Synthetic Gene-Transfer Vectors

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Gene therapy is "à la mode" and extends well beyond its own research fields, as is evidenced by special issues of multidisciplinary journals and frequent headlines in the media. This widespread interest has a number of origins. Most significantly, gene therapy is a conceptual revolution: for the first time, DNA is considered as a drug, providing a general framework for curing (and not only treating) thousands of hereditary diseases. But gene therapy is also a new type of weapon in the fight against acquired diseases of larger social incidence, either multigenetic disorders (e.g., cancer) or those resulting from foreign viral genes. Besides these concrete reasons, it also satisfies one of medicine's greatest dreams: molecular surgery at the root of life. Clinical attempts to transfer genes into humans have already begun (e.g., "Immunotherapy of patients with advanced melanoma using tumor-infiltrating lymphocytes modified by retroviral gene transduction" [1]).

Broadly speaking, gene therapy includes several approaches: molecular replacement of a mutated gene (by homologous recombination), addition of a new gene resulting in the synthesis of a therapeutic protein, and modulation of endogenous cellular (or possibly viral) gene expression by drugs. In this last approach, it has been amply demonstrated since 1987 that some double stranded DNA sequences can be recognized through triple strand formation with synthetic oligonucleotides,[2] or through strand replacement with entirely non-natural compounds.[3] While chemistry leads in this domain, it is almost absent from gene replacement and addition therapies, where the "drug" is DNA; yet here the power of organic synthesis could help in creating artificial drug carriers. Indeed, these therapies rely on gene transfer (transfection), i.e., harmless introduction of the selected gene into living cells (Figure 1). Most current vectors are engineered (recombinant) viruses[1] that involve complex technologies and suffer from severe limitations (see below). Synthetic gene-transfer vectors, although probably less efficient than viruses, do not raise the problems of working with biological and potentially infectious vectors.

Gene therapy has not yet stood the test of time. However, gene-transfer techniques are also among the most powerful tools of cell biology research (ranging from the study of intracellular gene/protein function and regulation to that of complex processes such as embryogenesis). Moreover, gene transfer has profound economic implications as a prerequisite to genetic engineering of microorganisms, plants, and animals (for crop and livestock improvement).[4] It is therefore not surprising that many gene-transfer techniques have been devised since the early 1970s, when gene manipulation emerged from research laboratories. Before reviewing the most common techniques, it is worthwhile considering how this problem has been solved in evolution.

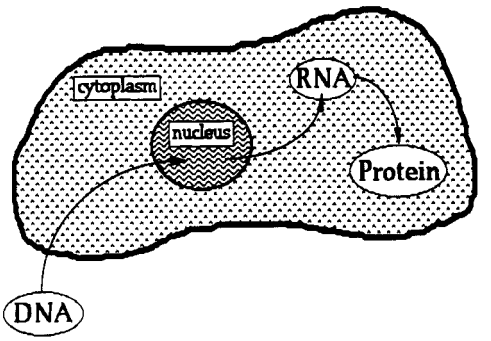

Figure 1. Gene transfer into a eukaryotic cell: a polyanionic macromolecule (DNA) is carried across the cytoplasmic lipid membrane and the nuclear membrane and finally is expressed into the corresponding protein.

Natural Gene Transfer Is a Highly Complex Process

Human fertilization has been studied in much detail for obvious reasons. Encounter of the parental genomes is more than pleasant: it is also a multistage, extremely complex process which prevents interspecies fertilization and polyspermy. Unfortunately, the key gene-transfer step is the least well understood: after receptor-mediated lateral binding of the sperm head to the egg cytoplasmic membrane, a fusogenic sperm protein (and possibly acrosomal enzymes) helps the membranes to merge. The sperm genome, which is highly compacted by polycationic proteins (protamins), can then penetrate into the egg.

Viral infection is another example: viruses are small (ca. 1000 Å) nucleic acid-containing particles, which

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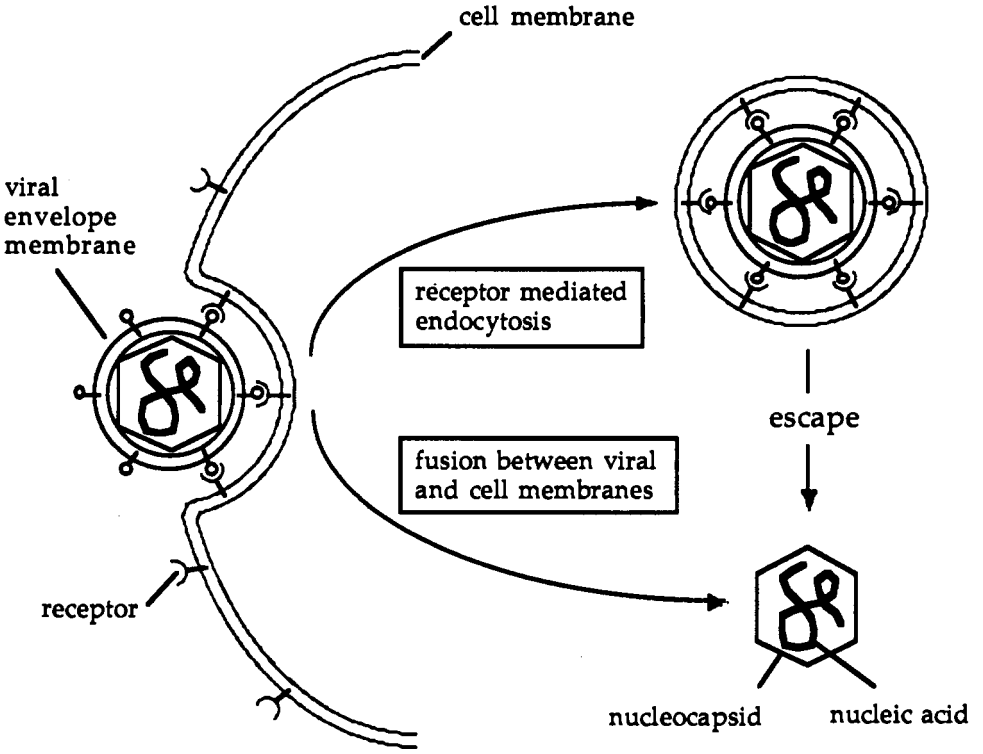

Figure 2. Cell infection by an enveloped virus begins with endocytosis or direct membrane fusion.

need the cellular machinery for multiplication (Figure 2). Infection begins by multiple binding to a cell surface receptor (protein or glycolipid) which triggers entry by direct membrane fusion, or alternatively by endocytosis and subsequent fusion-mediated escape. Although the structures of several fusogenic proteins are known, the molecular mechanism of fusion is poorly understood, mainly because it is a very short-lived event. Viral genomes, like sperm genomes, are also compacted by cationic proteins and by polyamines such as spermine, in order to fit into the nucleocapsid.

Thus, natural systems devoted to gene transfer have highly compacted silent genomes and show specific cell surface binding that triggers penetration through several possible pathways, all of which involve the rupture of a lipid membrane.

Gene-Transfer Techniques

The simplest method to introduce DNA into a cell is direct mechanical microinjection. However, for practical reasons, injection is only used to transfect eukaryotic germline cells for production of transgenic species. Other gene-transfer techniques are indirect and variously employ biological vectors (recombinant viruses) or model liposomes,[5] DNA coprecipitation with polycations, cell membrane perturbation by chemical (solvents, detergents, polymers, enzymes) or physical means (mechanic, osmotic, thermic, electric shocks). These techniques are of variable efficiency and complexity, and none could claim to cover the large range of cell types and environmental conditions encountered. However, for a given task, some techniques have become more popular than others.

Gene therapy relies almost exclusively on *recombinant viruses* (essentially retroviruses) which carry the gene of therapeutic interest into cells by the mechanisms described above (Figure 2); they are far more efficient than artificial vectors. The use of a biological carrier, however, raises two problems: to encapsulate the newly engineered genome into an empty viral particle and to prevent the new viruses from becoming infectious. Encapsidation is performed in genetically transformed animal cells maintained in culture and is complex and costly. Apart from the safety risks already mentioned, retroviral vectors also have other limitations: virus concentrations are less than ca. 10^6 particles/mL (by comparison, 1 μg of plasmid DNA/mL is 10^6-fold more concentrated); the retroviral genome is small (less than 10^4 nucleic base pairs = 10 kbp); hence, not more than 5 kbp of foreign information can be added. A search for larger viral vehicles is ongoing.[6]

In comparison with biological vectors, transfection by *DNA coprecipitation with calcium phosphate or cationic polymers*[7] looks amazingly simple. Although historically independent, these techniques share similar mechanisms and advantages and have quickly become the most popular ways of introducing DNA into cells of various origins. They consist of the formation of a

(5) Gene transfer with liposome-encapsulated nucleic acids has been extensively reviewed and will not be treated here; see, for instance: Straubinger, R. M.; Papahadjopoulos, D. *Methods in Enzymology*; Academic Press: New York, 1983; Vol. 101, pp 512–527. Mannino, R. J.; Gould-Fogerite, S. *Biotechniques* **1988**, *6*, 682–690. Nicolau, C.; Cudd, A. *Crit. Rev. Ther. Drug Carrier Syst.* **1989**, *6*, 239–271. Liposome encapsulation seems much less efficient than cationic lipid coating, which is discussed later: Legendre, J. Y.; Szoka, F. C. *Pharm. Res.* **1992**, *9*, 1235–1242.

(6) Currently, adeno and herpes viral vectors are being developed for this purpose. Why is there such pronounced interest toward the introduction of large pieces of genetic information into cells? Eukaryotic genes have a mosaic structure where the protein-coding information (exons) is interrupted by long stretches of noncoding nucleotides (introns). On the other hand, gene transcription is controlled by protein binding to defined nucleotide sequences, some of which may be very distant from the coding region. Regulation and stability of an exogene in a living context requires the entire gene, typically over 10^5 base pairs long.

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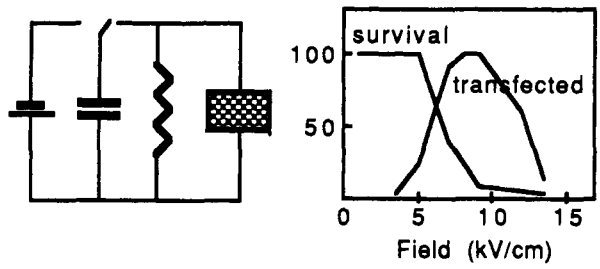

Figure 3. Left: Principle of electroporation of cells in suspension. Right: Typical profiles of percent cell survival and cells transfected (among the surviving), as functions of field strength.

finely divided precipitate of polyanionic nucleic acid with calcium phosphate or with a commercial high molecular weight cationic polymer (e.g., diethylaminoethyl-dextran or polybrene, a linear polymeric quaternary ammonium salt). The cationic precipitate is "eaten" by the cells (phagocytosis). The success of these techniques comes from simplicity and low cost, although they are neither very efficient nor reproducible, and often they are toxic to the cells. Efficiency is improved when the cells are incubated in the presence of glycerol or DMSO. The coprecipitation method is essentially restricted to the ex vivo transfection of phagocytic cells.

Electroporation (Figure 3) is based on the finding that when a strong electric field (typically kilovolts/centimeter) is applied to a cell suspension for a few microseconds, some regions of the cytoplasmic membrane undergo a slowly reversible breakdown, transiently allowing DNA to enter the cells.[8] Electroporation has become the method of choice in vitro, especially for cells which are resistant to the calcium phosphate technique. Many apparatuses have been designed, but the technique remains tricky to optimize (Figure 3, right) as the electric shock must be strong enough to perturb most of the cells, yet leave them viable.

The *particle gun* represents the most recent physical transfection technology:[9] micron-size tungsten or gold particles are coated with DNA and propelled onto cells (Figure 4). High-velocity microprojectiles reach tissues such as liver in vivo; this technique also seems very promising for use on eukaryotic cells that possess walls, such as plant cells.

Synthetic Gene-Transfer Vectors

In the numerous gene-transfer techniques mentioned previously, the most elaborate compounds that appear are commercial solvents (DMSO, glycerol) and polymers (polyethylene glycol, polybrene). Surprisingly, despite the creative power of chemistry, there has been no rational design of artificial gene-transfer vectors until recently. Within a few years, several groups reported the synthesis of molecules capable of compacting DNA and transferring it into cells.[10-21] Indeed, juxtaposition

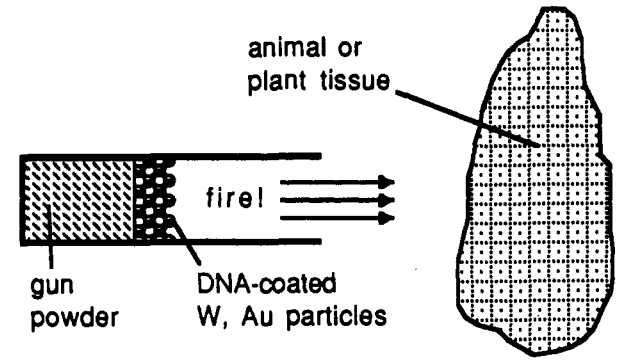

Figure 4. The particle gun technology.

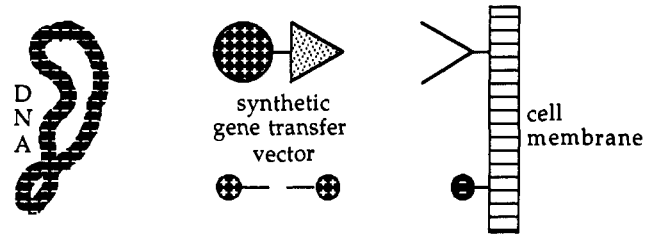

Figure 5. Current synthetic gene carriers are chimeric bifunctional proteins (upper) or cationic lipids (lower) able to compact DNA and bind it to the cell surface.

of a DNA molecule with a eukaryotic cell leads to the following common sense remarks with regard to transfection:

1. Plasmid DNA and the potential recipient cell are of similar size. Nature (see above) and spaghetti lovers both solved this contents/container puzzle in a similar fashion, namely, compaction.

2. A macromolecular polyanion (typically 10^4 charges) will not spontaneously cross an intact lipid membrane, nor will it even bind to the negatively charged cell surface. Therefore a synthetic vector should not only condense DNA but also mask its anionic nature. Furthermore, it should bind it to the cell surface in such a way as to trigger membrane destabilization or endocytosis.

Two classes of gene-transfer agents have been designed along these lines (Figure 5): hemisynthetic polypeptides,[12,15,17] where a DNA-binding polycationic protein (protamin, polylysine) is chemically linked to another protein (asialoorosomucoid, ferritin, insulin) whose recognition by a given cell surface receptor leads to active endocytosis; synthetic cationic lipids,[10,11,13,14,16,18,19,21] where hydrophobic effects provide a cationic glue which links the anionic DNA and cell surfaces together.

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Polylysine-Linked Proteins Carry DNA into Cells by Receptor-Mediated Endocytosis

Oppositely charged polymers such as DNA and polylysine condense each other into neutral soluble particles which should not bind nor enter efficiently into cells. However, the cationic polypeptide can be linked (usually through a sulfur bridge) to a protein which naturally enters a cell by endocytosis, and thus compacted DNA may be cotransferred with the protein. The corresponding receptor may be shared by many cell types as part of their general metabolism (e.g., ferritin,[15] which is an iron carrier), or alternatively DNA may be targeted to special cells which degrade asialoglycoproteins[12] or respond to insulin.[17] In any case, the nucleic acid must escape the degradative pathway of endosomes, as in the coprecipitation method discussed above. This has been shown for transferrin–polylysine gene delivery, which is only efficient in the presence of lysosomatropic agents.[22] However, when the complexes are linked to inactivated adenoviral particles which help them to escape from endosomes, transfection is enhanced by orders of magnitude.[23] This latter technique, although not straightforward, is among the most efficient to date.

Lipospermine-Mediated Gene Transfer Is Very Efficient When the Nucleolipid Particles Bear a Net Positive Charge

The smallest natural polycations able to compact DNA are the polyamines spermidine and spermine; this interaction is, however, quickly reversible in physiological conditions. *Lipopolyamines*, i.e., amphiphiles with a self-aggregating hydrocarbon tail linked to a cationic DNA-binding headgroup, have been shown to stably condense nucleic acids into discrete nucleolipidic particles[10] which may be further coated with an excess lipid layer (Figure 6).

Such polycationic particles, where the DNA charge has been reversed, bind cooperatively to anionic residues on the cell surface. Lateral diffusion and cell deformability result eventually in their spontaneous zipper-like "endocytosis" [13] (Figure 7), a mechanism formally reminiscent of viral entry.[24] The intracellular fate of the nucleolipidic particles is unknown, but some of them must end up in the cytoplasm, reach the nucleus, and become at some stage uncoated, since active exogene transcription is obtained.

This view is consistent with the following observations:

1. Cationic lipid-mediated gene transfer is efficient only when the particles bear a strong positive charge (Figure 8), irrespective of the individual charge of the lipid.[13]

2. Lipospermines with a single hydrocarbon chain have been synthesized. They form micelles instead of bilayers and still condense DNA. However, they do not provide a cationic surface for interaction with the cells, and as a result no transfection is observed.

3. Lipids with other strong DNA-binding headgroups (2 intercalates between base pairs; 3 binds into the DNA minor groove; see Figure 6) are unable to transfect cells.

More speculative arguments go along the same lines:

1. Lipospermines, as well as other cationic lipids able to transfect cells, do not form stable bilayer structures. They may therefore locally destabilize cellular membranes and help the particles to reach the cytoplasm.

2. Nuclear localization signals borne by endogeneous nuclear proteins and viral capsids contain an exposed stretch of at least five cationic amino acids which could make them accumulate in the nucleus after ionic binding to genomic DNA. Positively charged nucleolipidic particles could be caryophilic for similar reasons, provided their size is compatible with nuclear pore crossing.

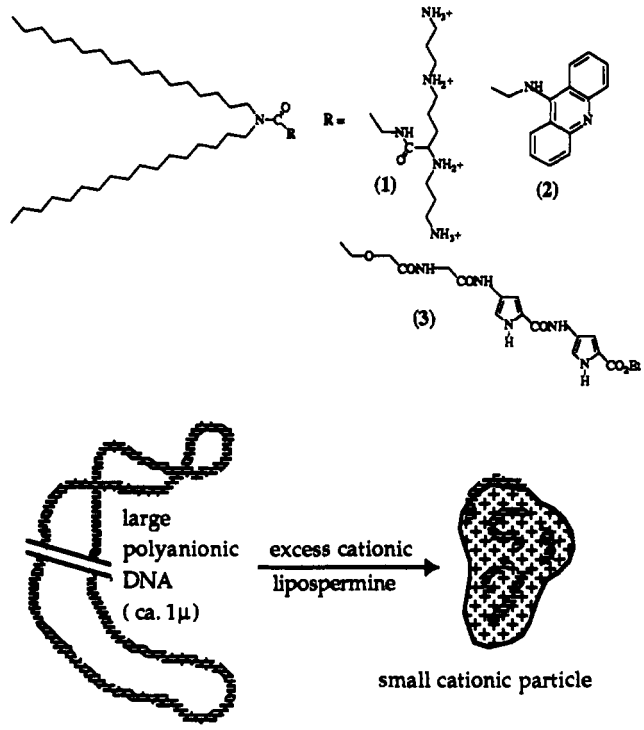

Figure 6. Upper: Structure of a lipospermine (1) and related acridine (2) and netropsin (3) analogs. Lower: Lipospermine-induced condensation of plasmid DNA.

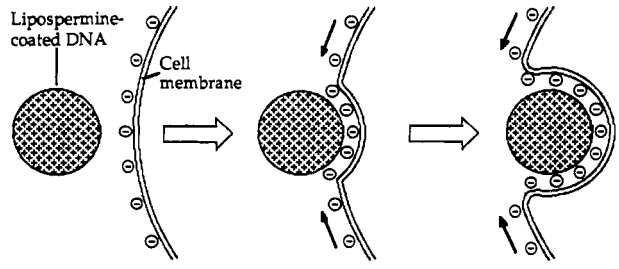

Figure 7. Spontaneous "zipper" endocytosis of a rigid cationic particle.

Cationic Lipids Are Attractive Alternatives to Classical in Vitro Techniques

When compared to the popular calcium phosphate method, transfection with cationic lipids is as straightforward (requiring only mixing of components), yet it is far more efficient. This new technique has a major

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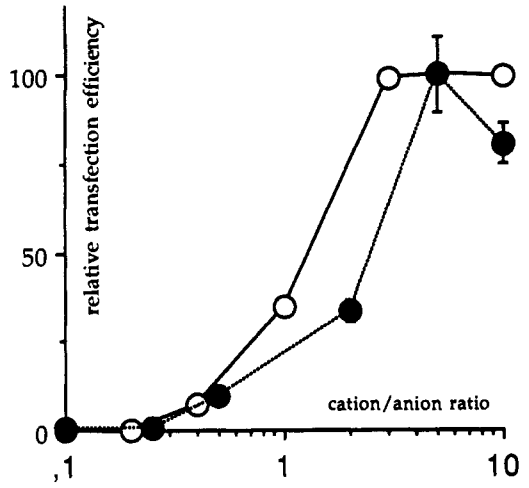

Figure 8. Gene-transfer efficiency as a function of the mean ratio of cationic lipospermine over anionic DNA charges. 3T3 rat fibroblasts (open circles) or rat cerebellar neurons (filled circles) were transfected with a plasmid containing the bacterial chloramphenicolacetyl-transferase (CAT) gene. This gene is absent from mammalian cells, so determination of CAT activity in cell extracts 48 h posttransfection provides a convenient measure of gene-transfer level.

advantage of being applicable to almost all animal cell types, as it is based on nonspecific ionic interaction (Figure 7). It is also of low toxicity, provided the chemical carrier is designed to be biodegradable. Therefore cells resistant to classical techniques, as well as fragile cells of various origins, can be efficiently transfected in vitro[25] (neurons, keratinocytes, lymphoid cells). Recently several synthetic cationic lipids have been commercialized for such gene-transfer purposes.

Future improvements with cationic lipids could come from a *modular transfection system* based on a neutral lipopolyamine–DNA core particle to which synthetic lipids with other functions could be anchored at will via their hydrocarbon backbone: Addition of a small amount of lipid with a given oligopeptide (or saccharide) headgroup should direct the nucleolipidic particle to the desired cell surface receptor; a lipid with a virus-derived fusogenic peptide headgroup could help DNA to enter the cytoplasm; similarly, a lipid bearing a nuclear localization signal would provide nuclear tropism to the core particle, resulting altogether in the designed modular construction of ecotrope virus mimics. Such improvements await further experimental work, but it may well be that the high transfection efficiency already observed with lipopolyamines alone is due to a unique (and fortunate) combination of properties such as DNA protection against nucleases, cellular membrane destabilization, and caryophily.

Synthetic polycation-mediated in vivo transfection has had only limited success.[26-28] This may be due in part to interference from circulating serum factors and cationic particle binding to the tissue matrix. The search for alternative synthetic vectors based on another principle has so far been fruitless (see, for instance, compounds **2** and **3**, Figure 6).

In conclusion, improved synthetic gene-transfer vectors could certainly replace recombinant viruses for gene therapeutical purposes. Indeed, besides the problems related to the genetic manipulation of potentially self-reproducing systems, viral vectors also suffer from severe cost, gene size, and concentration limits (see above) which are not shared by synthetic molecules. However, viral infection is almost perfect, requiring only a few particles per cell. On the lipopolyamine side, recent improvements resulted in a detectable transfection signal down to 25 ng of DNA.[29] Yet a rough calculation shows that there are still 3 orders of magnitude to go: chemists, back to the bench!

I thank my colleagues F. Barthel, B. Demeneix, J. P. Loeffler, C. Sirlin, and J. S. Remy for their intellectual and practical contributions. I apologize to other research groups which appear only once in the reference list: this Account was not intended to be a comprehensive review but rather highlights our own approach. This work has been supported by grants from the Centre National de la Recherche Scientifique, Association pour la Recherche sur le Cancer, Association Francaise contre les Myopathies, and Recherche & Partage.

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