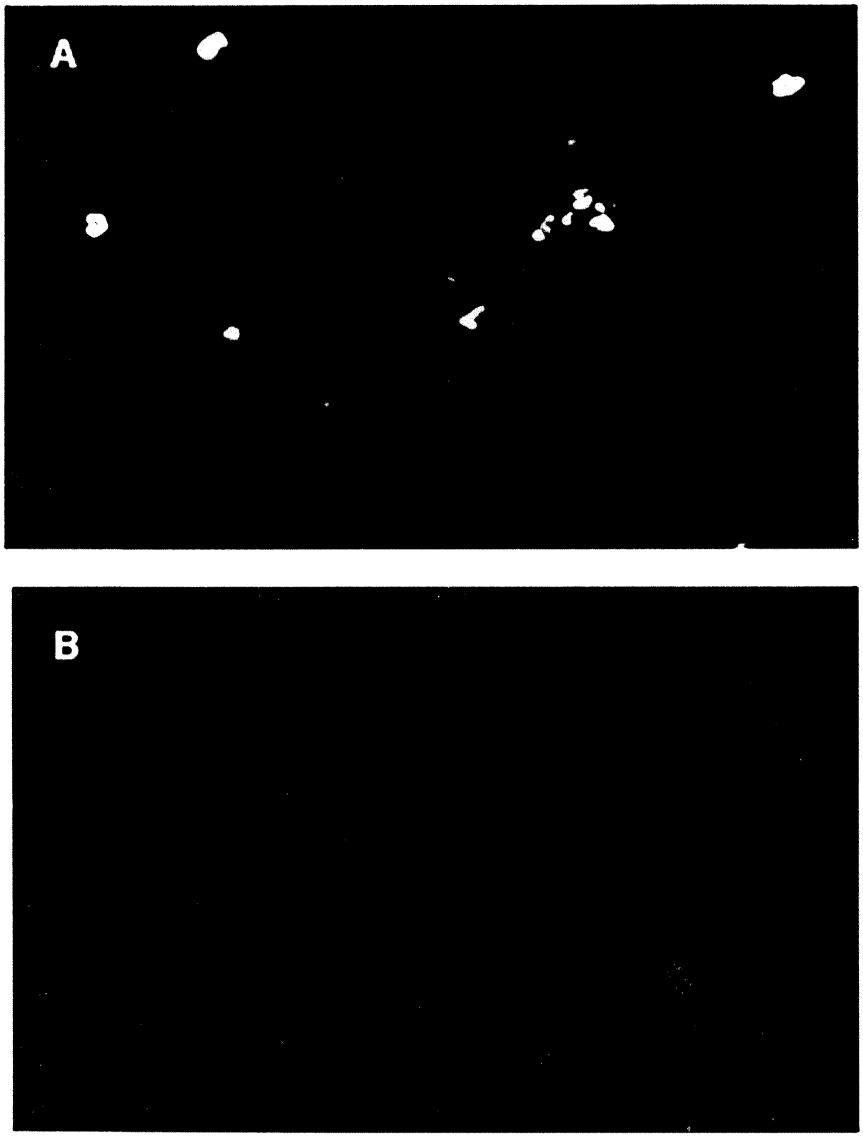


Figure 6. Effect of 35 mM chlorate on the binding of PLL-^{32}PDNA to cells at 4 °C. HeLa cells were grown for 48 hours in 35 mM chlorate or were left untreated as a control. PLL-^{32}PDNA complexes were prepared and incubated with HeLa cells for 4 hours at 4 °C to inhibit endocytosis. Cells were rinsed extensively in ice cold PBS and dissolved in 1.0 M NaOH. Aliquots of dissolved cells were added to 10 ml Scintillation fluid and counted for one minute in a Beckman model 2010 scintillation counter. Samples were assayed in duplicate. Error bars represent the range of the observed values.

Figure 6. Effect of 35 mM chlorate on the binding of PLL-^{32}PDNA to cells at 4 °C.

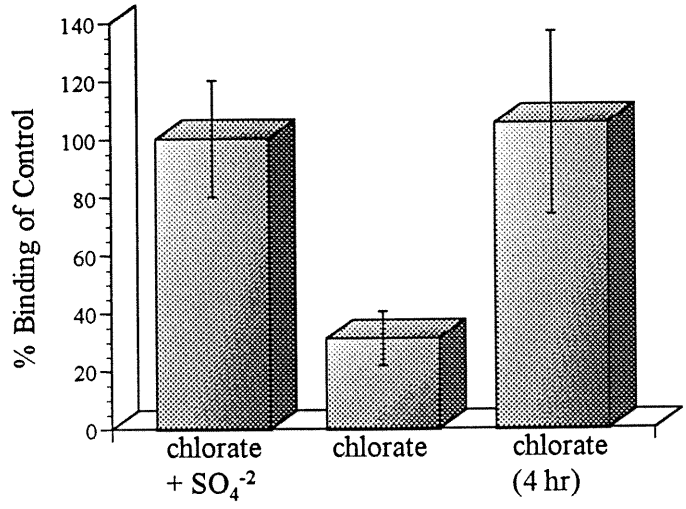


Figure 7. Effect of exogenous glycosaminoglycans on luciferase expression. HeLa cells were transfected with PLL-DNA in the presence of purified glycosaminoglycans (40 μg/ml) processed as described (Figure 3). Results are presented as a percentage of luciferase expression relative to untreated (control) cells. Error bars represent the range of the observed values over duplicate samples. *Legend: 1: heparan sulfate 2. heparin 3. chondroitan sulfate A 4. chondroitan sulfate B 5. chondroitan sulfate C 6. hyaluronic acid*

Figure 7. Effect of exogenous glycosaminoglycans on luciferase expression.

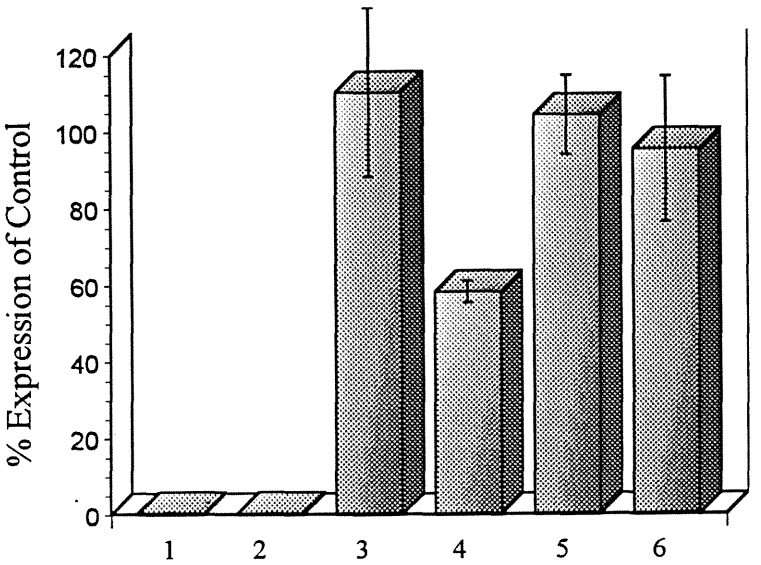


Figure 8. Effect of exogenous glycosaminoglycans on the uptake of PLL-YYDNA. HeLa cells were transfected with PLL-YYDNA in the presence of purified glycosaminoglycans (40 g/ml). Cells were prepared and imaged as described (Figure 5). *A: control;* B: chondroitan sulfate A; *C: chondroitan sulfate B*; D: chondroitan sulfate C; *E: heparin;* F: heparan sulfate. Each panel contains approximately the same number of cells.

Figure 8. Effect of exogenous glycosaminoglycans on the uptake of PLL-YYDNA.

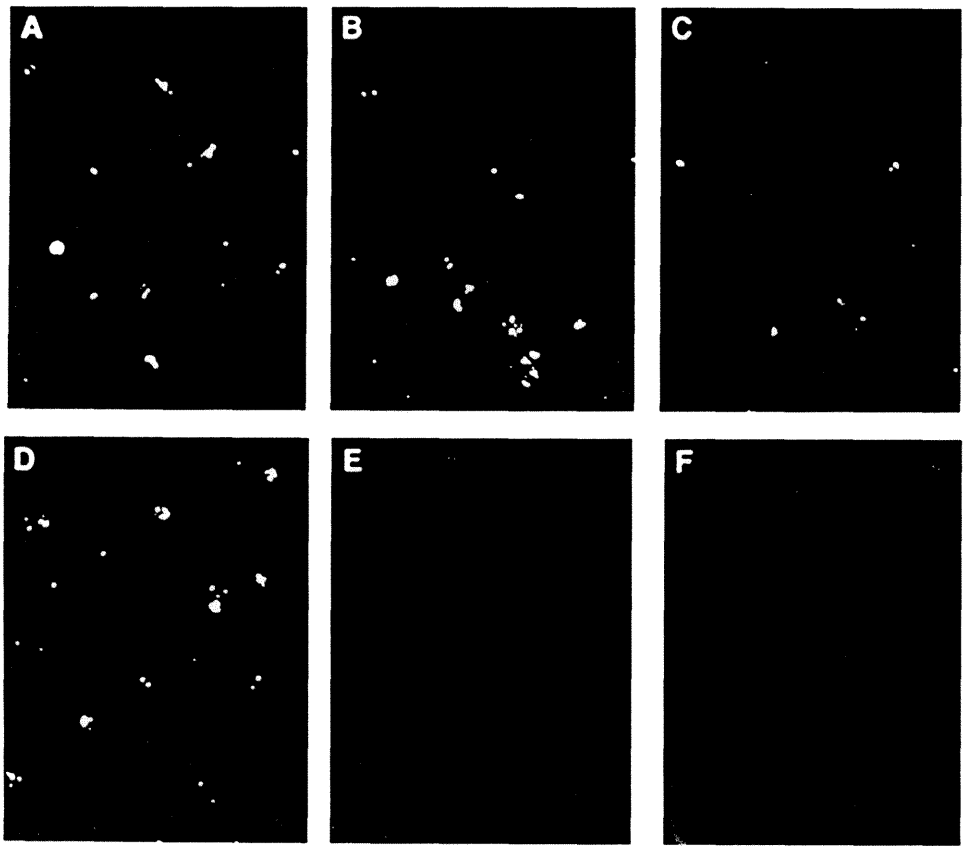


Figure 9. Effect of exogenous glycosaminoglycans on binding of PLL-^{32}PDNA. PLL-^{32}PDNA complexes were prepared and incubated with HeLa cells in the presence of glycosaminoglycans (40 g/ml) for 4 hours at 4 °C. Cells were rinsed extensively, dissolved, and quantitated as described (Figure 6). Results are presented as a percentage of binding relative to untreated HeLa cells. Error bars represent the range of the observed values over duplicate samples. *Legend: 1: heparan sulfate 2. heparin 3. chondroitan sulfate A 4. chondroitan sulfate B 5. chondroitan sulfate C 6. hyaluronic acid*

Figure 9. Effect of exogenous glycosaminoglycans on binding of PLL-^{32}PDNA.

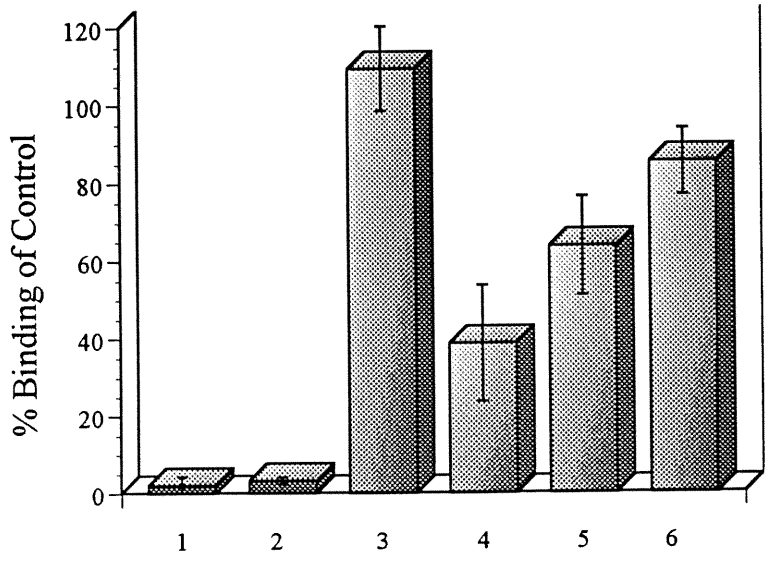


Figure 10. Uptake of PLL-YYDNA into wild-type and mutant CHO cells. PLL-YYDNA complexes were prepared and incubated with wild-type and mutant CHO cells for 4 hours at 37 °C. Cells were prepared and imaged as described (Figure 5). Each panel contains approximately the same number of cells.

Figure 10. Uptake of PLL-YYDNA into wild-type and mutant CHO cells.

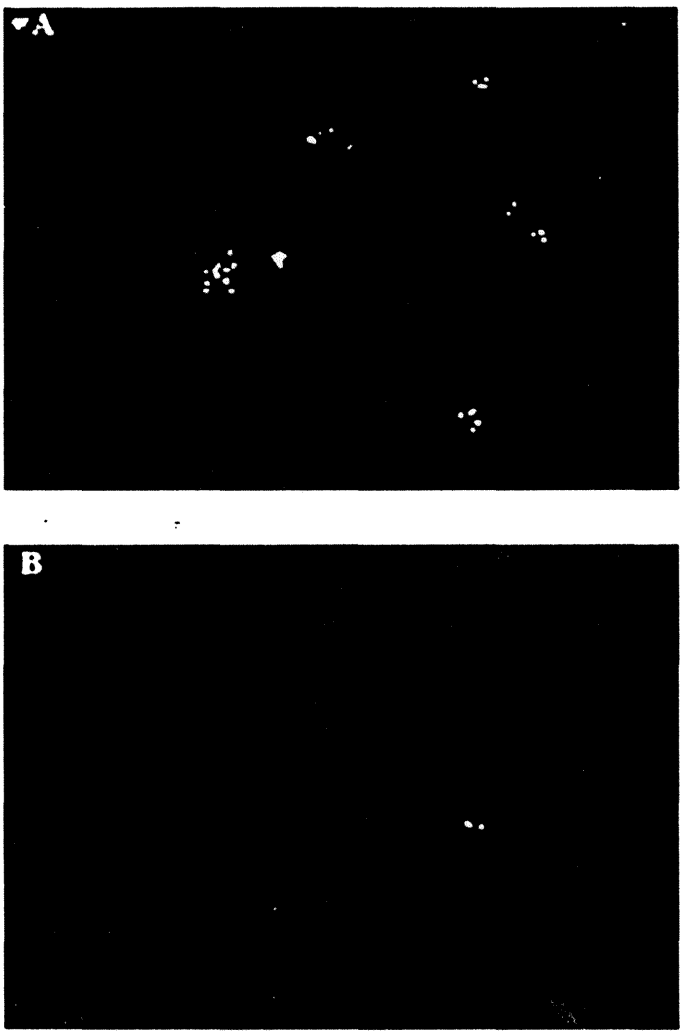


Figure 11A. Transfection of wild-type and mutant CHO cells by cationic lipids. Each lipid was transfected into CHO-K1 and CHO-*pgs745* cells (~2 x 10^5 cells/well) using 0.2 μg DNA and the optimum amount of lipid (as determined by the manufacturer). Cells were exposed to lipid:DNA complexes for four hours in serum free Ham's F-12 media, rinsed twice with PBS, and incubated in regular growth media for an addition 20 hours. Luciferase expression in cellular lysates was determined as described (Figure 3).

Figure 11B. Dependence of the correlation factor, *X*, on cationic lipids used for transfection. A correlation factor, *X*, defined as (RLU wild-type/RLU mutant) was calculated from the expression data in Figure 11A. *X* is plotted as a function of the type of lipid used.

Figure 11A. Transfection of wild-type and mutant CHO cells by cationic lipids.

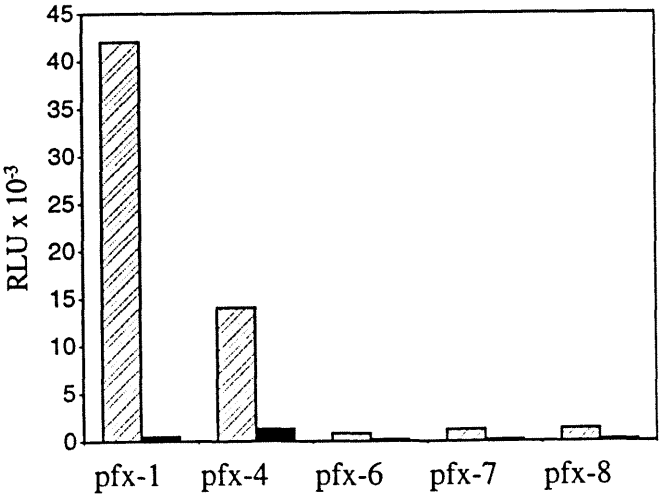
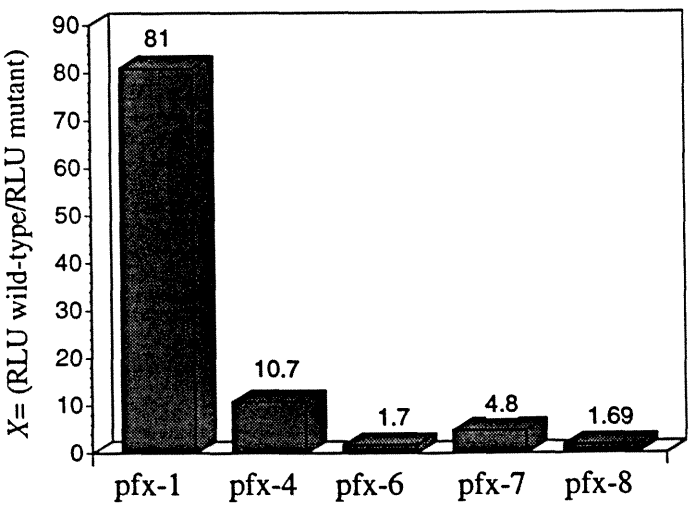


Figure 11B. Dependence of the correlation factor, *X*, on cationic lipids used for transfection.



Chapter 3: Transfection by Folate Receptor-Mediated Endocytosis: Evidence for Lysosomal Delivery

Abstract

We have utilized the folate receptor for the intracellular delivery of DNA. In this study, a series of folate-poly-L-lysine (FPLL) conjugates were synthesized and complexed with plasmid DNA encoding the firefly luciferase gene. The FPLL-DNA complexes were added to KB cells treated with chloroquine. Maximum luciferase activity resulted by titrating plasmid DNA to neutrality with FPLL conjugates consisting of approximately 8-14 folates covalently linked per polypeptide. At these optimized conditions, cells expressed eighteen times more luciferase than cells transfected with poly-L-lysine (PLL)-DNA, and the addition of excess, free folic acid competitively inhibited gene expression. Removal of chloroquine from the media significantly inhibited transfection efficiency of FPLL-DNA complexes. We conclude that PLL-DNA complexes are delivered more efficiently into the cell by folate receptor-mediated endocytosis but are brought into the cytoplasm by a lysosomal dependent pathway.

Introduction

Although gene therapy has progressed significantly in the past few years, problems associated with transferring foreign genetic material into cells remain. The delivery of genes across the cell membrane and into the nucleus is nontrivial since DNA is not readily endocytosed. Thus, DNA must be packaged into a vehicle capable of efficient entry into cells. Ideally, this vehicle possesses a mechanism to avoid lysosomal degradation and can be targeted to particular cells and tissues as well as receptors that are expressed on specific cell types.

Non-specific methods for delivering genes include the use of positively charged macromolecules such as polyamidoamines (1), polylysine (2), and lipid-based amphiphiles (3) to bind and electrically neutralize plasmid DNA. The DNA complexes attach to the cell surface and are internalized by adsorptive endocytosis. As a cell-specific alternative to these methods, receptor mediated polylysine constructs have been developed. A fraction of the ε -amino moieties of polylysine are covalently linked to a receptor ligand, and the modified polylysine is incubated with plasmid DNA. The resulting complex is delivered into the cell by receptor- mediated endocytosis (see Figures 4-6, Chapter 1). If agents such as chloroquine or viral membrane fusion components are included in the transfection to prevent lysosomal degradation, the gene is efficiently expressed. Using this technique, polylysine-DNA complexes have been transfected via the endocytosis of the asialoorosomucoid (4), transferrin (5), and mannose receptors (6). We are exploring the delivery of polylysine-DNA based on the cell-surface receptor for folic acid.

Folate compounds are a class of essential vitamins used in the biosynthesis of purines and amino acids. All folates are characterized by a pteridine ring, a p-aminobenzoate ring, and one or more glutamic acid residues (Figure 1). Of the existing folate derivatives, folate and 5-methyltetrahydrofolate are the principle physiological forms found at 25-50 nM in human blood (7). Folate deficiencies in pregnant women cause birth defects and are predispositional to several inherited diseases such as Fragile X Syndrome (8) and severe megaloblastic anemia (9). Defects in the cellular transport of folate can result in mental retardation (10). Recent evidence shows a link between folate deficiencies and the induction of carcinogenesis (11-12).

Over the past ten years, a copious amount of research has been devoted to the study of folic acid and its interactions with a high affinity binding site, or receptor, on cell membranes. The folate receptor is overexpressed on some neoplastic tissues in order to meet the increased nucleotide requirements of rapidly dividing cells. However, many tissues do not express an endogenous folate receptor and thus may acquire folic acid through a low-affinity plasma membrane carrier or nonspecifically by an as yet undefined mechanism (13). The possible utilization of both the folate receptor and carrier uptake mechanisms by some cells is still under debate (14-15). The distribution of the folate receptor among tissues *in vivo* and in common cell cultures is summarized in Tables 1A and 1B (16). KB cells, derived from a human nasopharyngeal carcinoma, are routinely employed for studying cellular folate

metabolism (17), because the expression of the receptor on this cell line is higher than on any other cell type (18).

The folate receptor is not an integral membrane protein but is attached to the cell membrane by a glycosylphosphatidyl inositol (Figure 2) (19). The receptor binds two monoclonal antibodies, MOV18 and MOV19 and 5-methf and folic acid ($k_d \sim 10^{-9}$-10^{-10} M) (20). Once the receptor binds a ligand, the receptor -ligand complex is shuttled to non-clathrin coated caveolae, or plasmalemmal microinvaginations approximately 50-100 nm in diameter (21). Caveolae are sequestered from the extracellular environment by pinching from the plasma membrane and are acidified through the action of an ATP dependent proton pump. The pH reduction in the caveolae induces folate-receptor dissociation, and free folic acid is transported into the cytoplasm via a low affinity-anion carrier in the caveolae membrane (Figure 3) (21). The mechanism of folic acid uptake is unique, because it does not merge with clathrin associated structures such as lysosomes (22).

The high density of folate receptors on neoplastic tissues and the novel intracellular pathway of folic acid suggested that folate receptor-mediated endocytosis might provide a useful method for the delivery of protein toxins into cancer cells. This therapeutic strategy had been attempted with conjugates of toxins and antitransferrin receptor antibodies as a way to kill cancer cells that upregulate the transferrin receptor. However, the degradation of toxin conjugates in the lysosome seriously limited the value of this therapy (23). Since the folate pathway was known

not to merge with lysosomes, Leamon *et al.* suggested that folate conjugated macromolecules might avoid lysosomal degradation.

To test this hypothesis, Low *et al.* covalently linked folic acid to ^{125}I-labelled bovine serum albumin (BSA) and incubated the protein conjugate with KB cells. While unmodified ^{125}I-BSA hardly entered cells, folate-^{125}I-BSA was efficiently endocytosed in a time and concentration dependent manner. Cellular uptake was effectively blocked by excess folic acid or anti-folate receptor antibodies (24). Although these results demonstrated that proteins could be more efficiently transported across the membrane by folate receptor-mediated endocytosis, conclusions regarding the intracellular destination of ^{125}I-BSA-folate conjugates could not be made. Since caveolae had been shown to remain associated with the plasma membrane during endocytosis (22), and an anion carrier protein would be unable to transport a folate conjugated protein, it was possible that ^{125}I-BSA-folate conjugates had remained trapped in sequestered caveolae. However, if folate-protein conjugates entered the cytoplasm following folate receptor-mediated endocytosis, then a lysosome independent pathway into the cytoplasm might exist.

To gain further insight into the destination of folate conjugates, Low *et al.* synthesized a conjugate of folate and the cytotoxin, momordin (25). This cytotoxin is a 31 kDa glycosylated protein that catalyzes the cleavage of N-glycosidic bonds on the 28S ribosomal subunit. Unlike other ribosome inhibiting proteins such as diphtheria toxin (26) and ricin toxin (27), momordin does not possess a receptor binding domain (28) and cannot enter mammalian cells (29). Low *et al.* demonstrated

that folate-momordin conjugates entered cells and also inhibited protein synthesis. The authors concluded that since momordin-folate could escape into the cytoplasm as an active toxin then, a non-lysosomal delivery pathway must exist.

Electron microscopy experiments were conducted to elucidate the intracellular pathway of folate conjugates. Although very few gold-labeled folate conjugates were found in lysosomal compartments, the mechanism whereby folate conjugates entered the cytoplasm was not found (30). Despite the inconclusiveness of these studies, the folate receptor was subsequently exploited for the delivery of other toxins (18), liposomes (31-32) and antisense oligonucleotides (33). None of these studies included assays to ascertain whether the intracellular pathway of these macromolecules merged with the lysosomal compartment.

The purpose of this study was to develop materials and methods for accomplishing gene delivery by folate receptor-mediated endocytosis and to evaluate whether lysosomal degradation of DNA complexes could be avoided by this pathway.

Materials and Methods

Cell Culture

KB cells derived from a human nasopharyngeal carcinoma, were generously donated by Professor Philip S. Low (Purdue University) and maintained at 37 ^{o}C in a 5% CO_2, humidified atmosphere. Cultures were allowed to propagate in folate free Dulbecco's Modified Eagle's Media (ffDMEM), supplemented with 10% fetal bovine serum (FBS, Gibco) and 40 mg/L gentamicin (Gibco). Monolayers were grown to confluency and subcultured once every seven days.

For transfection experiments, 2 mL of cell suspension were seeded at a density of 1 x 10^5 cells/mL into a 35 x 10 mm Falcon petri dish. After 24 hours, the media was decanted, and cells were rinsed twice in 2 mL Dulbecco's Phosphate Buffered Saline (DPBS, Gibco). Immediately prior to the addition of DNA complexes, ffDMEM supplemented with 10% dialyzed fetal bovine serum (dFBS, Gibco) and 100 μM chloroquine (Sigma) were added to cells. Folic acid (1 x 10^{-5} M) was added to the media of the control samples.

Plasmid Preparation

The PGL2 vector (6046 base pairs; Promega) encoding the genes for firefly luciferase and ampicillin resistance was amplified in competent JM109 *E. coli* (Promega). The plasmid was purified using a Maxi DNA Prep Kit (Qiagen) according to the manufacturer's instructions.

Preparation of Folate-Polylysine Conjugates

All materials and reagents were purchased from Aldrich and used as received. One hundred milligrams (0.23 mmol) of folic acid (pteroyl glutamic acid) was dissolved in anhydrous dimethylsulfoxide (5 mL) containing pyridine (18 μL). One equivalent each of N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were added to the dissolved folate. The reaction mixture was stirred continuously in the dark at 10 ^{o}C for 14 hours.

An aliquot of NHS-folate (5.3 x 10^{-5}; 5.3 x 10^{-6}, 7.95 x 10^{-4}, or 1.33 x 10^{-3} mmol) was added to poly-L-lysine (M_w= 95 k, 5.3 x 10^{-5} mmol) dissolved in 1 mL 50

mM $NaHCO_3$, pH 8.0. After three hours, the mixture was loaded onto a PD-10 G-25M column (Pharmacia) equilibrated in PBS. The sample was dialyzed against deionized water to remove unreacted NHS-folate and salts and lyophilized. The sample was redissolved to 100 μg/mL in sterile water and purified by size exclusion FPLC on a Superdex-200 column (Pharmacia). The mobile phase was 150 mmol NaP_i, pH 7.5 (peak retention times: PLL, 23 minutes, FPLL, 22 min.; unreacted small molecules 33 min.) and monitored at 214 and 280nm (~ 50% yield). No effort was made to separate the isomers of the modified derivative. Spectrophotometric analysis revealed four absorption maxima, λ_{max} = 220, 256, 283 and 368 nm. Polylysine content in purified samples was quantitated by the fluoraldehyde assay (Pierce) and compared to folate content (ε_{256}= 2692; (24)). In these experiments, conjugates consisting of 4, 8, 14, and 18 molar equivalents of folate per equivalent of polylysine were synthesized.

Preparation of DNA Complexes and Transfection

Polylysine-DNA complexes were prepared with minor modifications to published procedures (5). Three μg plasmid DNA (100 μg/mL) were added to 150 μL Hepes-buffered saline (HBS; 150 mM NaCl/10mM Hepes, pH 7.4). To the buffered plasmid was added varying amounts (1.5, 2.0, 2.5, or 3.0 μg of 100 μg/mL in sterile water) of poly-L-lysine (PLL) or folate-poly-L-lysine (FPLL). Samples were mixed, left to equilibrate for approximately 40 minutes at room temperature, and then added to cells. After a four hour incubation, transfection media was removed and

replaced with 2 ml fresh ffDMEM, 10% FBS. Cells were lysed and assayed for luciferase activity 22 hours later.

To harvest the cells, monolayers were rinsed three times in DPBS (calcium and magnesium free), and scraped with a rubber policeman into 1.5 mL eppendorf tubes. Cells were resuspended in DPBS and counted in a hemocytometer. Cell pellets were mixed with 120 µL luciferase assay lysis buffer (Analytical Luminescence Laboratories), and kept at 4 ^{o}C for 15 minutes. The lysate was combined with the assay substrates according to the manufacturer's protocol. Light units were integrated over a 10 second interval in a Monolight 2010 Luminometer (Analytical Luminescence Laboratories). A second group of cells was transfected and assayed for overall protein synthesis as described elsewhere (25).

Results and Discussion

Synthesis and Purification of Folate-Polylysine Conjugates

Figure 4 describes the synthesis used to prepare the folate-polylysine conjugates. The presence of two free, folate-associated carboxyl groups allows a straightforward amide linkage between folic acid and the polylysine backbone. In general, amide linkages are formed by a two-step process beginning with the formation of an "activated" carboxyl with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, water soluble) or dicyclohexylcarbodiimide (DCC). This reaction is favored at acidic pH. The activated species, an O-acylurea, is then added to the reactant containing primary amines at basic pH. Reactions involving

carbodiimides are limited by an extremely short half life of the activated species and by undesirable side reactions such as the hydrolysis of the O-acylurea to the starting material or by the formation of stable N-acylureas. These problems can be circumvented by reacting the carboxyl with N-hydroxysuccinimide and the carbodiimide to form a stable ester (34). This strategy was adopted for the preparation of folate polylysine conjugates.

Following the conjugation reaction, folate-polylysine was purified from small molecules by size-exclusion chromatography on a Pharmacia Fast Protein Liquid Chromatograph (FPLC). The retention times of PLL, FPLL, and unreacted small molecules were 23, 22 and 33 min, respectively (Figure 5). The absorbance spectra of the conjugates are consistent with the structure of the desired conjugate (Figure 6).

Optimization of Transfection by FPLL-DNA

In receptor-mediated gene delivery, two important factors are the amine/nucleotide ratio (charge ratio) and the number of receptor ligands conjugated per polylysine (conjugation ratio). To test both of these variables a series of FPLL conjugates with different conjugation ratios were synthesized and purified. DNA (3 μg) was then titrated with each of these conjugates and the resulting complexes were added to cells in the presence of chloroquine.

Figure 7 shows that for each conjugate, transfection was optimized at or near the charge neutralization point of DNA. Samples prepared with an excess of FPLL resulted in decreased transfection efficiency, presumably due to the presence of uncomplexed conjugate that can compete for cellular binding sites. This is in marked

contrast to dendrimers and cationic liposomes that require a net positive charge for optimum gene delivery. The optimum FPLL conjugation ratio was between 8 and 14. A single DNA complex made with these FPLL conjugates contains between 72 and 126 folic acid molecules.

A series of control transfections were performed to confirm that gene delivery occurs by folate receptor-mediated endocytosis. Transfection with native polylysine at the optimum charge ratio resulted in an 18 fold lower level of luciferase activity than with folate-polylysine. Moreover, the addition of free folate to cells incubated with PLL-DNA did not enhance the expression of luciferase indicating that a covalent linkage between folate and polylysine was required for enhanced transfection. Excess folate added to cells transfected with FPLL-DNA effectively reduced luciferase expression to levels obtained by PLL-DNA (Figure 8).

Since folate is an essential nutrient and cells were cultured in folate deficient media, it is possible that the relative differences in luciferase expression among the cells were merely caused by different amounts of folate containing compounds added to the cells. To test this hypothesis, protein synthesis was measured in cells transfected by each of the combinations in Figure 8. As determined by the extent of [^{3}H]-leucine incorporation into cellular proteins, protein synthesis was appproximately the same in all of the samples (Figure 9).

We also tested whether transfection inhibition by excess, free folate was caused by an inhibition of DNA uptake. This control has been typically performed by preparing DNA complexes with fluorescein labeled polylysine conjugates and adding

these complexes to cells incubated with an excess of free ligand. These controls are not ideal since the elimination of cellular fluorescence by the free ligand proves only that the polylysine conjugate enters the cell by the appropriate receptor. To show that the uptake of DNA is inhibited, fluorescently labeled complexes composed of FPLL and YOYO-DNA (Chapter 4) were prepared and incubated with KB cells in the presence of excess, free folic acid. After four hours, cells were fixed and evaluated with confocal microscopy. As demonstrated in Figure 10, free folic acid effectively reduced the uptake of DNA into the cell.

Through these experiments, we demonstrated that FPLL-DNA complexes are transported into cells by folate receptor-mediated endocytosis and lead to significant luciferase expression in the presence of chloroquine.. To learn about the natural pathway of FPLL-DNA, cells were also transfected under the optimized conditions in the absence of chloroquine. Transfection efficiency in untreated cells was approximately 30 times lower than in chloroquine treated cells (Table 2). These results indicate that the natural intracellular pathway of FPLL-DNA merges with the lysosomal compartment.

Discussion

The folate recepor has been exploited for the intracellular delivery of a number of macromolecules including liposomes (31-32), antisense oligonucleotides (33), and proteins (18). This chapter exploits folate receptor-mediated endocytosis for the transfection of DNA into KB cells by folate-polylysine conjugates. Expression was optimized when the negatively charged DNA was neutralized by the FPLL conjugates.

The most efficient transfection occurred using FPLL conjugates that consisted of 8 to 14 folates covalently linked per polylysine. At these optimized conditions, luciferase expression was nearly an order of magnitude greater than expression reported by transferrin-polylysine mediated transfection into K562 cells (35).

Maximum expression was obtained when transfections were performed in the presence of chloroquine. This compound has been shown to neutralize the pH of endosomes and lysosomes and to inhibit fusion of endosomes with lysosomes (36). Therefore, in the presence of chloroquine complexes probably languish in endosomes until the limited lifetime of the endosome enables the DNA complex to enter the cytoplasm.

Cells were also transfected with FPLL-DNA in the absence of chloroquine which led to a 30 fold decrease in luciferase expression. From these results, we suggest that DNA complexes delivered by folate receptor-mediated endocytosis merge with lysosomal compartments of the clathrin coated pit pathway. Although the effects of chloroquine on caveolae dependent pathways were not investigated, this is probably a reasonable conclusion.

These findings led us to question the conclusion made by Leamon *et al.* that folate receptor mediated endocytosis can be exploited for the non-destructive delivery of macromolecules. This conclusion was based largely on the observation that folate-momordin conjugates could inhibit protein synthesis in a time and concentration-dependent manner, although the effects of chloroquine on folate-momordin toxicity were not investigated (37). Since a small concentration of toxin could have lethal

consequences on a cell, it is possible that momordin-folate induced toxictiy was due to a small number of folate-toxins that had escaped lysosomal degradation. It is also plausible that once delivered into the endosome, the fate of the toxin conjugate was determined by the toxin, and not by the receptor or by folic acid. Momordin toxin has sequence homology with the catalytic portions of the ricin A chain which can translocate into the cytosol following delivery into an intracellular compartment (38). Finally, we have considered the possibility that our results are different from Leamon *et al.* because the pathway of FPLL-DNA is different from momordin-folate. FPLL-DNA complexes are multivalent and are much larger than toxin conjugates, which could route the complexes to lysosomes.

Lysosomal degradation remains a problem for receptor mediated gene delivery that cannot be solved by the use of the folate receptor pathway; however, methods to limit lyosomal degradation are currently being developed. Whole adenoviruses or adenoviral components included in receptor mediated transfection either by covalent attachment to the polylysine backbone or added free to cell culture media can increase gene expression dramatically by destabilizing the endosomal membrane (39). Recently, viral components were included in transfections by folate receptor-mediated endocytosis. Release of the DNA out of the endocytosed caveolae (potosome) was augmented which led to a signficant increase in transfection efficiency (40).

The delivery of polylysine-DNA complexes via receptor-mediated endocytosis *in vitro* has been well established by a number of laboratories. The crucial question that must now be answered is whether polylysine-DNA complexes can be delivered by

receptor-mediated endocytosis into specific tissues *in vivo*. The mode of administration of the complex may have a large influence on the answer to this question. PLL-DNA complexes injected intravenously will be partially protected from serum nucleases (41), but the presence of competitive serum polyanions such as heparin may destablize PLL-DNA complexes, rendering DNA susceptible to nuclease attack.

Additional factors will influence the fate of the complexes *in vivo*. Many years of research into the use of liposomes as drug carriers has demonstrated that both size and chemical composition determine whether a liposome is cleared by the liver and spleen or is able to pass into the vasculartur e of a tumor (42). The size of DNA complexes are between 10 and 150 nm (43-44), which is in the size range of liposomes that can be successfully delivered to tumors. Therefore, tumor targeting of polylysine-DNA complexes may be possible.

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Table 1A. Distribution of the folate receptor in normal tissues (16). The distribution of the folate receptor *in vivo* was determined by an immunoblot assay on tissues obtained from a cadaver of an 18 year old male. Signal: +++, very strong positive; +, positive; +/-, weak positive; -, negative. The choroid plexus was the only tissue with quantifiable folate receptor levels.

Table 1B. The quantity of folate receptor expressed on established cell lines (16). The quantity of folate receptor expressed on cell lines was determined by a radiolabelling assay using [^{3}H]folic acid. Values are expressed in terms of pmol folate receptor per 10^6 cells.

Table 1A. Distribution of the folate receptor on normal tissues.

Tissue	Folate Receptor
choroid plexus***	+++ (9 pmol/~10^6 cells)
kidney	+
lung	+
hyroid	+
liver	-
spleen	-
skin	+
aorta (artery)	+
cerebrum	+/-
muscle	+/-
pancreas	+/-
gut	+/-
heart	+/-

Table 1B. The quantity of folate receptor on established cell lines.

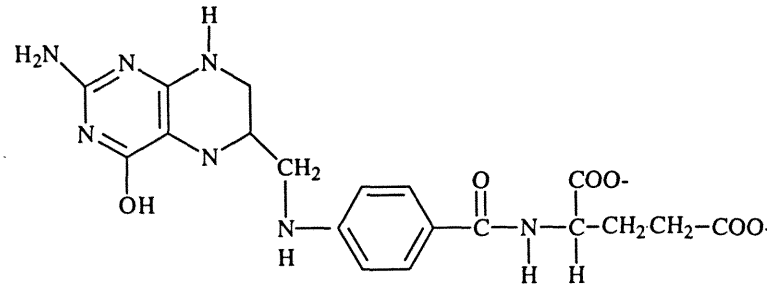
Cell Type	*pmol/10^6 cells*
fibroblasts	<0.1
CHO (hamster ovary)	<0.1
MA104 (monkey kidney)	1
IGROV-1 (human ovary, carcinoma)	20
Caco-2 (human, colon adenocarcinoma)	20
KB (human, nasopharyngeal carcinoma)	50-200
HeLa (human, cervical carcinoma)	15-60

Table 2. The effect of chloroquine on the transfection of FPLL-DNA complexes. Using the optimized conditions, a group of cells were transfected in the absence or presence of chloroquine (100 μM). Cells were treated as described in Figure 7. Results are the average of duplicate samples normalized per 10^6 cells.

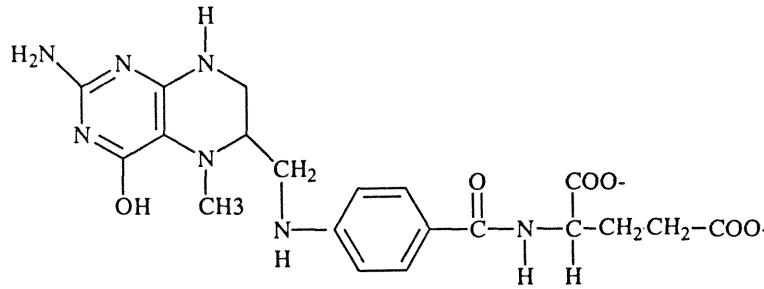
Table 2. Effect of chloroquine on the transfection of FPLL-DNA complexes.

Relative Light Units	*Chloroquine (100 μM)*
1.6×10^6	+
5.3×10^4	-

Figure 1. Structure of folate compounds.

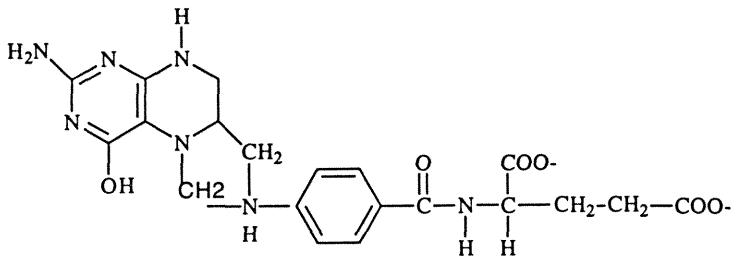


Tetrahydrofolate
(Folic Acid)

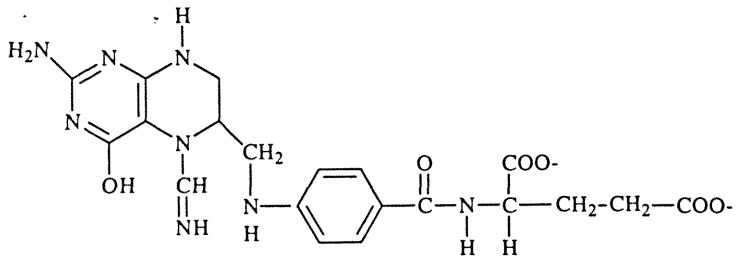


N^5 -Methyl-tetrahydrofolate

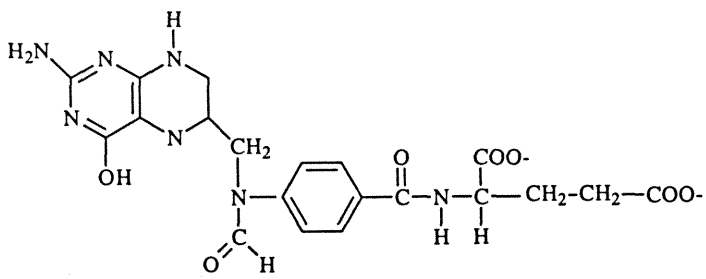
Figure 1. Structure of folate compounds cont'd.



N^5N^{10}-Methylene-tetrahydrofolate



N^{10}-Formimino-tetrahydrofolate



N^{10}-Formyl-tetrahydrofolate

Figure 2. Schematic of the folate receptor. Unlike integral membrane proteins that span the plasma membrane one or more times, the folate receptor is linked to the membrane by a glycosylphosphatidyl inositol (GPI). The C-terminal amino acid of the folate binding protein is linked to a phosphoethanolamine bridge. A glycan consisting of mannose and glucosamine is bound to a phosphatidylinositol-fatty acid inserted into the plasma membrane.

Figure 2. Schematic of the folate receptor.

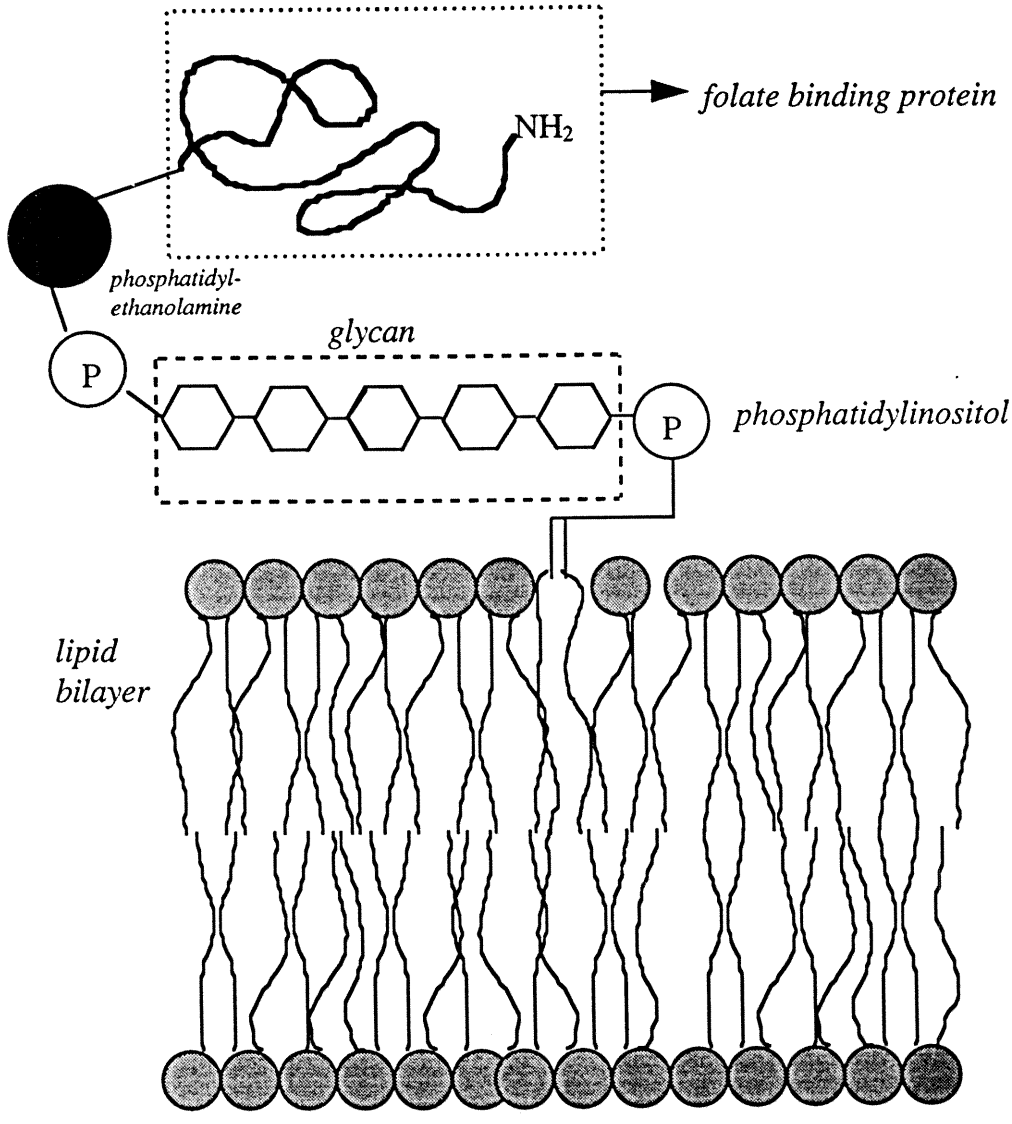


Figure 3. Endocytosis of folic acid by caveolae. Once the folate receptor binds a ligand, and the receptor -ligand complex is shuttled to non-clathrin coated caveolae, or plasmalemmal microinvaginations approximately 50-100 nm in diameter. Caveolae are sequestered from the extracellular environment by pinching from the plasma membrane and are acidified through the action of an ATP dependent proton pump. The pH reduction in the caveolae induces folate-receptor dissociation, and. free folic acid is transported into the cytoplasm via a low affinity anion carrier in the caveolae membrane. The mechanism of folic acid uptake is unique, because it does not merge with clathrin associated structures such as lysosomes.

Figure 3. Endocytosis of folic acid by caveolae.

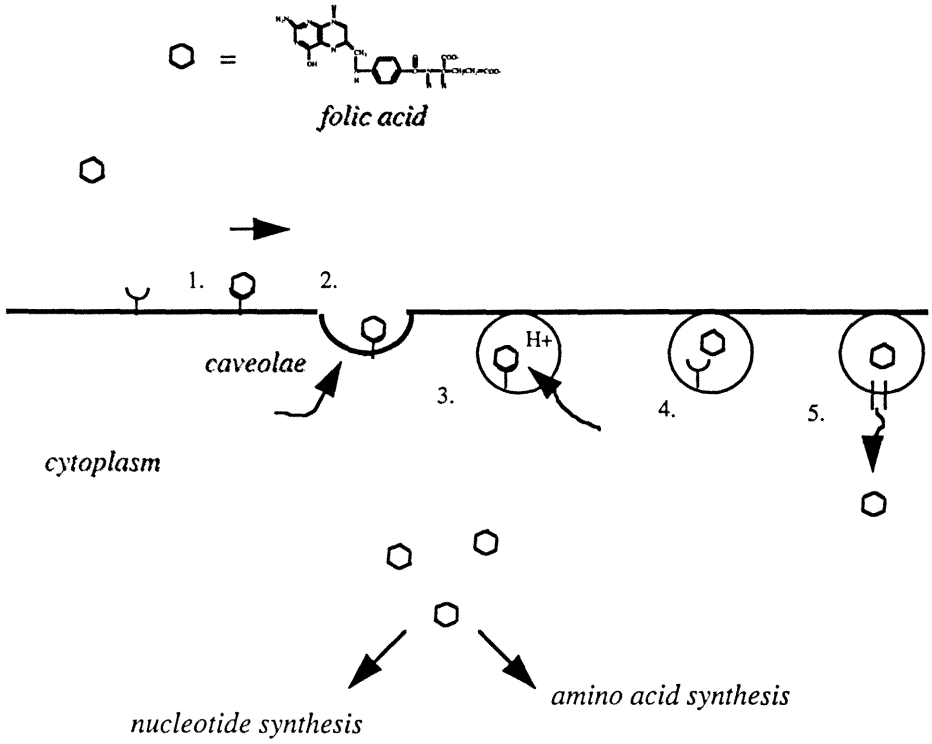


Figure 4. Synthesis of folate-polylysine conjugates. One hundred milligrams (0.23 mmol) of folic acid were dissolved in anhydrous dimethylsulfoxide (5 mL) containing pyridine (18 μL). One equivalent each of N-hydroxysuccinimide and EDC were added to the dissolved folate. The reaction mixture was stirred continuously in the dark at 10 ^{o}C for 14 hours. An aliquot of NHS-activated folic acid (5.3×10^{-5}; 5.3×10^{-6}, 7.95×10^{-4}, or 1.33×10^{-3} mmol) was added to poly-L-lysine (M_w= 95 k, 5.3×10^{-5} mmol) dissolved in 1 mL 50 mM $NaHCO_3$, pH 8.0. After three hours, the mixture was loaded onto a PD-10 G-25M column (Pharmacia) equilibrated in PBS. The sample was purified by size exclusion FPLC on a Superdex-200 column (Pharmacia).

Figure 4. Synthesis of folate-polylysine conjugates.

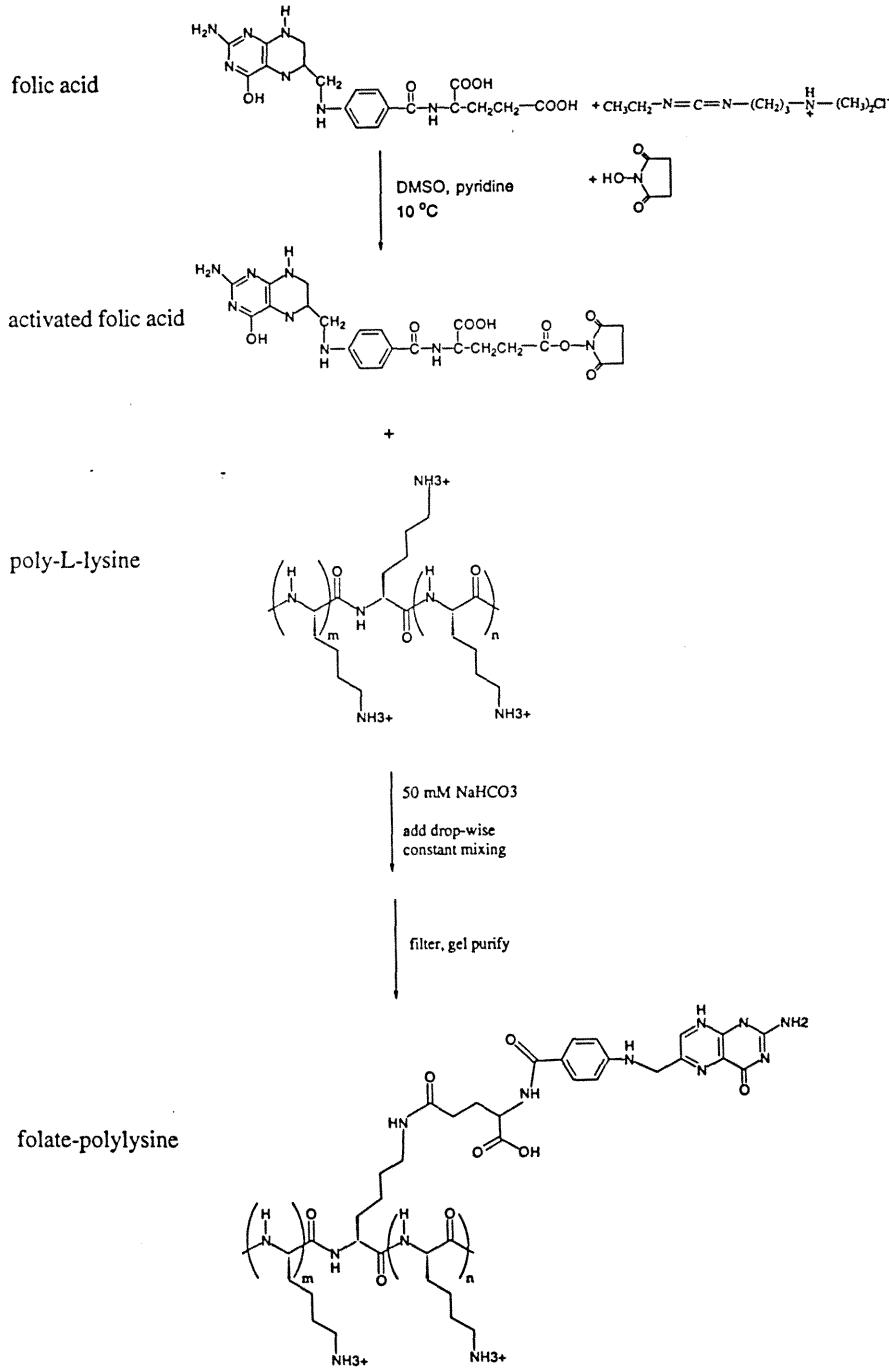


Figure 5. Elution profile of polylysine and folate-polylysine conjugates. Unmodified polylysine (Figure 5A) or folate-polylysine conjugates (Figure 5B) were filtered (0.45 um) and loaded onto a Superdex-200 FPLC column, previously equilibrated in PBS. The eluent was monitored by absorbance at λ =214 nm (dashed line) and λ =280 nm (solid line). Elution times were: polylysine, 23 minutes; folate-polylysine, 22 minutes; unreacted material, 33 minutes. Folate-polylysine conjugates had slightly longer retention times than unmodified polylysine and had an absorbance peak at λ =280 nm.

Figure 5A. Elution profile of polylysine.

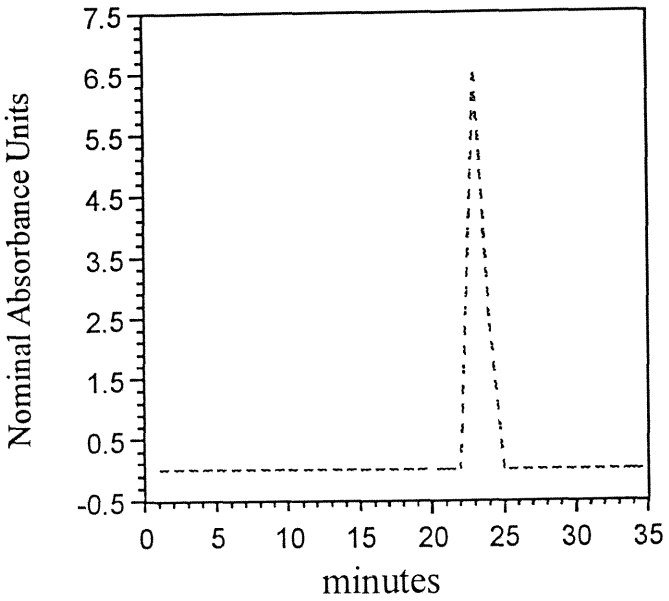


Figure 5B. Elution profile of folate-polylysine conjugates.

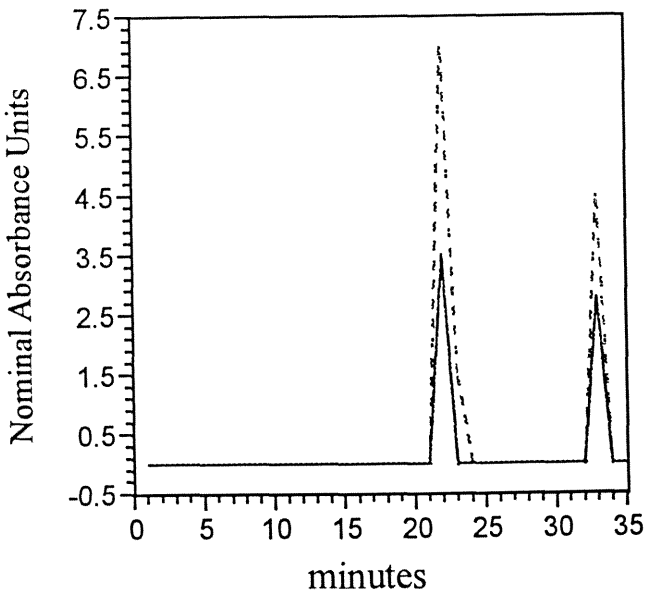


Figure 6. Absorbance spectra of folate-polylysine conjugates. Following purification by FPLC, conjugates were dialyzed against sterile, double deionized water for 6 hours. Samples were dried by lyophilization and dissolved to 1 mg/ml in autoclaved water. Spectrophotometric analysis revealed four absorbance maxima, λ_{max} = 220, 256, 283 and 368 nm that are characteristic for folic acid. Folate content (ε_{256}= 2692) in each of the conjugates was determined and compared to the content of polylysine. In these experiments, conjugates containing 4, 8, 14, and 18 molar equivalents of folate per equivalent of polylysine were synthesized.

Figure 6. Absorbance spectra of folate-polylysine conjugates.

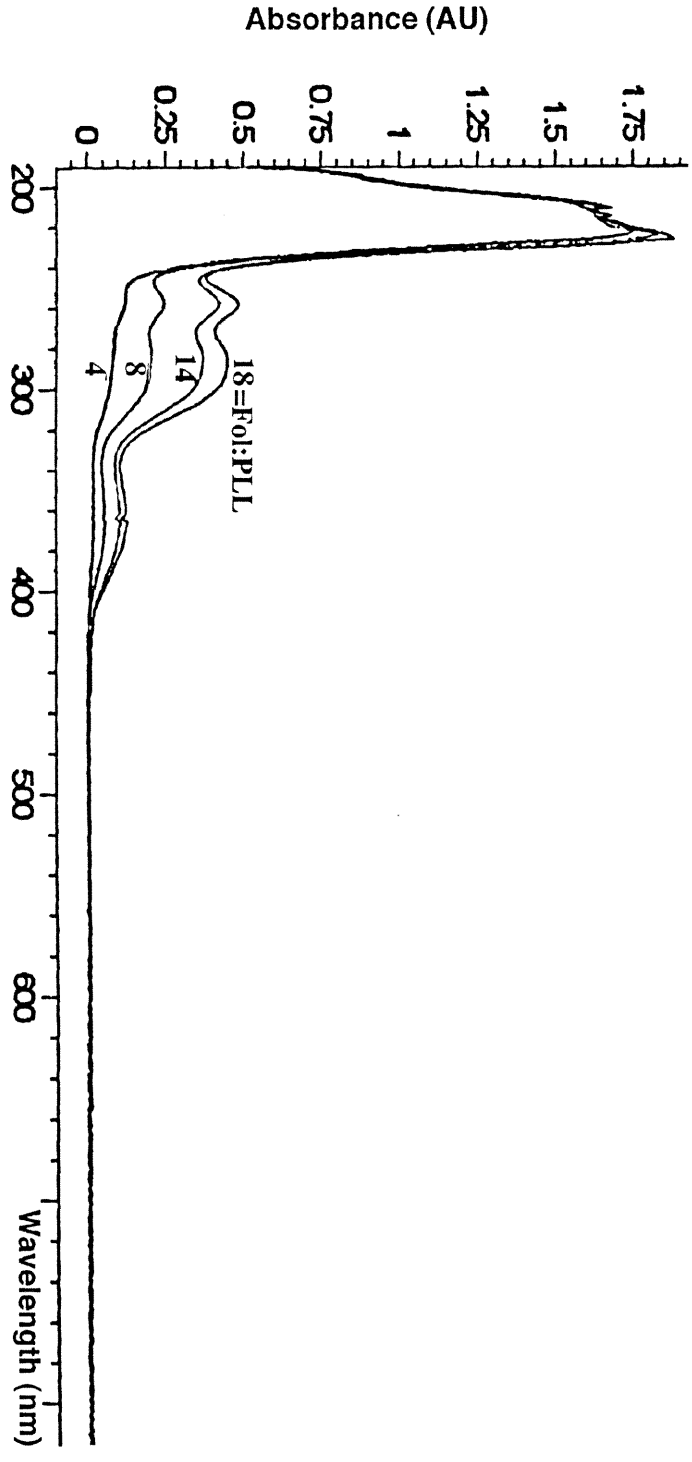


Figure 7. Determination of the optimum charge ratio (lysine:nucleotide) and conjugation ratio (folate:PLL) for luciferase expression. Varying amounts of each FPLL conjugate were added to 3 μg DNA (100 μg/mL H_2O) in 150 μL HBS, pH 7.4. Complexes were mixed gently, incubated at room temperature for 40 minutes and added to 2 x 10^5 KB cells growing in 2 mL ffDMEM 10% dFBS containing 100 μM chloroquine. After four hours, media was removed and replaced with fresh ffDMEM, 10% FBS. Twenty-two hours later cellular lysates were assayed for luciferase activity. Luminescence was integrated over 10.0 sec in an Analytical Luminescence Laboratories Monolight 2010 Luminometer. The data represents the mean of duplicate samples normalized per 10^6 cells.

Figure 7. Determination of the optimum charge ratio (lysine:nucleotide) and conjugation ratio (folate:PLL) for luciferase expression.

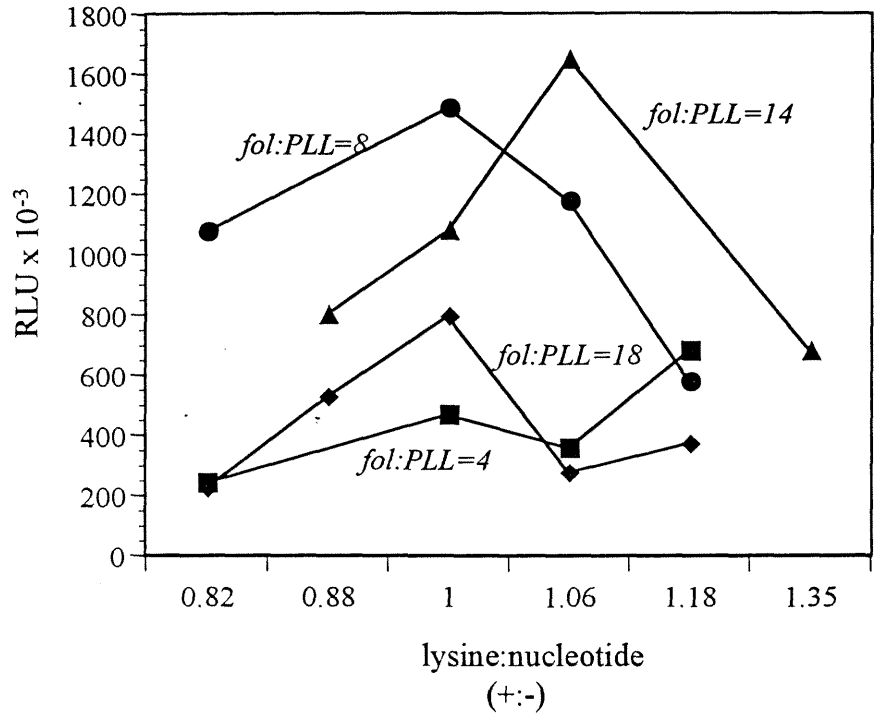


Fig. 8. Demonstration of folate receptor-specific gene delivery. Cells were transfected with an optimized amount of PLL or FPLL as described in Fig.7. Free folate was added (1×10^{-5} M) to the appropriate cells to demonstrate folate receptor specific transfection: (A) PLL-DNA; (B) Folate + PLL-DNA; (C) FPLL-DNA; (D) Folate + FPLL-DNA. Results are the average of duplicate samples normalized per 10^6 cells, and error bars represent the range of the observed experimental values.

Fig. 8. Demonstration of folate receptor-specific gene delivery.

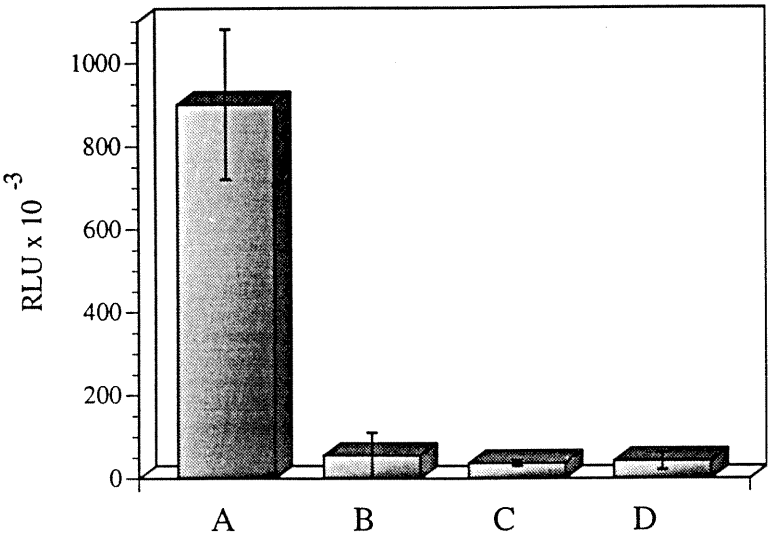


Fig 9. Effect of transfection conditions on protein synthesis. A separate group of cells were transfected using the optimum DNA to FPLL or PLL ratio as described in Fig. 7: (A) PLL-DNA; (B) Folate + PLL-DNA; (C) FPLL-DNA; (D) Folate + FPLL-DNA. Twenty four hours after the addition of complexes to cells, media was removed and replaced with 2 mL leucine free DMEM supplemented with 1μCi [^{3}H]-leucine. Two hours later, cells were rinsed 3 times in 2 mL DPBS and dissolved in 1 mL 0.1 N KOH. Cellular proteins were precipitated in trichloroacetic acid (1mL, 30%) and collected onto Whatman glass fiber filters. Dried filters were added to 5 mL Safety Solve scintillation cocktail, and radioactivity (counts per minute) was quantitated in a Beckman model LS 5000 TD scintillation counter. Results are normalized per 10^6 cells.

Fig 9. Effect of transfection conditions on protein synthesis.

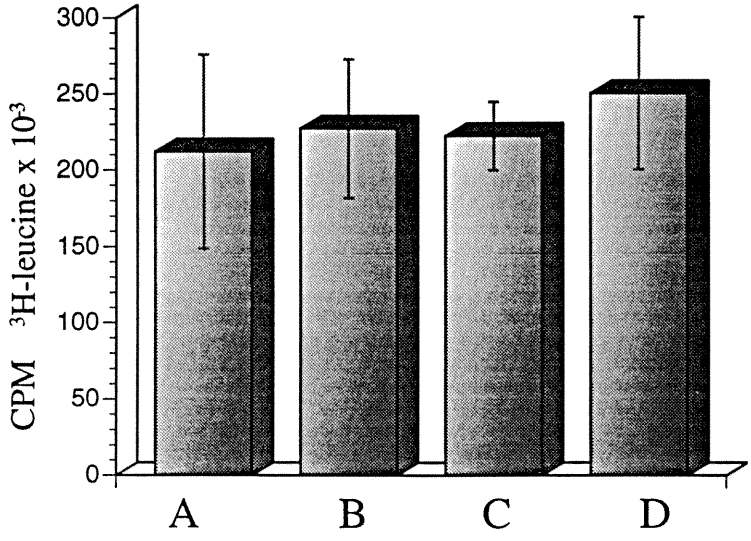
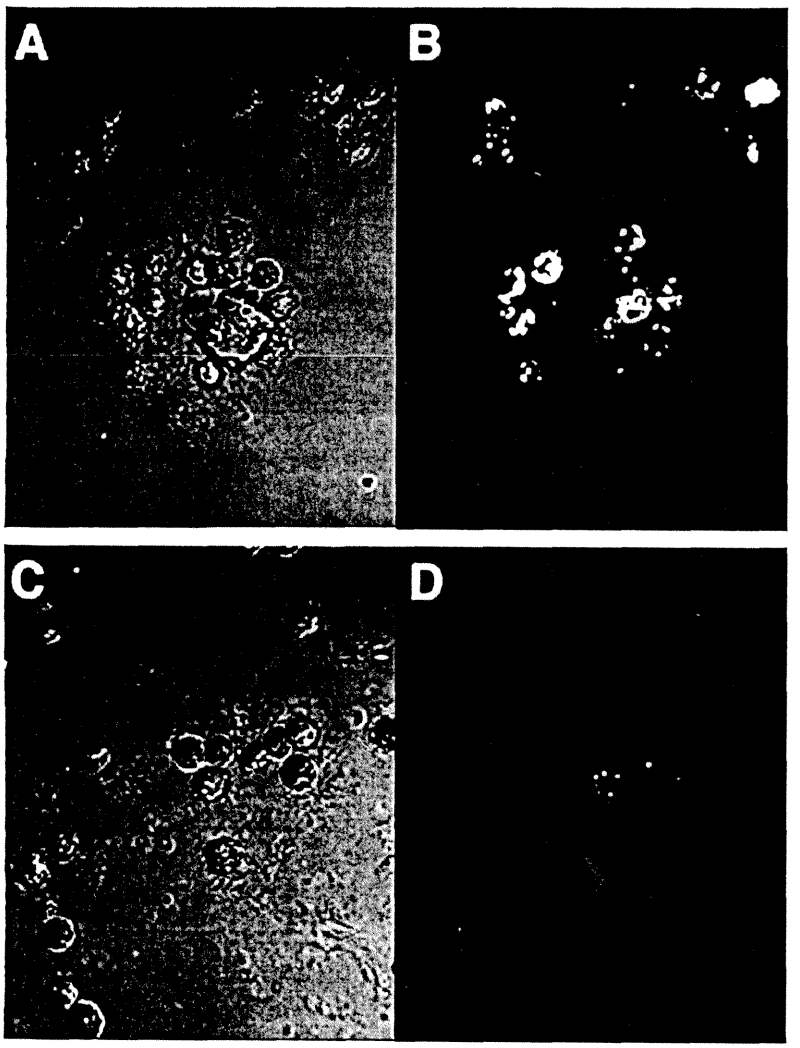


Figure 10. Competitive uptake of FPLL-YYDNA by excess, free folic acid. YOYO labeled DNA (YYDNA) was prepared as described in Materials and Methods, Chapter 2. KB cells were incubated with FPLL-YYDNA complexes for four hours and then rinsed three times in PBS. To remove surface bound DNA, cells were treated with DNAase (Sigma, 1 mg/ml) for 15 minutes and then detached by trypsin. Cell suspensions were pelleted, washed twice in PBS, and fixed in 4% paraformaldehyde/PBS for 10 minutes at room temperature. After a final rinse in PBS, cell pellets were resuspended in Biomeda/PBS solution (90:10) and mounted between a slide and coverslip to dry. Fluorescence and bright field images were obtained using a BioRad Confocal Microscope. A: bright field, FPLL-YYDNA, B: fluorescence, FPLL-YYDNA, C: bright field, FPLL-YYDNA + excess folic acid, D: fluorescence, FPLL-YYDNA + excess folic acid.

Figure 10. Competitive uptake of FPLL-YYDNA by excess, free folic acid.



Chapter 4: Characterization of Cellular Gene Delivery by Two-Photon Laser Scanning Microscopy

Abstract

In this study, two-photon laser scanning microscopy was used to characterize the transfection of DNA into a single cell by folate receptor-mediated endocytosis. The DNA was labeled with YOYO, a fluorescent cyanine intercalator and equilibrated with folate-polylysine (FPLL) conjugates. The FPLL-DNA complexes were added to cells and the transfection process was monitored over a 2 hour period. Preliminary results revealed that most of the DNA formed aggregates in solution that adsorbed to the cell surface but did not enter the cell interior. A fraction of the DNA entered the cell by folate receptor-mediated endocytosis. Surprisingly, a portion of the intracellular DNA was localized in the nucleus of the cell, although no signs of mitosis were observed. Thus, mitosis may not be a strict requirement for nuclear transport of transfected DNA.

Introduction

The plasma membrane is a formidable barrier to the delivery of foreign nucleic acids. Many agents that bind and promote the transfer of DNA across the membrane barrier have been developed (Chapter 1). The use of polylysine and polylysine conjugates for gene delivery has been the subject of many recent studies and is the major focus of this thesis. Methods have been described for augmenting the transfection of polylysine-DNA complexes into cells by covalently modifying the polylysine backbone with ligands for receptor-mediated endocytosis. The ligand-polylysine conjugates bind to DNA to form complexes that enter cells via receptor mediated endocytosis. Although gene delivery by receptor-mediated endoctyosis is inhibited by lysosomal degradation, techniques are currently being developed in a number of laboratories to minimize this problem. Thus, the cellular barriers to gene transfer by the plasma membrane and by lysosomal degradation are well characterized and are being overcome.

Despite these advances, transfection by receptor-mediated endocytosis is still far less efficient than by recombinant viruses, and it is likely that many of the molecular and physical barriers to efficient receptor-mediated gene transfer have not been elucidated. In part, the transfection process has not be thoroughly characterized because, only a limited number of biochemical and microscopic techniques are available for monitoring the transfection process. Electron microscopy can resolve the ultrastructural location of intracellular molecules and has recently been used to characterize cationic lipid-mediated gene transfer (1). However, electron microscopy

is not an ideal technique, because it can provide information only in the context of a large population of fixed cells. An ideal technique would enable one to observe the transfection process into living cells in real-time.

Fluorescent molecules are traced in live cells and tissues in real-time using two-photon laser scanning microscopy (TPLSM). This technique is based on the two photon effect, which predicts that two photons simultaneously interacting with a molecule can produce an excited energy transition equal to the transition of a single excited photon of half the wavelength (2). Unlike confocal microscopy, excitation of the sample is limited to the plane of focus, because the rate of two-photon excitation is dependent on the square of the instantaneous intensity of photons. This property minimizes phototoxic damage to regions of the specimen outside of the focal plane and also improves z-resolution (3). Images obtained from cross sections throughout the interior of the sample are compiled into a three-dimensional reconstruction of tissues and cells, but biological viability is maintained (4). With a heated stage and the proper microsocopic equipment, time-lapsed studies of cellular fluorescence uptake can be conducted.

In this chapter methods for using TPLSM to characterize the transfection of folate- polylysine-DNA complexes were developed. A significant amount of time was spent optimizing the experimental system and determining the best method for tracking the DNA. Labeling DNA by intercalation with YOYO produced the most promising results. The non-diffuse cellular staining pattern demonstrated that although YOYO was not covalently bound, it remained associated with the DNA and

provided a useful method for studying transfection by fluorescence microscopy. Preliminary results indicated that efficient transfection is inhibited by both the formation of large DNA aggregates in solution and by inefficient transport of DNA into the nucleus.

Materials and Methods

Cell Culture

Fetal bovine serum was purchased from Hyclone Laboratories (Utah). All other cell culture reagents were purchased from Gibco-BRL Life Technologies. KB cells were obtained from the American Type Culture Collection (ATCC) and cultured in folate deficient Dulbecco's Modified Eagle's Media (fdDMEM), 10% fetal bovine serum (FBS) for at least 12 weeks in order to maximize folate receptor expression. Twenty-four hours before an experiment, cells were plated (2×10^5/ml) into 35 mm culture dishes. As a control, folic acid was added to cells at the time of plating.

Preparation of Fluorescent Plasmids

The PGL2 plasmid (Promega) encoding the firefly luciferase reporter gene was amplified in competent JM109 *E. coli* (Promega) and purified by chromatographic methods (Qiagen). To prepare fluorescent DNA, plasmid (100 μ g/ml) was incubated with a fifty-fold molar excess of YOYO-1 (Molecular Probes) and incubated for two hours at 4 °C. The solution was loaded onto a Centricon- 30 desalting unit and spun for 3 hours at 6000 rpm and 4 °C to remove unbound dye. The retentate was diluted to its original concentration in sterile water. The molar concentration of YOYO per mole base pair was determined by spectrophotometric analysis to be 1:123. The

fluorescence of YOYO-DNA (YYDNA) was monitored (λ_{ex} = 488 nm, λ_{em}= 500-600 nm) in a Hitachi model F-4500 fluorescence spectrophotometer.

Synthesis of Folate-Polylysine Conjugate

Folate-polylysine conjugates were prepared and characterized as described (Chapter 3). Folate-polylysine with a conjugation ratio equal to 14 was used in these experiments.

Two-Photon Laser Scanning Microscopy

The two-photon laser scanning microscope is located in the Biological Imaging Center, California Institute of Technology. The instrument consists of a Ti:sapphire laser, an Argon ion laser, a scanner controller, and a Zeiss upright microscope. Data were acquired using Image Scan (Molecular Dynamics) image processing software. All images were obtained using a Zeiss 63x water immersion lens.

KB cells were incubated with DNA complexes and immediately placed on the microscope stage, heated to 37 °C. The microscope was insulated with a custom-designed mylar jacket. An individual cell was randomly chosen and brought into focus. Images were acquired for two hours beginning at t=45 minutes.

Results and Discussion

Fluorescence Characterization of YOYO--DNA and FPLL-YOYO DNA Complexes

The sensitivity of TPLSM is dependent on the the fluorophore employed. There are a number of methods for covalently linking a fluorophore to double stranded DNA. Fluorescent nucleotides can be incorporated into plasmids by nick-translation, but this method transforms the topology of the DNA from the supercoiled

to the closed circular form, and the efficiency of incorporation is small (5). Alternatively, DNA can be covalently linked to the photoactivatable intercalator, ethidum monoazide (6), but this dye has a low molar extinction coefficient, and photoactivation can result in the formation of cross-linked plasmids (7).

In this study, plasmid DNA was labeled by intercalation with a dimeric cyanine fluorescent dye, YOYO; 1,1'-(4,4,7,7-tetramethyl-4,7-diazaundecamethylene)-bis-4-[3-methyl-2,3,-dihydro-(benzo-1,3-oxazole). YOYO-1 has an extremely high molar extinction coefficient and is one of the most sensitive stains for the detection of DNA in electrophoretic bands (8). This dye avidly intercalates into double stranded DNA and partitions to excess unlabeled DNA by only 15-20% even after a 10 hours. The affinity for RNA is at least an order of magnitude smaller (9). The strength of the YOYO-DNA interaction is important, because the transfer of YOYO to intracellular RNA and genomic DNA would introduce artifacts in the microscopy data.

Plasmid DNA was incubated with a fifty fold molar excess of YOYO (dye:base pair = 1:120), and the resulting complexes consisted of a 49 molar excess of dye (dye:base pair =1:123). This ratio was not expected to induce structural changes caused by intercalation (8) but was large enough to give a strong fluorescence signal in microscopy experiments.

The fluorescence properties of YOYO-1 DNA folate-polylysine complexes were evaluated. YOYO-DNA was diluted in 500 μ l HEPES buffered saline (HBS, Chapter 2), and placed into a fluorimeter cuvette. The sample was excited at λ =488 nm and an emission spectra was obtained (λ =500-600 nm). A peak in the spectra was

observed at λ=510 nm. An aliquot of folate-polylysine (100 μ g/ml) was added to the YYDNA (Chapter 3) in order to condense and neutralize the plasmid, and the fluorescence at λ=510 nm was monitored over a two hour interval.

Within two minutes after adding the polylysine conjugate, the fluorescence intensity of the YYDNA dropped precipitously (Figure 2). We presumed that the decrease in fluorescence was due to quenching, because fluorescent dyes covalently bound to DNA have been shown to quench upon the formation of DNA complexes with polycations (10). The fluorescence intensity of FPLL-DNA complexes continued to fall until it reached quasi-equiibrium after 30 minutes. At this point, an equilibrium between the plasmid and the folate-polylysine conjugate was probably attained. The sample remained fluorescent for two additional hours which suggested that the fluorescence of FPLL-YYDNA complexes would remain stable througout the duration of a transfection experiment.

Determination of the Average Nuclear Diameter and the Optimum Z-Position

The goal of this experiment was to characterize the fluorescence associated with three main cellular compartments: the plasma membrane, the intracellular space, and the nucleus. To accomplish this goal, it was necessary to follow the fluorescence uptake into a cross section of the cell that included all three of these compartments. One caveat is that cross sections through the nucleus can be contaminated with cytoplasm if the scanner is placed within 1 μ m (the z resolution of the 63x lens) of the cytoplasm-nucleus interface. To avoid artifacts due to cytoplasmic contamination,

the optimum scanner position, defined as the center of the cell, was determined and used throughout the duration of the experiment.

To find the optimum scanner position, the average diameter of a KB cell nucleus and the average depth of the cytoplasm above the nucleus was determined. Cells were incubated with acridine orange, a cell-permeant nucleic acid intercalator that labels RNA in the cytoplasm but binds mainly to genomic DNA (9). Vertical scans (xz plane) through the centers of approximately 10-15 different cells were obtained (Figure 3 A and 3 B). One of these vertical scans is shown in Figure 4. On average, nuclei were 8-10 nm in diameter and asymmetrically located towards the top of the cell. Approximately 3-4 nm of cytoplasm separated the top of the nucleus from the membrane-media interface.

From these experiments, we determined that the optimum scanner position was approximtaely 7-8 μ m below the membrane-media interface.

Time Dependent Transfection of FPLL-DNA

Cells were transfected with FPLL-DNA and placed immediately onto the heated microscope stage. The culture dish and objective were enclosed in a plastic sheath to minimize evaporation, and the entire microsocpe was enclosed in mylar insulation. After approximately 40 minutes, cell-associated fluorescence was bright enough to begin monitoring transfection.

A single, isolated cell with normal morphology was chosen and brought into focus. The cell was scanned vertically, and the membrane-media interface, indicated by the highest z position with a fluorescence signal, was located. The scanner was

then positioned approximately 8 μ m below the membrane-media interface, where the center of the nucleus would be expected. At this position, horizontal scans were acquired over a 120 minute period (Figure 5 A-C). The raw data images were processed by an imaging filter to eliminate noise.

A significant fraction of DNA formed aggregates and adsorbed to the cell surface but did not enter the cell interior. The formation of these aggregates surprised us, since electron microsocopy studies have shown that polylysine-DNA complexes are between 20-150 nm in diameter (11-12). However, complexes used for EM studies are usually prepared in HEPES buffers, because phosphate buffers precipitate the complexes. The aggregates seen in these experiments may have formed after they were added to phosphate-buffered media. The formation of aggregates may significantly inhibit transfection by receptor-mediated endocytosis and may be toxic to cells by blocking the entry of growth factors and other important cellular constituents.

A very small amount of DNA in the form of punctate points was found in the intracellular space. This staining pattern suggested that labeling the DNA with YOYO was a valid method for tracing gene delivery, since a diffuse pattern would indicate that the dye could partition to cellular RNA and genomic DNA. We could not ascertain whether the complexes were in vesicular compartments or were free in the cytoplasm, because a counterstain for intracellular organelles or membranes was not employed in these preliminary experiments.

To verify that intracellular YYDNA was transfected by receptor-mediated endocytosis, FPLL-YYDNA was added to cells exposed to excess folic acid in the

media. Membrane-associated aggregates were still observed, but intracellular fluorescence was much lower than in folate deficient cells. This control was repeated with approximately 5 other randomly chosen cells (not shown).

A very small fraction of intracellular fluorescence was found in the nucleus. This result was repeated with three other randomly chosen cells on different days. Nuclear fluorescence was in the form of punctate points similar to those observed in the cytoplasm. Although fluorescence accumulated in the nucleus throughout the transfection period, the images indicated that nuclear transport is a significant barrier to efficient gene delivery.

The localization of even a small amount of DNA in the nucleus was surprising since, it has been hypothesized that DNA is too large to enter the nuclear pore and can enter the nucleus only during mitosis when the integrity of the nuclear membrane is compromised. Although the results are suggestive of nuclear specific fluorescence, we cannot conclude from these experiments that fluorescence is not due to cytoplasmic contamination. Control experiments using diacetate and coumarin cytoplasm dyes were used as counterstains, but unfortunately, the fluorescent product was able to diffuse into the nucleus, thereby destroying the delineation between cytoplasm and nucleus. More experiments should be conducted to optimize the counterstain for these experiments.

Keeping in mind the limited interpretations, we also suggest that this cell did not appear to be in mitosis. At 45 minutes, the outline of the nucleus was clearly defined, and the morphology of the cell was flat and dendritic. During mitosis, cellular

morphology changes into a more rounded shape. In addition, mitosis requires a total of about 80 minutes during a typical 24 hour cell cycle (13). Based on the cell morphology and the total amount of time the cell spends in M-phase, the cell was probably not dividing.

Future experiments to monitor transfection by TPLSM should include controls to verify that a cell is not in mitosis. Mitosis could be characterized by the absence of a nucleolus, a round, distinctive structure in the nucleus that is involved in ribosome formation (13). The nucleolus could be localized by staining the cell with acridine orange (unpublished observation). For future experiments, cells should be transfected, monitored by TPLSM, and stained at the end of the experiment in order to ascertain the presence of the nucleolus. Staining the cell following the transfection could also serve as a control to ensure that the nuclear region of the cross section was not contaminated by cytoplasm.

In conclusion, we suggest that the transport of FPLL-DNA into the nucleus may not be strictly limited by mitosis. DNA might be able to enter the nucleus by a nuclear pore dependent mechanism. Nuclear transport of macromolecules occurs by an energy dependent transport mechanism through the nuclear pore complex (14). A region of the macromolecule contains a nuclear localization sequence (NLS) characterized by one or more clusters of basic amino acids. The NLS binds to the cytoplasmic heterodimer, importin which in turn docks onto the nuclear pore complex and facilitates nuclear transport (15).

Supposedly, the nuclear pore complex can only accommodate macromolecules that are smaller than 25 nm (15), but recent evidence suggests that adenoviruses (65-80 nm) may use the nuclear pore for entry. An NLS, located on the adenoviral major coat protein, is required for nuclear transport (16). Since adenoviruses infect non-dividing cells (17), the adenovirus is a useful model for investigating and possibly improving the nuclear transport of FPLL-DNA. Patches of exposed lysine residues on the FPLL-DNA complex may serve as a nuclear localization sequence for importin binding and subsequent transport of FPLL-DNA into the nucleus. The covalent attachment of a nuclear localization sequence to the polylysine backbone might improve the transfection efficiency of non-dividing cells.

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Figure 1. The 2-photon effect in laser scanning microscopy. Fluorescent molecules are traced in live cells and in animals, such as the zebra fish embryo. A pulsed IR laser beam is focused on a region of the embryo. Two photons simultaneously interact with a fluorophore within the specimen to produce an excited energy transition equal to the transition of a single excited photon of half the wavelength. Unlike confocal microscopy, excitation of the embryo is limited to the plane of focus, because the rate of two-photon excitation is dependent on the square of the instantaneous photon intensity. This property minimizes phototoxic damage to regions of the specimen outside of the focal plane and also improves z-resolution. Images obtained from cross sections throughout the interior of the sample are compiled into a three-dimensional reconstruction of the embryonic tissue, but biological viability is maintained.

Figure 1. The 2-photon effect in laser scanning microscopy.

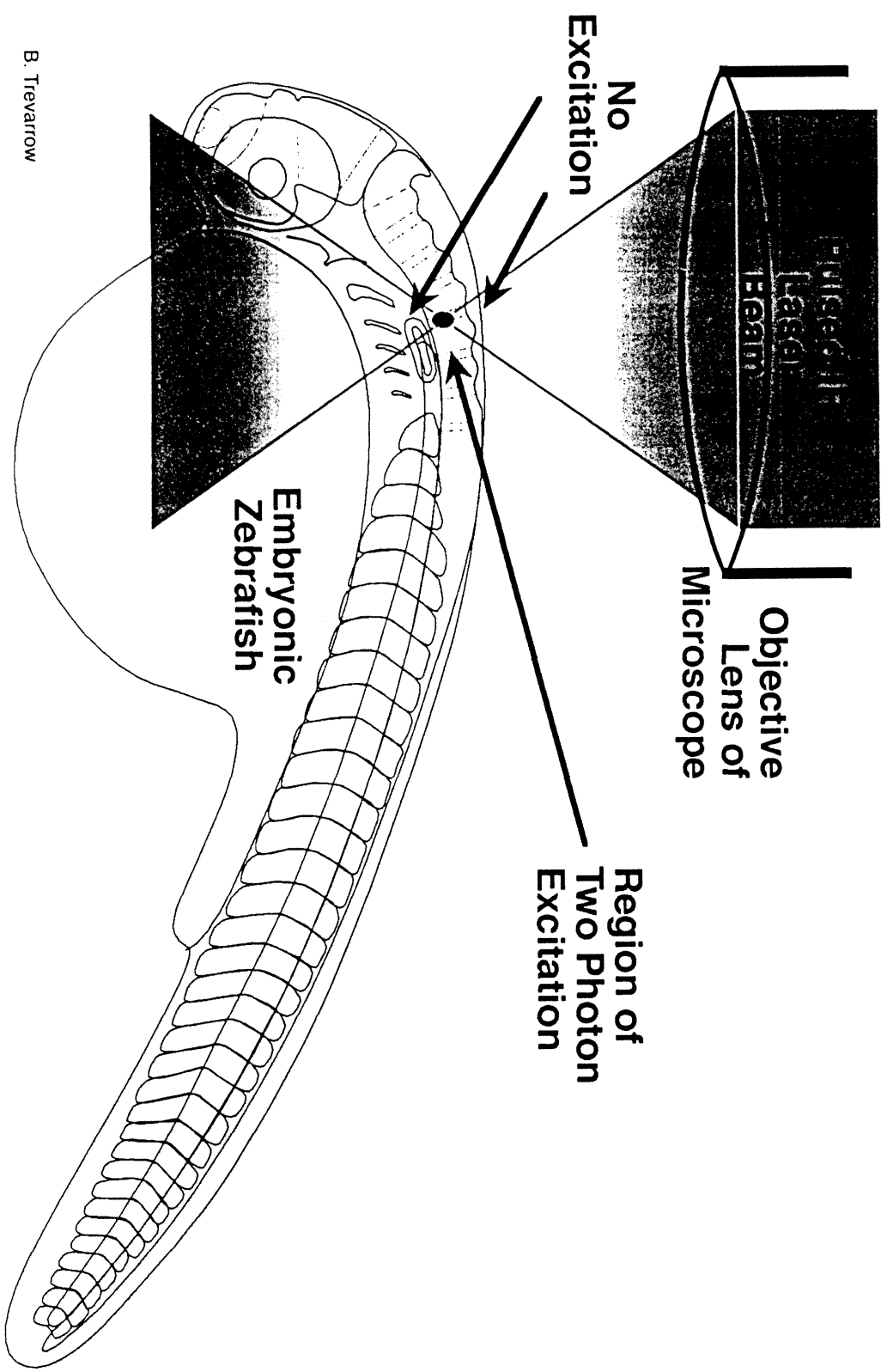


Figure 2. Fluorescence of FPLL-YYDNA complexes. The fluorescence properties of YOYO-1 DNA folate-polylysine complexes were evaluated. YOYO-DNA was diluted in 500 μl HEPES buffered saline (HBS, Chapter 2), and placed into a fluorimeter cuvette. The sample was excited at λ 488 nm and and an emission spectra was obtained (λ =500-600 nm). A peak in the spectra was observed at λ =510 nm. An aliquot of folate-polylysine (100 μ g/ml) was added to the YYDNA (Chapter 3) in order to condense and neutralize the plasmid, and the fluorescence at λ =510 nm was monitored over a two hour interval.

Figure 2. Fluorescence of FPLL-YYDNA complexes.

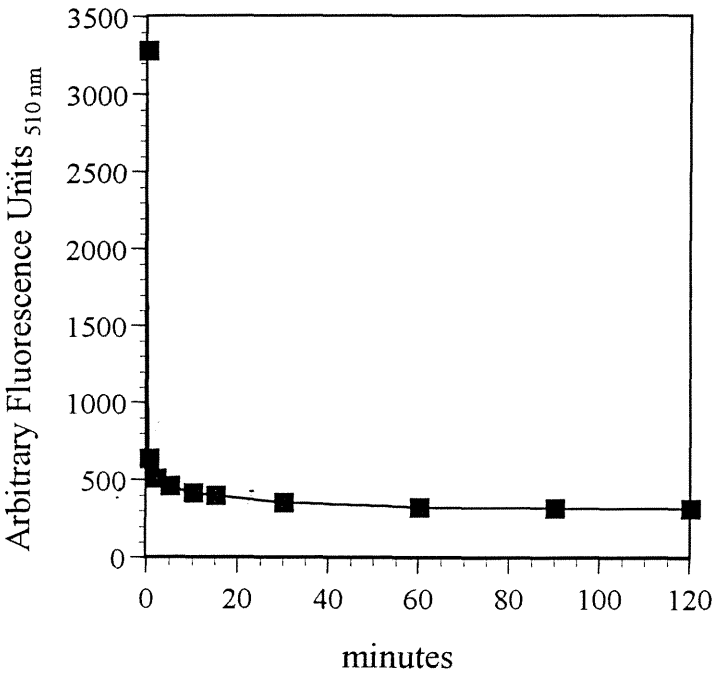
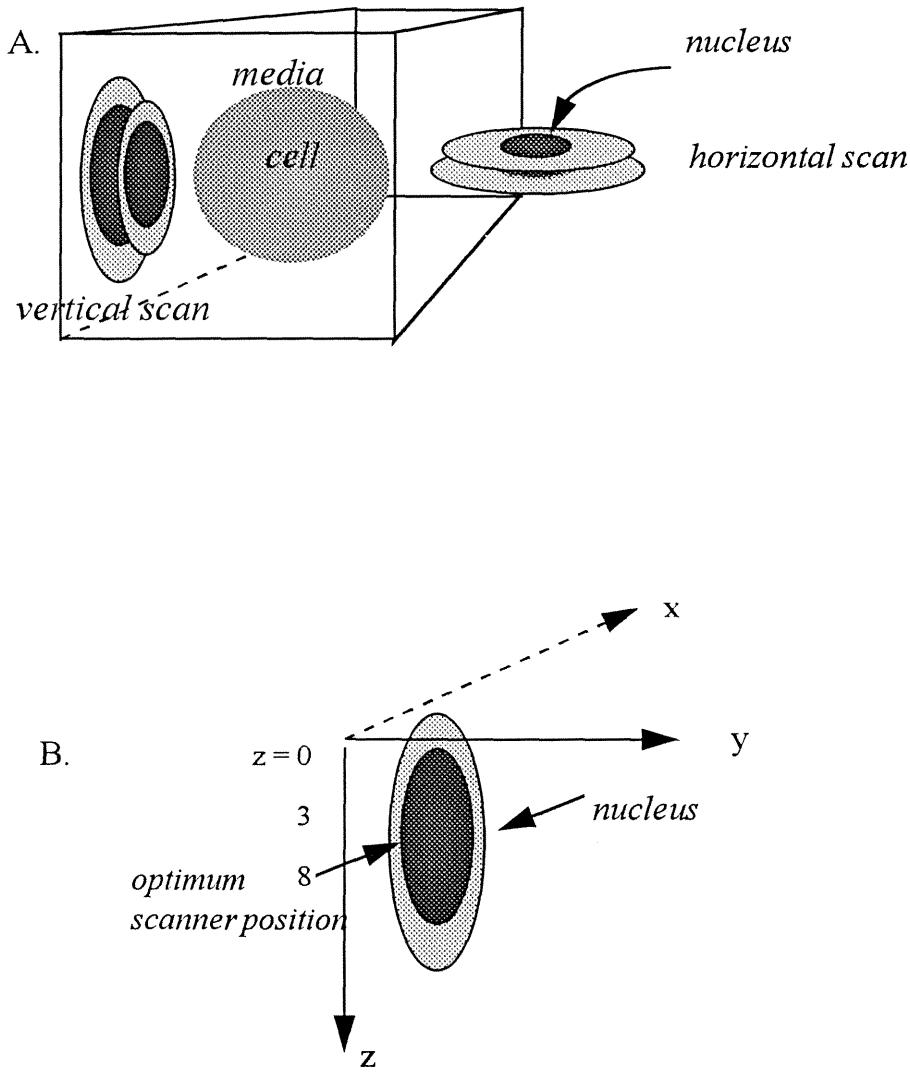


Figure 3. Determination of the optimum scanner position. A. Using the two-photon laser scanning microscope in the Beckman Institute, cross sections through tissues and cells in both the vertical and horizontal directions can be obtained. B. A vertical slice through the center of a cell can be used to determine the optimum scanner position for transfection experiments. The top of the vertical scan, which is defined as the highest z position with fluorescence signal, is designated as z=0. The cytoplasm-nucleus interface is found by locating the z position that marks the beginning of high fluorescence intensity due to nuclear staining. Using these defining points, the mid-point through the nucleus i.e. the optimum scanner position was determined to be z=8 μ m.

Figure 3. Determination of the optimum scanner position.



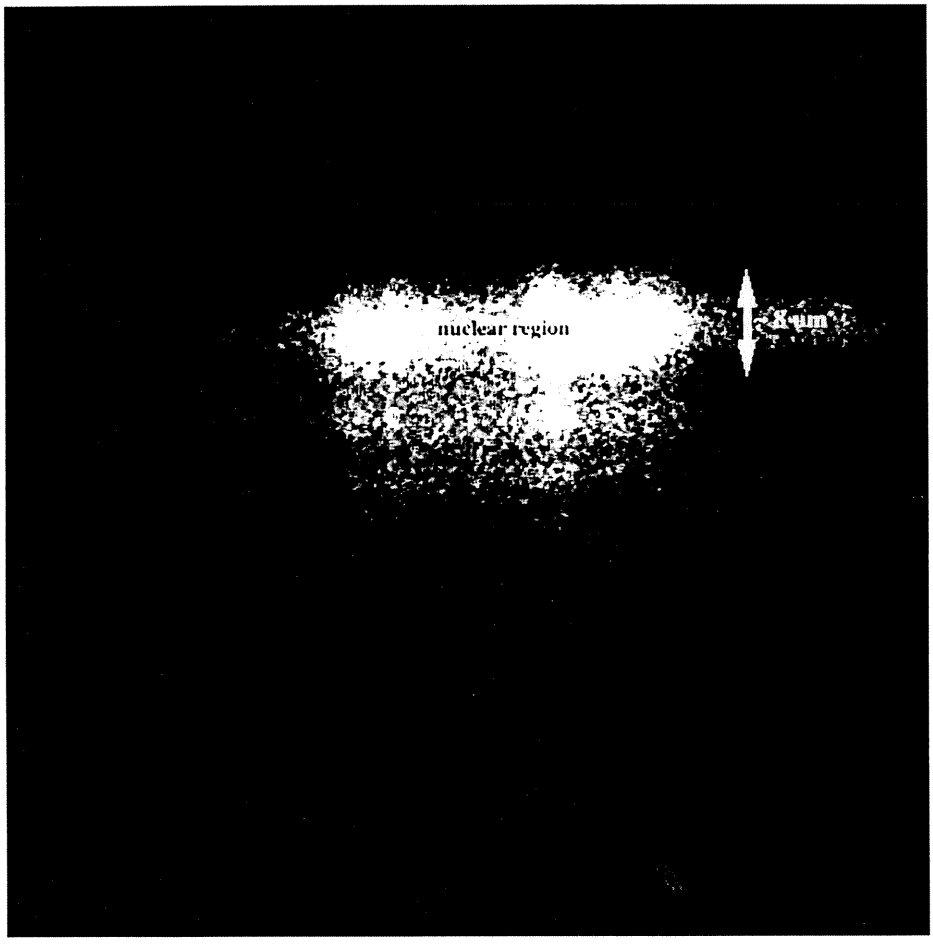
z=0, membrane-media interface

=3 nucleus-cytoplasm interace

=8 ~ center of nucleus

Figure 4. Vertical scan of a KB cell. Cells were incubated with acridine orange. A vertical scan was obtained through the center of a randomly chosen KB cell. The brightest fluorescence was found in the nucleus. Using the Image Scan software, the distance between the top of the bright fluorescent area to the bottom of the area was determined to be approximately 8 μ m.

Figure 4. Vertical scan of a KB cell.



Figures 5 A-C. Uptake of FPLL-YYDNA into KB cells. Complexes were prepared as described in Materials and Methods and added to KB cells. After approximately 40 minutes, a single, isolated cell was chosen for imaging. Cellular morphology was normal and no signs of cell division were observed. The cell was scanned, and the top of the cell, indicated by fluorescence at the highest z position was located. The top of the cell was used as a reference point (z=0) to position the scanner approximately 8 μ m below the cell surface where a non-contaminated cross section of the nucleus would be expected. At this position, horizontal scans were obtained over 120 minutes. The raw data images were processed by an imaging filter to eliminate noise.

Figure 5 A. Uptake of FPLL-YYDNA into a KB cell: 45 minutes.

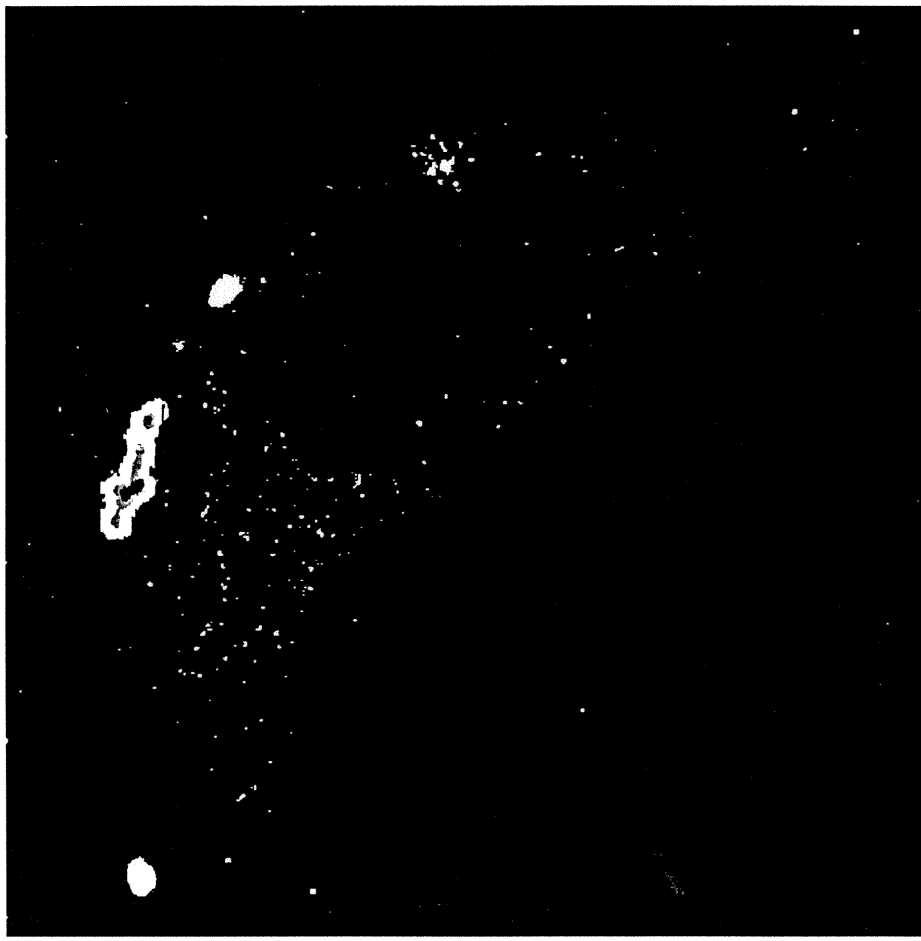


Figure 5B. Uptake of FPLL-YYDNA into a KB cell: 90 minutes.

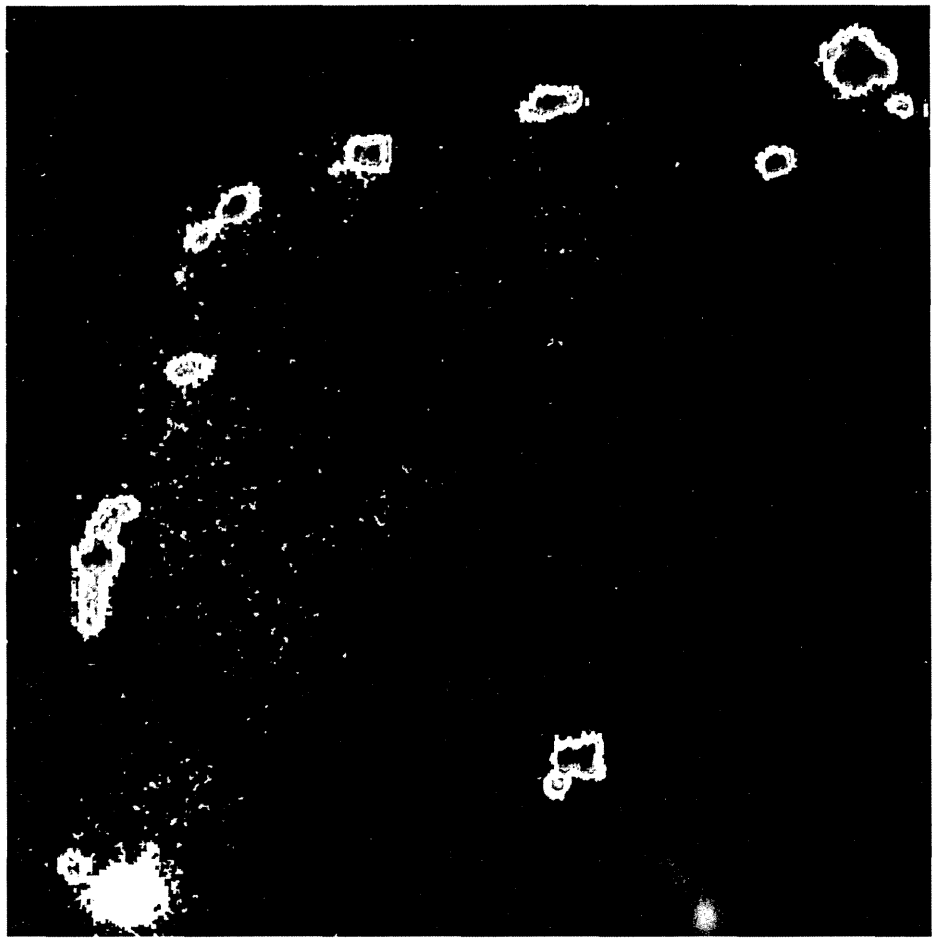
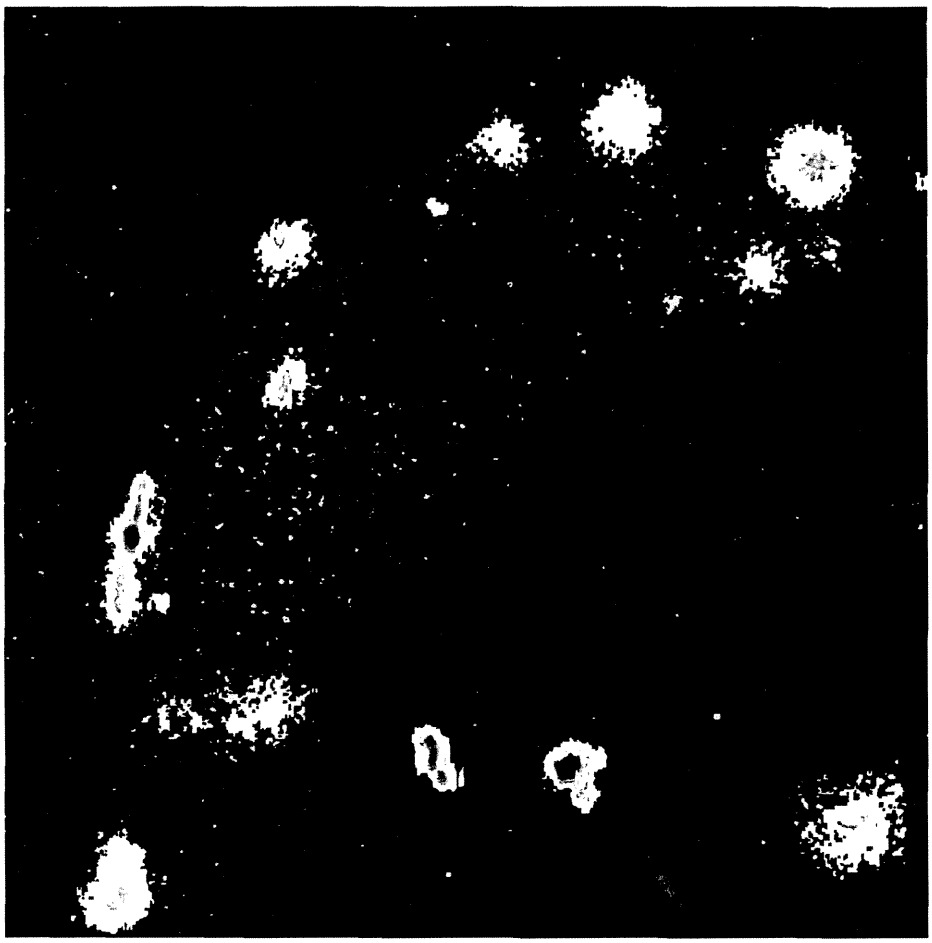


Figure 5C. Uptake of FPLL-YYDNA into a KB cell: 120 minutes.



Chapter 5: Summary and Conclusions

In this thesis, experiments were conducted to identify and eliminate the barriers to efficient, cation-mediated gene delivery.

Chapter 2:

Many investigators have reported that transfection efficiency varies dramatically among different cell types. Transfection efficiency is especially low in hematopoietic cells. Unfortunately, not even the manufacturers of the cationic agents can provide a scientifically based explanation for this observation. Some researchers have speculated that transfection by certain cell lines is limited by unknown, intracellular factors. However, many of these same cell types can be transfected successfully by receptor-mediated endocytosis.

This implies that gene delivery into "nontransfectable" cells is transport limited; the number of anionic sites on the cell membrane that mediate transfection might be the defining variable for cellular transfectability. Membrane phospholipids are the putative binding sites for cationic DNA complexes, but since all cells possess membrane phospholipids, I proposed that other membrane-associated molecules mediate transfection. In Chapter 2, I hypothesized that membrane-associated proteoglycans mediate the intracellular uptake of cationic-DNA complexes.

The results in Chapter 2 demonstrate that proteoglycans play a role in the uptake of cationic-DNA complexes. Since transfection is essentially abolished in mutant, proteoglycan deficient cells and hematopoietic cells are known for lacking proteoglycans, it is possible that gene delivery into these cells could be improved by

increasing the expression of cellular proteoglycans. Experiments to test this hypothesis are currently underway.

The implications of these results on the prospects for cation-mediated gene delivery *in vivo* are grim. In the circulation, cationic DNA complexes will be neutralized by heparin and other charged components. The effective concentration of cationic-DNA complexes in the circulation will be reduced by deposition onto the extracellular matrix and onto the basement membranes of non-targeted tissues. Hence, the characteristics that make gene delivery by cationic methods so successful *in vitro* may lead to their demise *in vivo*. Gene therapy trials using cationic methods *in vivo* will determine whether non-viral gene therapy agents need to be redesigned.

Chapter 3:

By 1993, receptor-mediated gene delivery of polylysine-DNA complexes was a novel concept that had been demonstrated in practice by less than a handful of laboratories. Because the technique promised to be able to target specific cells and receptors *in vivo*, receptor- mediated gene delivery was appealing. However, the degradation of DNA in the lysosomal compartment limited the use of receptor-mediated endocytosis for *in vivo* gene therapy. Methods or receptor systems that could be exploited to avoid the intracellular destruction of the transfected DNA were of keen interest to the gene therapy field.

In the early 1990's, the intracellular pathway of folic acid was elucidated. It was shown that folic acid was endocytosed into an acidic compartment but never merged with lysosomes. The unique intracellular pathway of folic acid motivated me

to develop a polylysine based method for delivering DNA into cells by folate receptor-mediated endocytosis.

Folate-polylysine conjugates were synthesized, characterized and used to transfect DNA into KB cells. The role of the folate receptor in the uptake of FPLL-DNA into KB cells was rigorously demonstrated. In the presence of chloroquine, the efficiency of folate receptor-mediated gene delivery was excellent when compared to other techniques, but this efficiency dropped dramatically when chloroquine was removed from the media. These results showed that although gene delivery through the folate receptor was possible, the folate receptor did not confer any obvious intracellular advantage over the other receptors studied. Since then, the development of methods to avoid lysosomal degradation using viral fusion peptides has demonstrated some success *in vitro* and *in vivo.*

Chapter 4:

In this chapter, methods for using two-photon laser scanning microscopy to characterize the transfection process were developed This research was motivated by my interest in the nuclear transport mechanism of transfected DNA and the possible barriers that the nucleus presented to gene delivery. Many investigators had speculated that transport of DNA into the nucleus was possible only in dividing cells, whose nuclear membranes were disrupted. If this was true, then methods to increase delivery into the nucleus would have to be based on synchronizing cells in the cell cycle, but these methods could be impractical *in vivo.* However, if nuclear transport

was not strictly limited by a mitotic event, then nuclear transport might be a surmountable barrier to gene delivery.

Two-photon laser scanning microscopy was the best method for studying nuclear tranport since the transfection process could be followed in real-time into a single, non-mitotic cell. This technique also allowed me to identify other barriers to transfection. While conducting time-dependent transfection experiments, I found that transfection was inhibited by the formation of aggregates on the cell surface, which would likely be problematic *in vivo*. The results also revealed that DNA could enter the nucleus of a non-dividing cell but, the process was very inefficient. Therefore, it might be possible to increase the delivery of DNA into the nucleus by attaching nuclear localization sequences to the DNA complex.

The Future of Gene Therapy

Gene therapy has progressed significantly over the last decade, but the field has a long way to go before it can provide practical, safe methods for treating human disease. The transfection process must be characterized more thoroughly, and the rate limiting steps of gene delivery and expression must be clearly defined. Unfortunately, this theme has not permeated the gene therapy literature. In my opinion, engineers can contribute significantly to this field by developing mathematical models to describe gene delivery on a cellular and *in vivo* scale, so that the critical parameters to efficient gene delivery can be elucidated and modulated.