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Nucleic Acid Cellular Delivery

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1. Introduction

The potential of using nucleic acids to correct a malfunctioning, nonexpressed gene or to supplement the production of a natural occurring protein has generated much enthusiasm from researchers and the lay public. Even though the potential for medical advances exists, there are still obstacles that must be overcome before this gene-therapy paradigm becomes a viable option. One of the more substantial obstacles is the delivery vector for the nucleic acids (e.g., plasmid DNA, oligonucleotides, siRNA, ribozymes, etc.). As a whole, nucleic acids offer unique challenges in the design and development of drug-delivery systems because of their poly anionic nature and their lack of resistance to metabolic degradation. Historically, the major theme in the design of modern nucleic acid delivery systems is to mimic the viral method of nucleic acid transfer.

In this chapter, the adeno-associated virus (AAV) will be presented as a model system for understanding efficient particle based delivery of nucleic acids. This virus was chosen because it is one of the simplest ones that is currently being evaluated in gene-therapy paradigms. In light of the mechanism AAV uses to gain intracellular access and movement, a review of the similarities and differences between AAV and nonviral systems will be presented. In closing, an outlook will be provided for what the future might be for nonviral gene systems.

2. AAV-2 As a Model for Nucleic Acid Transfer

AAV has demonstrated a broad tropism in terms of infected cell types (1) and is a very effective gene transfer cassette for the potential treatment of a variety of diseases (1–3). AAV, composed of a single strand of DNA encapsulated with a protein shell, belongs to the parvovirus family. It is an extremely simple and small virus. Its viral protein coat is composed of three proteins and the viral particle size is in the range of 20–30 nm. It has been shown that the AAV enters cells via clathrin-coated pits, and that this process requires dynamin, a 100-kDa cytosolic GTPase (4,5). Three cellular receptors have been identified for AAV to enter its host cells. Heparin sulfate proteoglycan (HSPG) is necessary for a stable contact with the cell surface (6,7). Efficient cell internalization of AAV, however, requires another cellular receptor, such as integrin $\alpha_v\beta_5$ receptor (8,9) or fibroblast growth factor receptor 1 (FGFR1) (10). It is

likely that there may be other co-receptors. The AAV cytoplasmic trafficking events, viral uncoating, and nuclear translocation, although under extensive studies (9,11–14), are still not fully understood. In order to gain a greater understanding of AAV-mediated nucleic-acid transfer, we studied the infectious entry process of AAV-2 in HeLa cells by tracing AAV proteins, viral DNA, and intact viral particles using fluorescent-dye labeled AAV, A20 antibody immunocytochemistry, and subcellular fractionation techniques. The focus of these studies was to obtain a better insight into how this extremely simple virus deposits its DNA into the nucleus, in order to design more efficient nonviral delivery systems.

The first step in AAV cellular entry is the virus' interaction with the extracellular matrix (ECM). AAV can only transduce particular cell types and has tropism for particular cell types depending on the composition of this matrix. Previously, investigators (15,16) have used fluorescent-labeled viruses to study the early stages of the viral-infection pathway, AAV in particular. In our experiments, a green fluorescent dye, Alexa Fluor™ 488 (Molecular Probes), was used to covalently label AAV serotype 2. Before incubation, cells were pulsed by labeled AAV for 37°C for 10 min to allow internalization. Cell samples were counter-stained with red SYTO 64® dye, which provides the shape of the whole cell (Fig. 1A,B), and nuclei position was detected by DAPI staining (data not shown). In agreement with a previous report (4), fluorescent virus (yellow) demonstrated a gradual perinuclear accumulation during the first 4-h infection period (Fig. 1A). Furthermore, the majority of the viral signal maintained this perinuclear pattern up to 12 h postinfection before diffusing into the nuclear area at 16 h postinfection, as shown in Fig. 1A. A large amount of fluorescence could be detected inside the nuclei, 24 h postinfection, and after 48 h postinfection, fluorescent signals could be detected solely in the nuclear region. We also performed a free-dye control experiment to eliminate the possibility of free-dye artifact (Fig. 1B). In contrast to AAV infection, the majority of free Alexa Fluor™ 488 dye rapidly entered cell nuclei from 2 h to 8 h postinfection when labeled virus still resided outside the nuclei. This distinguished distribution pattern of free dye from labeled AAV suggests that the nonconjugated dye molecules did not bring contamination to the AAV results.

Fluorescent-dye-labeled virus can only provide information about viral proteins. Whether those fluorescent signals represent intact viral particles or dissociated capsid proteins, however, is unknown. To address this question, we performed an immunocytochemistry assay using A20 monoclonal antibody (MAb) (Fig. 2A,B). A20 antibody has been demonstrated to be able to specifically recognize the intact AAV particles with a defined three-dimensional (3D) structure and is used to detect intact AAV particles in immunocytochemistry (17,18). In agreement to fluorescent virus data (Fig. 1), AAV showed a gradual perinuclear accumulation pattern during its early infection (up to 12 h postinfection). Figure 2B shows a DAPI-stained nucleus with the co-stained A20 viral particles. In contrast to fluorescent virus results, however, intact AAV particles continuously remain outside the cell nuclei throughout the 48-h experimental period, although some small clusters of intact AAV particles might be observed inside nuclei after 24 h postinfection (Fig. 2A). Whether or not this small amount of intact AAV particles found in nuclei was caused by AAV nuclear translocation, cell division, or capsid reassembling is unknown. However, the seemingly co-localization of AAV signals with nucleoli suggests the last possibility, because empty AAV capsids are first assembled in nucleoli before spreading to the whole cell area. Collectively, these data and fluorescent virus data indicate that the nuclear entry of AAV follows a slow and

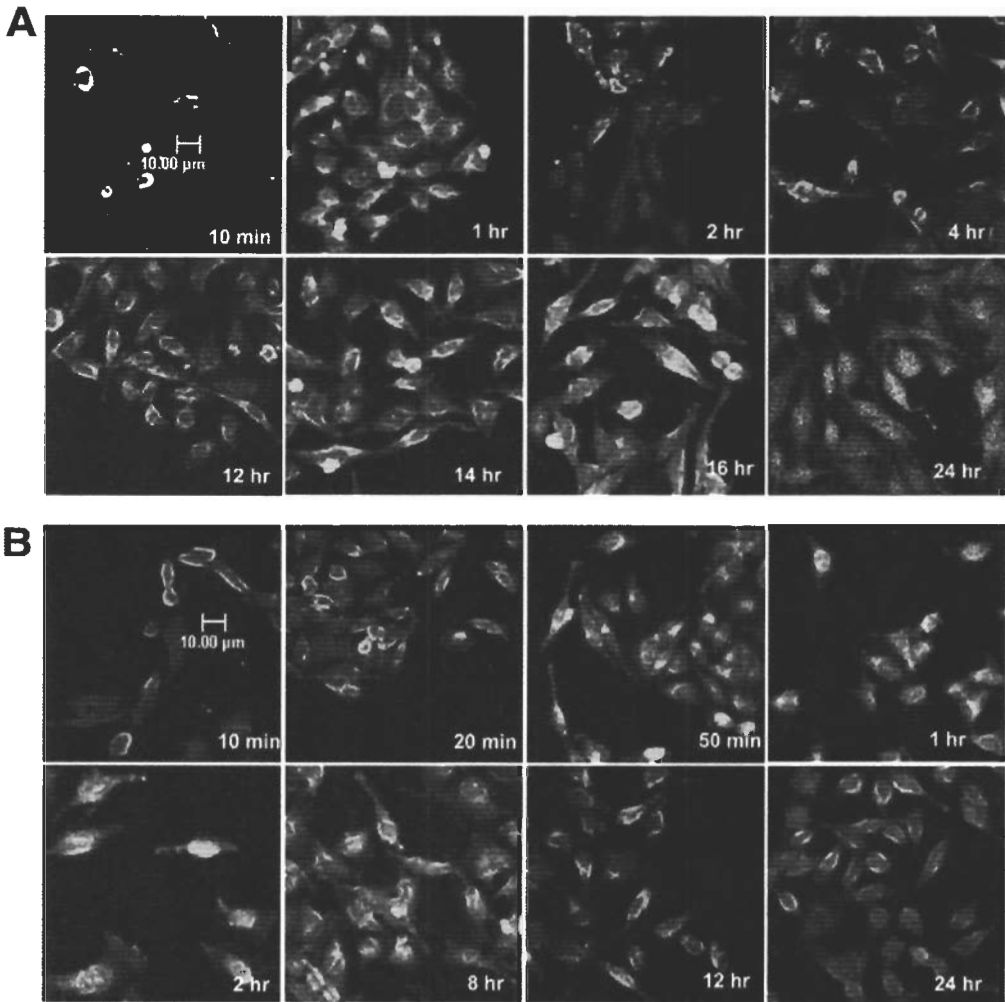


Fig. 1. Fluorescent-dye-conjugated AAV infection. (A) Infection of HeLa cells using fluorescent AAV. (B) Incubation of HeLa cells using free fluorescent dye.

regulated mode. A control experiment was performed to show only when both AAV and A20 are present can we observe the signal (Fig. 2C).

With the knowledge of the migration of viral proteins (Fig. 1) and intact viral particles, we further studied the viral infection by tracing the viral DNA using a nucleic acid slot-blot method. Cells were fractionated into cytoplasmic and nuclear parts, and no cross-contamination was confirmed by the fact that no histone H3 was shown in the cytoplasmic part (Fig. 3C) and less than 0.5% acid phosphatase activity was in the nuclear fraction (data not shown). As supplementary control experiments, the presence of proliferating cell nuclear antigen (PCNA) and heterogeneous nuclear ribonucleoprotein complex (hnRNPC) in each fraction was also examined by Western blotting (Fig. 3C). PCNA is normally found in cell cytoplasm, and is only present in the nuclei of dividing cells, such as HeLa cells. hnRNPC shuttles between the cytoplasm and nucleus, but predominantly resides in the nucleus. Typical distributions of PCNA and

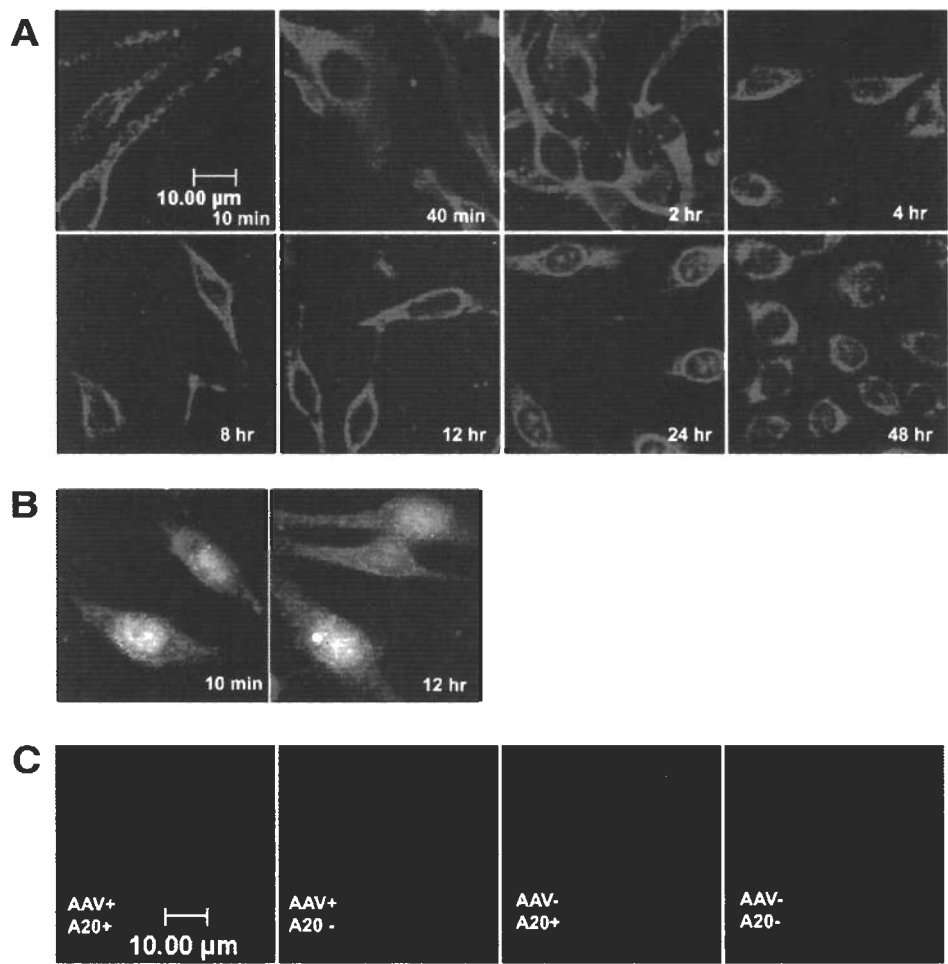


Fig. 2. A20 immunocytochemistry detection of intact AAV particles. (A) Immunocytochemistry detection of AAV intact particles at different time points. (B) Nuclear staining of 10-min and 12-h time points. (C) Control experiments showing the specificity of A20 antibody.

hnRNPC further prove the integrity of the cytoplasmic and nuclear fractions. As for the slot-blotting results, no observable amount of viral DNA can be seen in the nuclear fractions until 16 h postinfection, and the amount of DNA inside cell nuclei then gradually increased to a significant level at 48 h postinfection (Fig. 3A). The nuclear translocation of viral DNA coincides with that of the viral proteins (Fig. 3A), while intact AAV particles remain outside nuclei. Figure 3B shows the ratio of nuclear viral DNA to input DNA over time. These data suggest that the AAV, after accumulating perinuclearly, uncoats prior to or during the entry into cell nuclei.

AAV enters cells through a receptor-mediated endocytosis pathway via clathrin-coated pits. In order to evaluate the intracellular disposition of the viral DNA the cells were fractionated after infection and the cytoplasm separated from the nucleus. The cell postnuclear supernatants (PNS) contents were fractionated by a continuous iodixanol gradient centrifugation method (19). After an initial 10-min incubation with

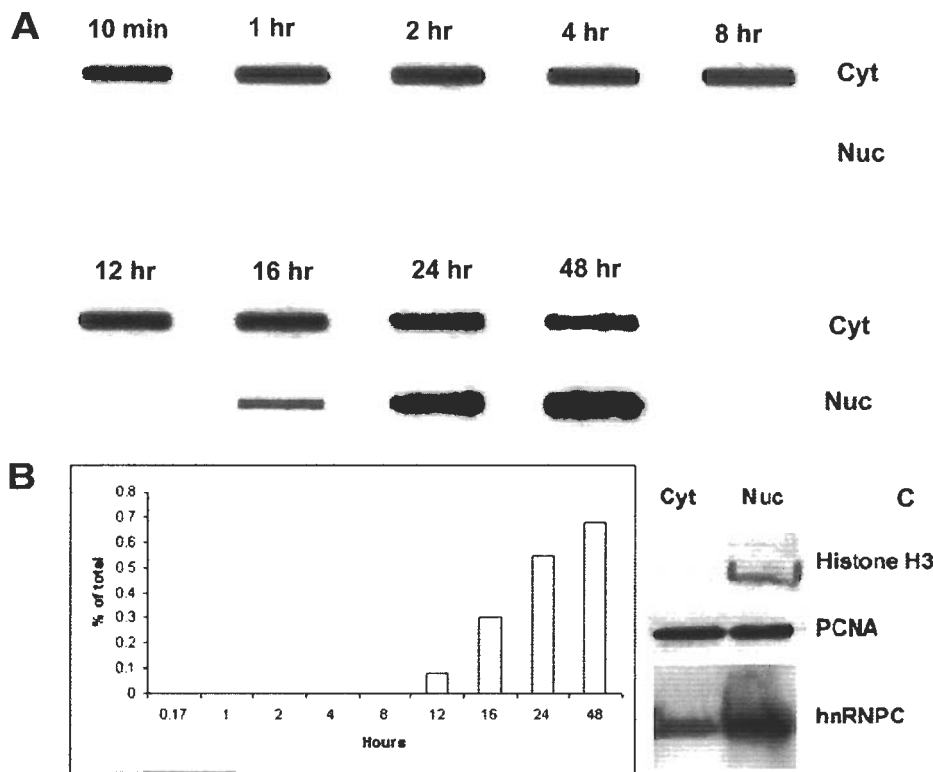


Fig. 3. Slot-blot detection of AAV DNA. (A) Slot-blot results of AAV DNA extracted from cytoplasmic and nuclear fractions after indicated periods. (B) Quantification of nuclear DNA vs total DNA. (C) Western control experiments showing the purity of cytoplasmic (cyt) or nuclear (nuc) fractions.

HeLa cells, AAV was readily detected in the cytoplasm in both early-endosome-trapped and endosome-escaped forms (Fig. 4A, “37°C 10 min” to “37°C 8h”). As the infection proceeded, increasing amounts of virus left the early endosomes as shown by an elevated ratio of escaped vs endosome-associated AAV (Fig. 4B). Incubation of virus with cells at 4°C for 1 h showed no free virus in this gradient (Fig. 4A, “4°C entry”), indicating the virus, attached to the cell surface, was efficiently removed by trypsinization and the free virus signals mentioned earlier truly belong to the virus escaped from endosomes. However, virus in this treatment was found in early endosomes (Fig. 4A, “4°C entry”), suggesting a rapid viral endocytosis between the addition of virus to cells and transfer of cells to 4°C. When both cells and virus were precooled to 4°C before incubation at 4°C for 1 h, no signal was identified (Fig. 4A, “4°C no entry”), indicating no viral entry occurred. No significant amount of AAV was detected in dense-endocytic-vesicle region throughout the research courses. Our data, similar to the results of a study that demonstrated canine parvovirus co localizes with transferrin—an early endosome marker (5)—agree with the early-endosome-escaping model, and more than half of the entered AAV could be found in the cytosol as early as 10 min after the incubation (Fig. 4A,B).

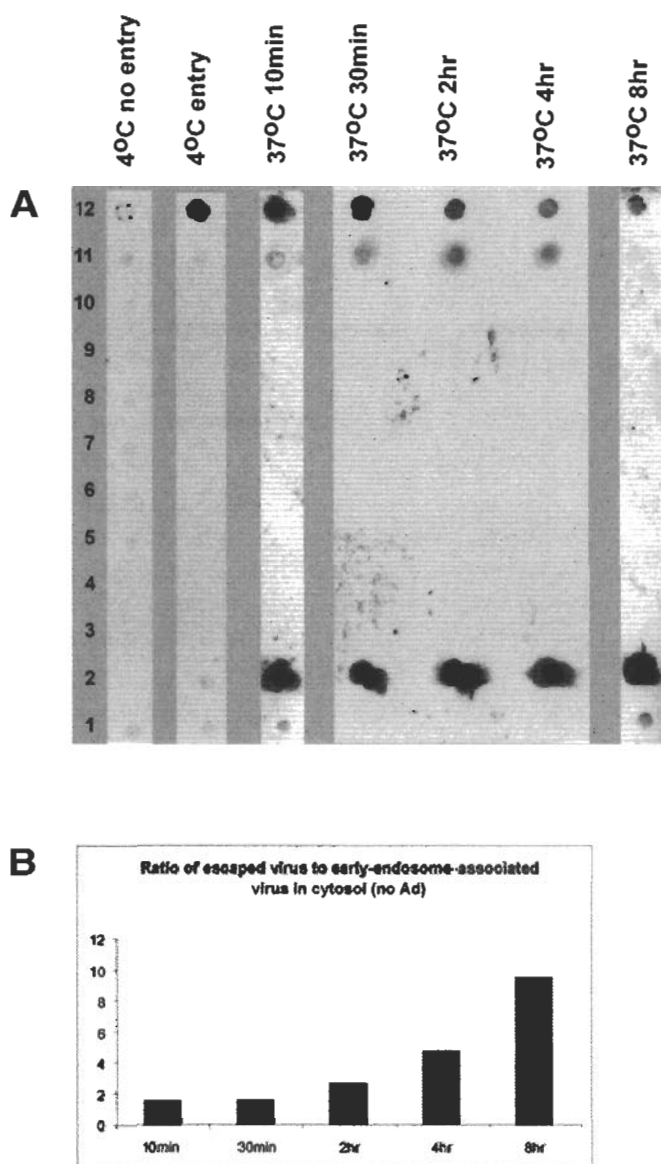


Fig. 4. Subcellular fractionation of AAV. (A) Dot-blot detection of AAV DNA from the fractionated PNS of AAV infected HeLa cells at the indicated conditions. (B) Intensities of escaped virus signals (fraction 2) and early-endosome-associated virus signals (fraction 11 and 12) were plotted as the ratio of escaped to endosome-associated virus.

From prior studies it is clear that AAV-2 has a highly regulated method of cellular entry and subcellular distribution within HeLa cells. The first step is a somewhat selective interaction with cells expressing particular extracellular ligands. This is followed by a signaling event that facilitates the endocytosis of the AAV-2 particles within the cell. Endocytosis is followed by a rapid viral particle transverse from the endosome

into the cytoplasm by a poorly defined mechanism. Once the AAV-2 particles are in the cytoplasm, there is gradual accumulation around the nuclear membrane.

Based on our experiments and the results of other researchers, it appears the AAV protein coat has several important functions, because it is unlikely that the single-stranded DNA of the virus impacts on these steps: (1) it protects the viral nucleic acid from metabolic degradation; (2) it allows for the interaction of the viral particle with the cellular membrane allowing attachment and entry; (3), it may facilitate endosomal escape of the viral particle; (4) the viral proteins likely have roles in shuttling the particle to the nuclear membrane. The viral coat of AAV-2 is comprised of three proteins and it appears these proteins work in harmony to facilitate viral particle internalization and intracellular disposition. AAV is one of the simplest and smallest of the viral vectors being used in gene-delivery studies. It is reasonable to assume that this virus is an excellent model for nonviral gene-delivery scientists to model in the crafting of their delivery vectors.

3. Nonviral Mediated Gene Transfer

Nonviral systems for nucleic-acid delivery have not been viewed as effective as viral delivery for numerous reasons, including poor cellular uptake, inefficient nucleic acid transfer from endosomes, metabolic degradation, and limited nucleic acid entry into the nucleus. Recently, advancements have been made in these areas to improve transfection reagents. Researchers are utilizing a variety of approaches for nucleic acid transfer, from using naked plasmid DNA to the crafting of highly advanced supramolecular complexes. In this chapter, we will focus on two of the more studied cationic vector systems, looking for similarities and differences with AAV.

Cationic molecules, either liposomes or polymers, demonstrate several viral-like properties when combined with nucleic acids. After the nucleic acid interacts with the cationic material, a particle is formed that is resistant to nuclease degradation (2). Often an excess of cationic charges is required for efficient protection. These transfection particles are thought to be able to interact with extracellular proteoglycans, thus serving in the initial step of cellular entry (21,22) leading to transfer across the cell membrane. Currently, the exact mechanism of cellular entry of the nonviral particles is not clear, and it is unknown whether or not entry differs between cell type and cell cycle.

After entry into endosomes, the gene-transfer complex needs to exit the endosome and gain entrance to the cytoplasm. As described earlier, AAV has an effective, yet unclear mechanism: to accomplish this feat. Other virus, such as adenovirus, can use the gradual drop in pH of endosome as a trigger mechanism for part of its viral protein coat to facilitate endosomal escape (23,24).

Different mechanisms have been proposed for nucleic acid escape from endosomes with cationic macromolecules. Cationic lipids can destabilize lipid bilayers by promoting the formation of nonbilayer lipid structures. Mixtures of cationic lipids and anionic phospholipids preferentially adopt the inverted hexagonal (H_{II}) phase. Furthermore, the presence of "helper" lipids such as dioleoylphosphatidylethanolamine or cholesterol—lipids that enhance cationic lipid-mediated transfection of cells—also facilitate the formation of the H_{II} phase (25–27). It is suggested that the ability of cationic lipids to promote nonbilayer structures in combination with anionic phospholipids leads to disruption of the endosomal membrane following uptake of nucleic acid-cationic lipid complexes. Szoka and colleagues have proposed a more extensive membrane model in

which the anionic lipids on the external leaflet of the endosome membrane interact with the cationic molecules of the transfection complex, resulting in movement of the nucleic acids into the cytoplasm (28,29). Of course, the lipid composition of the target cell membrane and fusogenicity of the lipoplex also impacts on transfection efficiency, the latter in turn is influenced by the structure of the lipid and by the structure and relative amount of the neutral helper lipid used in lipoplex formulation.

For example, mixtures of the cationic lipid *N,N*-dioleoyl-*N,N*-dimethylammonium chloride (DODAC) with the anionic lipid cholesteryl hemisuccinate (CHEMS) can form nonbilayer structures such as the hexagonal H_{II} phase under conditions of neutral surface charge (30). Similarly, mixtures of the cationic lipid 3α -[*N*-(*N*',*N*'-dimethylaminoethane)-carbamoyl] cholesterol hydrochloride (DC-Chol) and dioleoylphosphatidic acid (DOPA) also exhibit H_{II} -phase preferences (30). The addition of these helper lipids alters poor transfecting cationic lipids to highly effective transfection complexes. These studies demonstrate the importance of membrane fusion events on nucleic-acid transfer.

As with the AAV particles, at some point the transfer vector is required to release its nucleic acids. It appears for AAV that this occurs at the nuclear membrane or within the nucleus. It is unclear where release of the DNA occurs for the two types of cationic transfer vectors or if there is an advantage of release site. Several nucleases are in the cytoplasm that could degrade noncomplexed plasmid DNA (31), however, so there is an advantage to maintaining the complex. After vesicles escape from the endosome, the nucleic acid must traffic to and enter the nucleus for gene expression. The mechanism by which polyethylenimine (PEI) and DNA polyplex arrives in cell nuclei has yet to be elucidated. Lipoplexes, polyplexes, and plasmid DNA are too large to enter the nucleus through the nuclear-pore complex. Furthermore, it is well-known that the expression of plasmids microinjected into the cytoplasm of cultured cells is poor, whereas the expression of those microinjected into the nucleus is high. These results and others raise the issues with regard to plasmid trafficking in cells that are not dividing. During mitosis, the loss of the nuclear membrane could eliminate these trafficking barriers. Consistent with this postulate, gene transfer in cultured cells is greatly enhanced by mitotic activity for both lipoplexes and polyplexes. For the polymer system (PEI), there is evidence that the entire particle may be transferred to the nucleus (32) via specific mechanisms, which may be one of the reasons this vector is so effective.

4. Specific Examples on Transfection Complexes

Nonviral vectors are composed of nucleic acid, usually a plasmid and the transfer vector. Plasmid DNA is extrachromosomal segments of nucleic acids that bacteria use for genetic information storage. Plasmids can easily be manipulated with current molecular-cloning methods into useful mammalian expression vectors. The second component is the DNA carrier. Commonly used systems include cationic lipids; and cationic polymers such as poly-lysine, and synthetic cationic polymers. Cationic liposomes employ specific types of cationic lipids (33–35) as the functional component to transport DNA. These vesicles are usually not in the classical bilayer structure of liposomes. This is particularly true after cationic lipids have interacted with nucleic acids (36) resulting in a variety of lipid/DNA shapes. Nevertheless, the term cationic liposome is used because they are based on lipids. Synthesized cationic lipids show activity in delivering genes into the lung, liver, and other tissues (33). Although these compounds

are diverse in chemical structure, they do have common features. In most cases, there is a cationic head group composed of primary, secondary, tertiary, or quaternary amines, which is attached to a hydrophobic tail group. In plasmid-mediated delivery studies, there is often a relationship between the net charge of the plasmid-DNA/cationic liposome and its effectiveness in transgene expression. Generally, a small net positive charge is required for transfection. Charge ranges from 1:1 to 1:20 (charge ratio) have been reported in the literature (36–39). A slight positive charge of the complex enhances the interaction with the net negative charge of cellular membranes with anionic lipids and carbohydrates, especially in tissue-culture studies.

Cationic liposomes remain promising nonviral systems for use in gene delivery. Although the exact biochemical and biophysical mechanisms of cationic liposome-assisted gene transfection and expression are not thoroughly understood, the barriers involved in the transfection process *in vitro* generally include the following events: (1) formation of the liposome/DNA complex; (2) entry of complex into the cell; (3) escape of DNA from the endosome; (4) dissociation of DNA from the liposome; (5) entry of DNA into the nucleus; and (6) DNA transcription. Some of the barriers are approached by rational design of the delivery systems (1–5) while others (6) will relate to the innate properties of the plasmid itself (e.g., promoters and enhancers). The cationic lipid assists in protection from nucleases, increases cellular uptake, and escape from the endosome. In a majority of reported studies, the cationic liposomes function most efficiently when the cationic lipid is combined with a second lipid known as a helper lipid.

Several cationic polymers, such as poly(L-lysine), PEI, and polyamidoamine dendrimers, have been shown to form complexes with DNA and thus facilitate gene transfer. These cationic polymers bear groups that are protonated at physiological pH (Fig. 5). Figure 6 is a scheme demonstrating the transfer of nucleic acid with PEI. The transfecting unit-polyplex is formed by electrostatic attraction between the cationic charge on the polymer and the negatively charged DNA.

Polylysine was used to condense DNA as early as 1969 and was initially used merely as a model for the interaction of biopolymers such as DNA and histone proteins (40). Poly-L-lysine conjugated with asialoorosomucoid was the first polycation employed for gene delivery (41). The gene-transfer efficiency of these early poly-L-lysine polyplexes was very low (42); however, with the conjugation of targeting ligands, the gene-transfer activity of poly-L-lysine polyplexes was enhanced (43). Copolymers of poly-L-lysine and other amino acids have also been shown to transfer genes into mammalian cells. The conjugation of histidine to poly-L-lysine produced a transfecting polyplex that was more efficient than a poly-L-lysine-chloroquine mixture (44,45). The toxicity of poly-L-lysine was reduced with poly(lactic-co-glycolic acid) (PLGA)-grafted poly(L-lysine) (PLL) (PLL-g-PLGA) and the cationic PLL-g-PLGA polymeric micelles exhibited better gene-transfer efficiency than PLL (46).

Of the cationic polymers, PEI is the most popular for gene delivery, because DNA/PEI complexes display high transfection efficiency in cell culture and have potential for gene delivery *in vivo* (47,48). PEI shows efficient gene transfer without the need for endosomolytic or lysosomotropic agents. The molecular weight of PEI can affect its gene-delivery efficiency. Some reports revealed an increase in gene-transfer activity with a decrease in molecular weight (from 100 to 11.9 kDa) (49), whereas others demonstrated a decrease in activity on decreasing the molecular weight (from 70 to 1.8 kDa) (50). It appears that an optimum transfection result can be obtained with molecu-

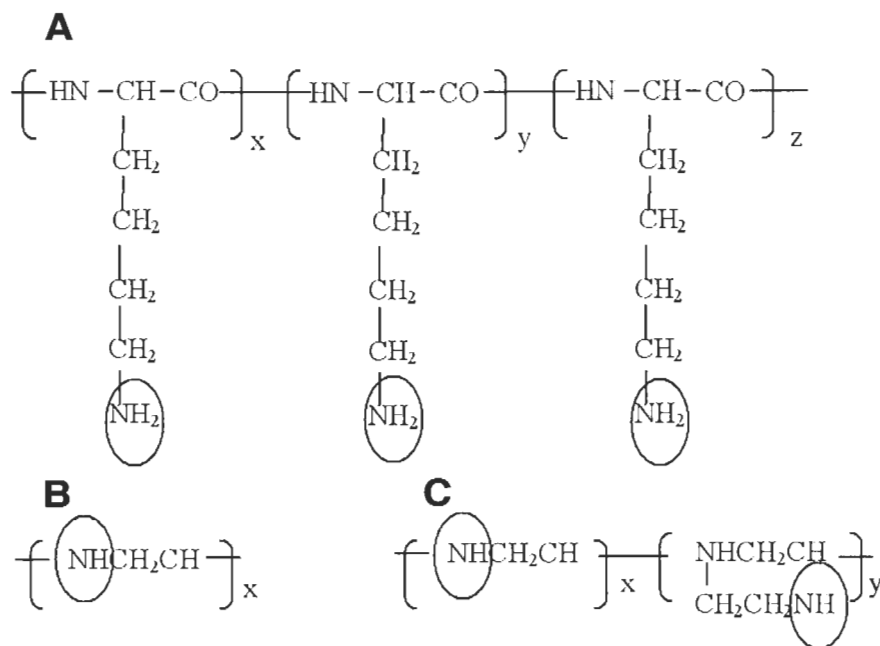


Fig. 5. Examples of cationic polymers used for gene delivery. (A) Poly L-lysine. (B) Linear polyethylenimine. (C) branched polyethylenimine.

lar weight between 11.9 and 70 kDa. High efficiency gene transfer has been obtained without the help of nuclear localization signals with linear PEI as a DNA-complexing agent. However, the coupling of targeting ligands to PEI enhances gene transfer in some cell lines (51). Recently, complexes were generated by the mixing of plasmid DNA, linear polyethylenimine (PEI22, 22 kDa) as the main DNA condensing agent, PEG-PEI [poly(ethyleneglycol)-conjugated PEI] for surface shielding, and Tf-PEG-PEI (transferrin-PEG-PEI) to provide a ligand for receptor-mediated cell uptake (52). These new DNA complexes offer simplicity and convenience, with tumor targeting activity *in vivo* after freeze-thawing.

Encouraging *in vivo* results of PEI polyplexes mediated gene expression were obtained in experimental animals. The examined animal anatomical sites included mouse lung (53), rat kidneys (54), and mouse brains (55) by intratracheal administration, intrarterial injection, and with direct injection. For intravenous injection of PEI polyplexes, transfection is found predominantly in the lung endothelium (56). However, with PEG-coated PEI, gene expression is observed in spleen, heart, lungs, and liver 24 h after intravenous injection of this complex in mice (57). Furthermore, the combination of a PEG-coated PEI and the transferrin targeting ligand resulted in reporter gene expressed in tumor without significant toxicity (58). Thus it appears that a simple polymer has some of the attractive attributes associated with AAV particles.

5. Conclusion

In summary, we have presented information describing gene-transfer vectors. This was accomplished by first addressing how one of the simplest viral vectors (AAV)

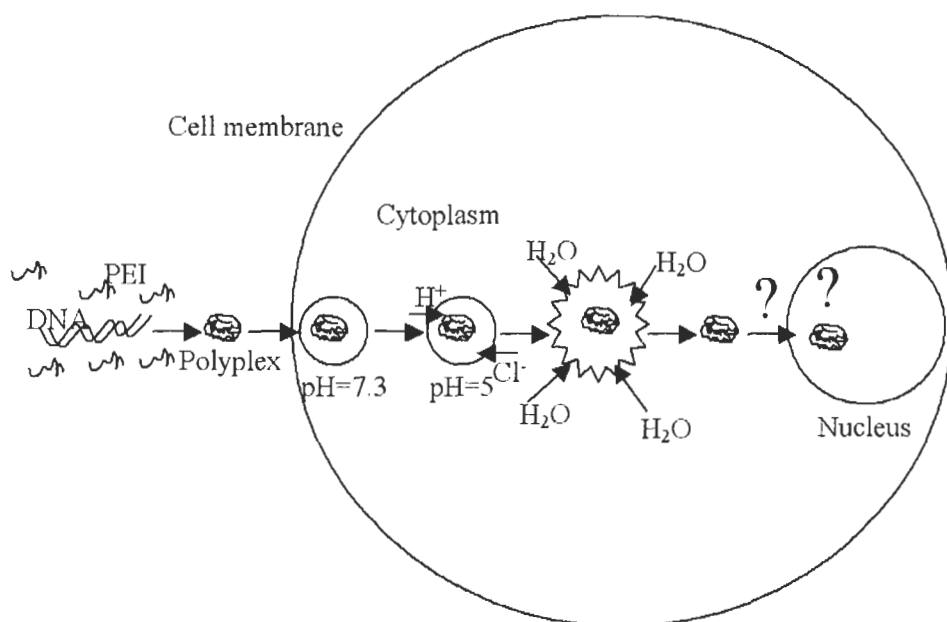


Fig. 6. Schematic representation of PEI mediated DNA uptake by mammalian cells. DNA is compacted in the presence of polycations into a transfection particle. This particle interacts with the cell membrane and is internalized within endosomes. The transfection particle is then released via a proton sponge effect resulting in an osmotic opening of the endosome. The transfection particle then progress to the nucleus.

facilitates nucleic acid movement into the cell. Several distinct stages of this transport were identified, including membrane binding, viral-particle internalization, viral-particle translocation from endosomes, and finally viral accumulation within the nucleus. The only factor that governs these processes in AAV-2 would be the three viral proteins occurring in the viral coat. At this time it is not clear how these proteins are operating, but through site-directed mutagenesis studies (59) it is hoped that these protein roles can be teased out. The current similarities between most nonviral vectors systems and how viruses function are amazing. The main difference is that most current nonviral vectors have not been designed with the entire transportation process in mind. In the early stages of their utility, the biggest concern was creating a system that could protect DNA, could transfer it to the cell demonstrating gene expression, and would be nontoxic. Currently efforts are underway to create supramolecular transfection systems that can mimic the viral pathway in several manners. These include systems that have select tropism for specific cells, nonviral vectors with built in enhanced endosome escape mechanisms, and systems that can target the nuclear membrane. Its clear we still have several lessons to learn from this simple virus.

Acknowledgments

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