Review

Transfer and Expression of Foreign Genes in Mammalian Cells

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ABSTRACT

The transfer of foreign genes into eukaryotic cells, in particular mammalian cells, has been essential to our understanding of the functional significance of genes and regulatory sequences as well as the development of gene therapy strategies. To this end, different mammalian expression vector systems have been designed. The choice of a particular expression system depends on the nature and purpose of the study and will involve selecting particular parameters of expression systems such as the type of promoter/enhancer sequences, the type of expression (transient versus stable) and the level of desired expression. In addition, the success of the study depends on efficient gene transfer. The purification of the expression vectors, as well as the transfer method, affects transfection efficiency. Numerous approaches have been developed to facilitate the transfer of genes into cells via physical, chemical or viral strategies. While these systems have all been effective in vitro they need to be optimized for individual cell types and, in particular, for in vivo transfection.

INTRODUCTION

Developments in molecular biology over the past two decades have led to the cloning of numerous mammalian genes. The study of mammalian gene structure and function has been greatly enhanced by the use of eukaryotic expression vector systems. These vector systems facilitate the introduction of specific genes into mammalian cells, the transcription of these genes and the subsequent production of proteins that are appropriately modified to reflect native molecular structure and biological function. These mammalian expression vector systems have advantages that include but are not limited to *(i)* the ability to constitutively and inducibly express proteins, *(ii)* the ability to produce a large quantity of protein that is post-translationally modified and appropriately folded, *(iii)* the ability to characterize the impact of specific mutations on cell metabolism and *(iv)* the ability to stably alter cellular phenotype as a function of transgene expression. Recently, these mammalian expression vector systems have become the basis for defining the appropriate level of gene expression in vivo and are also used as the tools for gene therapy approaches in vivo. Although this discussion of different expression vector systems will focus on the transfer of genetic material in vitro, some of these principles have already been applied to the development of gene therapy strategies for cystic fibrosis (CF) (13,49,73).

When designing a DNA transfer and expression study, the primary factors concerning the expression vector are *(i)* the type of promoter/enhancer sequences, *(ii)* the type of expression (transient or stable) and *(iii)* the amount of desired expression. In addition, the efficiency of transfection must be taken into consideration. Efficiency is primarily influenced by vector purity and the method of DNA transfer. These methods include chemical (phosphate precipitation, cationic polymers, liposomes, molecular conjugates) and physical (electroporation, biolistic, microinjection). The strategies discussed in this paper will focus on elucidating the parameters of mammalian expression vector systems and gene transfer methods.

EXPRESSION VECTORS

Basic Construction

Numerous vector constructions have been used to deliver genes to and express genes in human cells. Lists of mammalian expression vectors can be found in a variety of texts and manuals concerned with recombinant DNA techniques, gene transfer and/or gene therapy (81,123). They can generally be divided into two broad categories: viral vectors and plasmid vectors. Viral vectors are essentially inactivated viruses into which genes are cloned. Plasmid vectors are more complicated and their functional components include prokaryotic, eukaryotic and viral sequences. The prokaryotic elements facilitate bacterial propagation and the maintenance of the vector, while the eukaryotic and viral elements comprise transcriptional elements and sequences coding for selectable markers. Both types of vectors are generally classified with respect to their viral backbone or the sequence elements that regulate the expression of the gene to be studied or utilized (Table 1).

Promoters/Enhancers

The regulation of gene expression in

Table 1. Mammalian Expression Systems

Expression Vector Base	Features
Adeno	Broad mammalian host range; tropic for airways but infects other organs; generally a nuclear episome; high level of expression; high-titer recombinant virus stocks; used for gene therapy; immunogenic.
Adeno-associated	Broad mammalian host range; requires adenovirus for packaging; wild-type virus integrates into specific site on human chromosome 19, but integration of recombinant expression virus is random; limited insert size (about 4.5 kb) for packaged recombinant virus; nonpathogenic; potential for gene therapy if transgene is small.
Epstein-Barr	Retained as a nuclear episome in human, monkey and dog and not generally in rodent; episome replicates autonomously; potential for gene therapy.
Herpes Simplex	Broad mammalian host range; lytic; carries large (> 5 kb) fragments of exogenous DNA; packaged virion can accommodate 150 kb DNA; viral genomes are stable in serial propagation.
Papilloma	Retained as a nuclear episome in rodent (BPV) or human and monkey (HPV); episomal nature of vector/transgene is unpredictable; must be empirically assessed for each transgene; used to study gene regulation and for high-level transgene expression.
Polyoma	Broad mammalian host range; replicates best in murine cells; can integrate; will be episomal with polyoma ori and large T antigen in *cis* or *trans*.
Retro	Host range dependent on virus coat—ecotropic (species from which wild-type virus was isolated) and amphotropic (broad mammalian); RNA virus; integrates into DNA as a reverse transcribed provirus; difficult to get high titer of recombinant virus; limited insert size (about 8.5); used in gene therapy of small genes.
SV40	Broad mammalian host range; replicates readily in simian cells; can integrate; will be episomal with SV40 ori and large T antigen in *cis* or *trans*.
Vaccinia	Broad mammalian host range; viral infection shuts down host synthesis; lytic; used primarily for transgene protein overexpression.

This list represents some of the common viruses that are the basis for most mammalian expression systems.

either a plasmid or viral vector is controlled by promoter/enhancer sequences as well as signal sequences that are necessary for efficient processing of the transcripts. The most common promoters/enhancers are of viral origin. However, bacteriophage, cytoplasmic, nuclear, constitutive, cell or tissue type-specific and inducible promoters can also be used (Table 2).

In addition to the enhancer/promoter sequences that can regulate gene expression, locus control regions (LCRs) can be used for expression in particular cell types. An LCR located 50 kb upstream of the globin gene locus has been identified and shown to be effective for regulating gene expression in erythroid cells (59). This sequence appears to control chromatin structure and increases the transcriptional activity of the β-globin gene. Expression studies have shown that different genes, such as thy-1 and thymidine kinase (tk)-neo, linked to a heterologous LCR, are highly expressed when transfected in erythroid cells, regardless of the genomic integration site (12). Similar LCR elements associated with other tissue-specific genes could provide a means to develop expression vectors that are position independent and tissue specific (67,76,89).

Transient/Stable Expression

Another factor in defining a eukaryotic expression vector system is whether the expression is to be transient or stable. Transient expression systems are extremely useful for studying elements that regulate gene expression or when it is important to have experimental results within a short time frame. Transient expression studies are predicated on a burst of gene expression between 12 and 72 hours after transfection. This burst of gene expression is followed by a rapid deterioration in expression of the transgene because of cell death or loss of the expression plasmid. The optimal time to assay expression needs to be determined empirically and will depend on the cell type, the cell doubling time and the characteristics of the vector regulatory elements. Transient expression systems can be evaluated in terms of the protein products synthesized in the transfected cells, such as the activity of a reporter gene that is not endogenously expressed in the cell type used. The most common reporter gene systems include bacterial chloramphenicol acetyltransferase (CAT) and β-galactosidase (β-gal), firefly luciferase (Luc), human placental alkaline phosphatase (AP), β-glucuronidase (GUS) and more recently, green fluorescent protein (GFP) (2,5).

Table 2. Commonly Used Promoter/Enhancer Elements

Promoter/Enhancer	Characteristics
β-actin	Moderate to strong constitutive cellular transcriptional enhancer.
Adenovirus inverted terminal repeats (ITR)	Weak constitutive viral transcriptional enhancer.
Cytomegalovirus (CMV)	Strong constitutive viral transcriptional enhancer.
α-fetoprotein	Three tissue-specific transcriptional cellular enhancer elements.
γ-globin/β-globin	Tissue specific and developmentally directed enhancers for fetal (γ) and adult (β) erythroid cells.
β-Interferon	Virus inducible; enhancer under negative control; strong constitutive expression element that is derepressed during induction.
Metallothionein II (MT II)	Inducible by heavy metals, phorbol esters (TPA), and glucocorticoids; tends to be "leaky".
Mouse mammary tumor virus (MMTV)	Glucocorticoid-inducible viral transcriptional enhancer; weak to moderate level of induction of transcription.
Murine leukemia virus (MuLV) long terminal repeat (LTR)	Moderate to strong viral transcriptional enhancer.
Rous sarcoma virus (RSV) LTR	Strong viral transcriptional enhancer.
SV40	Moderate to high-level constitutive viral transcriptional enhancer; inducible with phorbol ester (TPA).

Stable expression systems can obtain moderate to high levels of gene expression when coupled with an enrichment or selection scheme. These systems are particularly useful when large quantities of protein are required. To achieve stable expression, the expression vector can either integrate into the host cell genome or be maintained as an extrachromosomal (episomal) element, under conditions of chronic selection. The selectable marker on the expression vector facilitates enrichment of cells that contain the transgene of interest. If the promoter/enhancer complex can modulate transcription of the transgene, then expression can be modified (e.g., enhanced or decreased) by compounds (e.g., hormones, metals, antibiotics) that are added to the growth medium.

A typical vector used to enrich for successfully transfected cells will carry a gene essential for the survival of a given cell line that is either defective in the gene or void of the gene altogether. Classic selectable markers such as the herpes simplex virus tk, dihydrofolate reductase (dhfr), hypoxanthine guanine phosphoribosyl transferase (hprt) and adenyl phosphoribosyl transferase (aprt) genes can only be used in cells deficient in TK, DHFR, HPRT or APRT, respectively (5). Alternatively, genes that confer resistance to cytotoxic drugs are quite effective in situations when the cell line is not defective in one of the endogenous genes mentioned earlier. The most common selection marker is the aminoglycoside phosphotransferase (aph) gene that confers resistance to antibiotics such as kanamycin, neomycin (Neo^r) and geneticin (G418). Genes encoding either for hygromycin B phosphotransferase (HygB), xanthine-guanine phosphoribosyl transferase (XGPRT), Zeocin™ (Zeo) or blasticidin (Bsd) have also been effectively used (5). Because each cell line is different in its sensitivity to these compounds, the optimal drug concentration must be empirically determined for each cell type. The selection typically requires long-term (about 10 days) in vitro cultivation in the presence of a drug.

An additional approach to select or enrich for transfected cells relies on the fluorescence-activated cell sorting (FACS) analysis. Cell selection is based on expression of cell surface proteins that bind specific antibodies (e.g., human and murine CD24, human CD4ζ and murine CD2, the multidrug resistance gene *mdr-1* and the human low-affinity nerve growth factor receptor, LNGFR) that will facilitate FACS identification of cells carrying the expression vector. The genes used to mark the cells indicate gene transfer (44); however, at present, this method requires refinement to eliminate false-positive cells.

Degree of Gene Expression

In general, the level of transgene expression is influenced by *(i)* the number of gene copies within the cell, *(ii)* the rate of transcription of the gene, *(iii)* the stability of the mRNA transcript and *(iv)* the position of integration with regards to the genomic environment and the flanking DNA. The number of gene copies within a cell depends, in part, on the number of copies that enter the cell during transfection. If the vector contains or is co-transfected with a gene for a drug resistance marker, the rate of expression of the gene of interest can be amplified under increasing concentrations of the selective drug. This process can occur if the vector is integrated into the DNA or if it is contained as an extrachromosomal particle within the cell. The DHFR gene has been extensively used in this fashion. Amplification of stably integrated dhfr DNA is associated with expanded chro-

mosomal regions called homogeneous staining regions. The amplification can also be unstable and will result in extrachromosomal units that autonomously replicate, called double minute chromosomes (4,104). In the case of episomal vectors, the episome copy number can also be amplified by increasing the selective pressure on the cells because the episome can replicate autonomously. As long as the selective pressure is maintained or increased, the episome will be retained and the copy number will be increased.

Another method of increasing the level of gene expression in a cell is through enhancer-dependent induction or through the 3′ untranslated region (3′ UTR) stabilization of the mRNA transcript. Certain enhancer elements contain sequences that can be stimulated by hormones or hormone-like compounds such as steroids (Table 2). A common steroid-inducible enhancer that is used is the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) (94,112). Other inducible elements are the metallothionein promoter/enhancer that is inducible by divalent cations such as cadmium or zinc (74), as well as a glucocorticoid-inducible element (78). Unfortunately, many of these compounds are not specific for the gene of interest. To overcome the resulting pleiotropic effects, different inducible systems have been developed. They rely on prokaryotic control elements or on mutated versions of endogenous hormonally regulated elements and include the tetracycline-, FK506/rapamycin-, RU486/mifepristone- and the ecdysone-inducible systems (120,122).

Enhancement of gene expression through the stabilization of mRNA has not been fully explored, although there is now evidence that mRNA levels can be modulated by specific 3′ UTR sequence elements (53,85,142). There is evidence that the 3′ UTR contains sequences that are hormone inducible (107) and that, in some cases, the 3′ UTR also appears to regulate cell-specific expression of a given gene (126). These findings indicate that development of eukaryotic expression vectors requires that both the enhancer sequences and the UTRs be considered to provide optimal expression of a given gene.

Episomal Vectors

While most expression vectors have the ability to integrate into the host genome at some point in the transfection process, a class of vectors has been developed to avoid complications that arise from integration. Most notable is the Epstein-Barr virus (EBV)-based system (134,150). This system is designed for enhanced expression and maintenance as an episomal element. EBV-based vectors require two viral elements, the origin of replication (OriP) and the viral EBNA1 gene that facilitates the extrachromosomal maintenance of the vector. Another feature of this vector is that it readily accepts large pieces of DNA, allowing the insertion of very large fragments of genomic DNA, up to several hundred kilobases (9). Because they are retained as extrachromosomal replicating elements within the nucleus, transgene expression is independent of position effects observed when DNA vectors are randomly integrated. In addition, because the episome is often present as multiple copies, the expression of the transgene can be amplified (1). Because these vectors cannot rely on viral envelope or DNA coat protein elements to enter cells, artificial delivery vehicles must be used to introduce the vectors into cells. Long-term expression in the absence of selection has been shown in cultured epithelial cell lines using an EBV vector expressing the CF gene (88).

Expression Vector Purification and Modification

A critical parameter for achieving successful transfection is the quality of the expression vector DNA. To optimize transfection efficiency, plasmid vector DNA should be free of protein, RNA and chemical contamination. Usually, the DNA is purified either by two rounds of CsCl gradient centrifugation, by anion-exchange chromatography or, more recently, by triple-helix affinity chromatography (145). Furthermore, removal of bacterial endotoxins or lipopolysaccharides from the plasmid DNA has been shown to increase transfection efficiency in cultured cells (144).

A recent strategy for increasing the efficiency of gene transfer relies on the incorporation of targeting moieties into plasmid DNA. For example, the addition of simian virus 40 (SV40) nuclear localization signal (NLS) sequences to plasmid DNA has facilitated nuclear uptake of DNA in digitonin-permeabilized HeLa, COS7 and HepG2 cells (127). Another innovation is the development of modified plasmid vectors, called minicircles, by site-specific recombination (33). These supercoiled minicircles lack both the bacterial origin of replication and the antibiotic resistance marker and have been shown to increase gene transfer efficiency, in vitro and in vivo (34). The increased efficiency seems to be related to their small size or to the absence of bacterial sequences.

DNA DELIVERY

There are many different methods to transfer expression vectors into cells. The efficiency of transfection for naked

Table 3. DNA Transfer Strategies

DNA Transfer/Protocol	Mammalian Cell Type
PO_4 Precipitation **$CaPO_4$** **BES-$CaPO_4$** **$SRPO_4$**	Most mammalian cell types; most effective with adherent cells; epithelial cells are more susceptible to the cytotoxic effects from $CaPO_4$ than from $SrPO_4$.
Electroporation	Primarily used for cells in suspension (e.g., hematopoietic cells) and embryonic stem cells; has also been successfully used for transfection of fibroblasts and airway epithelial cells.
Microinjection	Used for oocyte or zygote transfection; effective for embryonic stem cells and airway epithelial cells.
Liposome/Dendrimer-DNA Complexes	Most mammalian cell types; more effective for adherent cells than cells normally grown in suspension.
Recombinant Virus	Most mammalian cell types; tropism for specific cell types is dependent on genes inserted into viral coat.

Cell specificity using transfection strategies other than liposome or recombinant virus requires the construction of expression vectors that contain cell-specific transcriptional regulatory RNA degradative elements.

plasmid vectors is quite low, and these vectors require transfection vehicles (Table 3). These vehicles can be classified as either chemical (phosphate precipitation, cationic polymers, liposomes, molecular conjugates and others) or physical (electroporation, biolistic and microinjection). The choice of vehicle protocol depends, in part, on the cell type and number of cells to be transfected, and on the desired transfection efficiency. For example, there is a discrete difference in transfection efficiency between attached cells and cells in suspension when cells are transfected by calcium phosphate ($CaPO_4$) precipitation. Fibroblasts and epithelial cells can have a transfection efficiency as high as 10^{-1} (24), while lymphocytes transfected by $CaPO_4$ precipitation show a gene transfer frequency of only approximately 10^{-8} (116). To circumvent this reduction in transfection efficiency, lymphocytes have been stably transfected at higher efficiency (10^{-2}) by electroporation (116). Another key to effective transfection is the growth state of the transfected cells. The best results are achieved when the cells are in log phase growth. This is absolutely essential if the cells are to be stably transfected. In the case of transient transfection, cells can be quiescent because integration into the cell genome is not necessary for the expression of the transfected gene. Another factor to consider in transfection is that uncut, supercoiled DNA is optimal for transient expression systems, while linear DNA tends to be more recombinogenic and will facilitate stable transfection (97). Finally, the choice of the transfection vehicle will be critical when defining the appropriate system for in vitro versus in vivo DNA transfer.

Viral expression vectors do not require a delivery vehicle for DNA transfer because viruses infect cells as part of their natural biological function. However, the mode of entry for each virus is different. For example, retroviral vectors have been used for delivery into hematopoietic cells (11) and have been engineered to facilitate cell type-specific infection into erythroid cells in vitro (75). Adenovirus-based viral vectors have been used in vivo for the delivery of the CF transmembrane conductance regulator (CFTR) gene, in part, because of the tropism of the adenovirus for the airways (30,152).

Chemical Methods

$CaPO_4$ precipitation. $CaPO_4$ coprecipitation of DNA has been successfully used for transfection of different types of mammalian cells (24,27,29, 61–63). This technique has an efficiency that varies with the cell type but is most commonly used for stable transfection efficiencies of 10^{-3} to 10^{-5}. There are several variations of this technique involving different buffer solutions. One variation uses HEPES buffer (27,29,61–63), while another uses N, N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) (24). In the case of airway epithelial cells, there is evidence that under certain growth and culture conditions, $CaPO_4$ precipitation can cause cell lysis or squamous differentiation (16). Consequently, an alternative to transfection by $CaPO_4$ precipitation was developed for airway epithelial cells and uses strontium phosphate ($SrPO_4$) precipitation (16,119).

A key factor affecting the efficiency of transformation appears to be the pH of the phosphate buffer solution. A pH of 7.1 (HEPES method) or a pH of 6.95 (BES method) appears to be optimal for $CaPO_4$ precipitation, while $SrPO_4$ has a pH optimum of 7.6–7.9. Some studies also show a higher efficiency of transfection if the plasmid to be transfected is linearized (131), while others show that the circular form is more effective (24).

After application of the precipitate, cells are incubated at CO_2 levels ranging from 3%–5% (16,24,27,29,62) under conditions that facilitate pinocytosis. The agents that are most effective are glycerol (146), dimethyl sulfoxide (DMSO) (90), chloroquine (93) and sodium butyrate (58). Since these agents are toxic to cells, transfection

conditions for individual cell lines require prior optimization of dose and exposure time. Key elements of this transfection strategy are the quality of the precipitate, which must be fine and uniform, and the removal of the precipitate following incubation. This is especially critical for primary cells that must be thoroughly washed with serum-free medium to remove all traces of the DNA/PO_4 precipitate observed when cultures are viewed by phase contrast microscopy. If the precipitate is not removed, cells will undergo terminal squamous differentiation and will no longer proliferate and/or they will lyse. It is also important to keep detachment of adherent cells to a minimum during the washing process. When the washing is completed, the cells can be placed in growth medium for further growth and analysis.

Cationic polymers. Among the cationic polymers used for the transfection of cultured mammalian cells, DEAE-dextran was one of the first studied. This reagent has been successfully used to deliver nucleic acids into cells for transient expression and short-term expression analyses but is not suitable for stable transfection studies (56). The primary drawback of the DEAE-dextran system is the cytotoxicity. Therefore, the effects of concentration and exposure times need to be determined before transfection for individual cell lines. Other synthetic cationic polymers have been used to transfer DNA into mammalian cells. The polymers include protamine, intact and fractured polyamidoamine dendrimer (64,82,136,137) and, more recently, polyethylenimine (PEI) (6,14), which appears to be better than cationic liposomes at delivering DNA across the nuclear envelope (110,153).

Liposomes. DNA or RNA can also be transferred into cells by encapsulation in liposomes. These are artificial membrane vesicles that either fuse with the cell membrane and deliver their contents into the cell for expression (96) or enter the cell through an endocytic pathway (52,153). Since the first lipids were described, several hundred new cationic lipids that range greatly in transfection efficiency have been developed for local and systemic gene transfer (54,84).

Liposome-mediated gene transfer offers several advantages over other chemical transfection systems. Among these are a relatively high efficiency of gene delivery, ability to transfect various cell types resistant to $CaPO_4$ or DEAE-dextran, successful delivery of a wide range of DNA (86), effective delivery of both RNA (95) and protein (36). Disadvantages include the cytotoxicity of the liposome formulation and the need to optimize various parameters for each formulation, such as the DNA-to-liposome charge ratio, the amount of DNA, cell density and the transfection period (54). Other critical factors that must be optimized for effective DNA delivery are the size and homogeneity of lipid-DNA complexes (26).

Transfection of human airway epithelial cell lines that display differentiated features, such as cell polarity, has shown the importance of matching the liposome formulation with cells relevant to the organ to be transfected. Studies with one airway epithelial cell line, 16HBE14o- (28), comparing transfection efficiency based on relative fluorescence have shown that DOTAP > > DC-Cholesterol > Transfectam > LIPOFECTAMINE™ > naked DNA (data not shown). However, it is important to recognize that, although the relative efficacy of these compounds indicates that DOTAP is the most appropriate for the 16HBE14o- cells in culture, there may be conditions under which one of the other liposomes might have proven better. The growth state of the cells, their state of differentiation and cell number (i.e., total amount of cellular lipid) may also play an essential role in transfection efficiency because they affect the total net charge (Figure 1). This adds another element in the equations when devising a transfection scheme, taking it from a two-component (liposome and DNA) to a three-component system that must also take into consideration the total amount of cellular lipid in determining the optimal DNA:lipid ratio.

Studies with different cationic formulations indicate that the amount of DNA taken up by the cells does not necessarily correlate with transfection efficiency (42). This finding suggests that the cellular uptake of the lipid-DNA complex is not a limiting step for transfection. In cultured cell lines, most cells are able to take up the cationic lipid-DNA complexes, but only a small proportion are effectively transfected. An important step in nonviral gene transfer is the entry of DNA into the nucleus. Cationic lipids appear to be limited in their ability to deliver plasmid DNA from the cytoplasmic to the nuclear compartment. The intracellular trafficking of the lipid and plasmid DNA is still rather poorly understood; however, several recent studies investigating intracellular trafficking of oligonucleotides indicate that the cationic lipid-mediated delivery of oligonucleotides to the nucleus can be quite effective (71,156).

Cationic liposomes have been successfully used for the transfer of genes both in vitro and in vivo (20,87,102, 103). They have already been tested in clinical trials for in vivo gene therapy, mostly of cancer and CF (20,25,55,113, 125,132). However, these studies have been inconsistent in demonstrating long-term transgene expression and effective delivery of plasmid DNA.

Molecular conjugates. Recent studies with cationic amino acids such as polylysine that form conjugates with the DNA have been very intriguing. These studies have indicated that the polylysine/DNA conjugates can be effective for in vitro and in vivo DNA transfer (31,98,108,133,143). A primary advantage of these amino acid conjugations is their ability to condense DNA. Condensation is critical for the effective uptake of the DNA and its transfer to the nucleus. Conjugates between 25 and 50 nm appear to be ideal (41,46). The size of the conjugate must be closely monitored to ensure size uniformity and efficient delivery. These factors are highly dependent on the salt solution in which they are made and can thus be modulated by altering the character of the salt solution. Toxicity can be a factor with the polylysine conjugates, so alternatives such as lysine-rich molecules have been developed (22,141). Conjugates can also be easily modified with specific proteins that facilitate cell- and organelle-specific delivery (41,46,47,79,99,141). These include modification of the polyamino acid to include nuclear localization proteins, antibodies to the immunoglobulin receptor or cell surface sugars. These modifications appear to enhance the conjugate specificity and to increase the efficiency of gene transfer. The in vivo immune issues may compromise some of the enhanced effectiveness derived from conjugate modification, but these systems may yet prove superior to other artificial DNA transfer vehicles because of their ability to condense DNA.

Other chemical delivery systems. A new generation of gene delivery products is being developed (45). One such compound is a gramicidin S-DNA-lipid complex (87). This complex has the added advantage that the gramicidin S helps permeabilize the cell membrane, thereby facilitating DNA uptake. Other novel DNA delivery vehicles include lipid systems that have been combined with viral systems (43,68,151). However, because such systems are chimeric complexes that combine lipids with viral proteins, they have a tendency to be immunogenic. The ultimate application of these systems in vivo or therapeutically will require further study.

Physical Transfection Strategies

Physical methods for DNA delivery

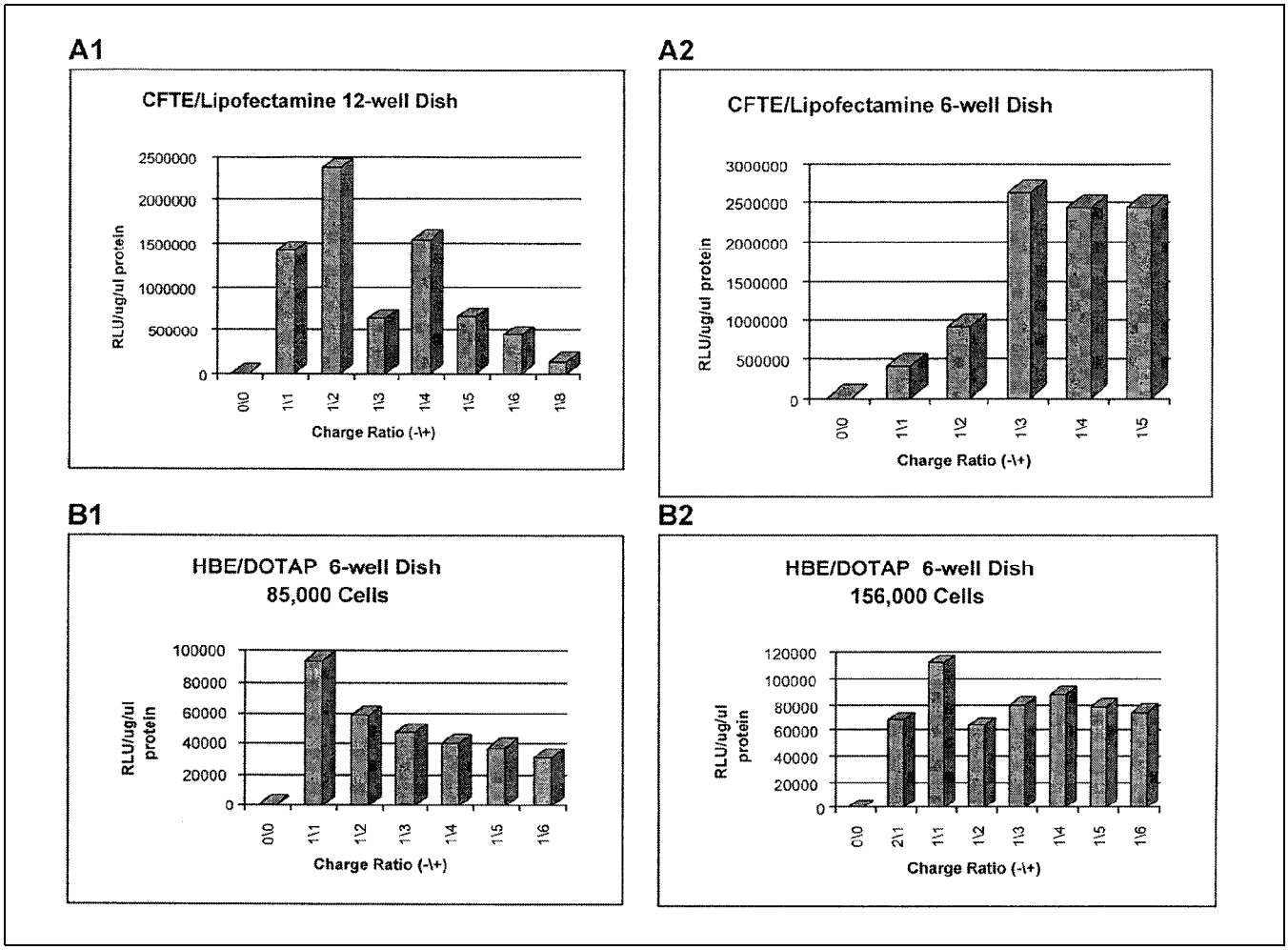

Figure 1. Effect of changing the amount of transfected DNA and cell number on transfection efficiency. CFBE41o- cells were transfected with 1 and 3 µg DNA in 6- and 12-well dishes. A decrease in the amount of DNA transfected (i.e., an increase in the ratio of cellular lipid to cationic lipid) led to a shift in the optimal charge ratio range towards a more net positive charge. Data represent mean (A1 and A2) $n = 3$; (B1 and B2) $n = 2$.

depend neither on viral vehicles and their receptors nor on biochemical structures or features of target cell membranes required for uptake of a vehicle-DNA complex.

Electroporation. Electroporation is one of the most widely used physical methods and is based on exposing cells to a pulsed electrical field of sufficient strength to cause a reversible permeabilization of the cell membrane in localized areas (3,129). Two distinct types of apparatus are available commercially and differ in how they control pulse strength and length, the electrical parameters that cause cell permeabilization. The most critical parameters for successful electroporation with such a device are the magnitude and length of the current pulse (i.e., decay time or pulse width if an exponential wave pulse or a square wave pulse is used, respectively).

For mammalian cells, the conditions for electroporation involve either high field strength coupled to a low capacitance or low field strength coupled to a high capacitance. Low voltage (about 1000 V) and high capacitance (> 500 μF) conditions have been more commonly used for transfection and improve cell survival and efficiency. However, the optimal electrical conditions (voltage and capacitance settings) are cell type dependent. Optimal conditions require the assessment of cell viability, which should be in the range of 10%–50%. Electroporation guidelines, including parameters that have been successful for other cell types, have been reviewed elsewhere (23,115). As is the case with other transfection methodologies, the efficacy of gene transfer is increased when the cells are proliferating. Mid-log phase cells are generally electroporated as a suspension of 10^6 to 10^7 cells/mL. The use of overgrown cultures significantly decreases efficiency and survival after pulsing (8). Transfection efficiency can also be optimized by increasing the DNA concentration to the range of 10–40 μg/mL for transient expression assays or decreasing it to 1–20 μg/mL for stable transfection.

One drawback of electroporation is the detachment of adherent cells from their substratum and the resultant trauma. This produces undesired physiological effects that can undermine cell viability. Recent studies have proposed in situ electroporation using Petri dish electrodes and electrodes that attach to the surface to be electroporated in vivo (Genetronix, a BTX Instrument). Other methods include cells grown on microcarriers (115), on a glass surface coated with an electrically conductive material (118), on microporous membranes of polyethylene terephthalate or polyester (149) or on glass coverslips mounted in a specific chambers (157).

Electroporation has been most useful for gene targeting studies and for

transfer into embryonic stem (ES) cells in the development of transgenic animals (129,139) to facilitate the introduction of a low number of copies. This approach has also been effectively used for the transfection of airway epithelial cells (72,83). Recently, a modified electroporation method using microelectroporation chambers and plating the electroporated cells at optimal density was described for mouse ES cells; a frequency of 1% was achieved (138).

Biolistic (gene gun). A relatively new physical method of gene transfer is the biolistic particle bombardment (or gene gun) technique (77,148). Biolistics have been applied to deliver foreign DNA to a broad range of somatic tissues and cells, including brain, skin, mammary gland, liver and tumor cells, as well as human epithelial, endothelial, fibroblast and lymphocyte-derived cell culture lines (147,155). The biolistic technology involves accelerated delivery of plasmid DNA (coated with high-density metal particles) into cells using one of several acceleration instruments. Gold particles are preferred to tungsten because of their size uniformity and spherical shape. The basic instrument has a plastic membrane (macrocarrier) that carries the microparticles, a macrocarrier acceleration mechanism and a stopping screen that blocks the macrocarrier and allows the microparticles to reach the cells. The different acceleration instruments use a variety of systems to provide the physical force that accelerates the microparticles. These systems include shock waves created by the sudden release of compressed air (nitrogen or helium), an electric discharge through metal wire (135) or a high-voltage discharge (147). A primary consideration is the derivation of the appropriate instrument settings for optimal bombardment. The major advantages of this gene delivery strategy include the relatively low level of cell damage and its potential application for in vivo transfection (124,154). Drawbacks include poor tissue penetration (≤ 500 μm), the manipulations required for microparticle preparation and the initial cost of the acceleration instrument.

After bombardment of cultured cells, the medium can be replaced immediately or within 1–2 min after bombardment (124,140). Compared to electroporation, smaller amounts of DNA (submicrogram quantities per dose) and fewer target cells are required for the DNA transfer. Identical experimental conditions can also be used for transient or stable transfection. The most critical parameters affecting DNA transfer efficiency include target distance and air pressure, as well as particle size, density and velocity (66).

Microinjection. Another approach that has been used for high efficiency gene transfer is intranuclear microinjection (17,37). This approach has been used to transfer genes into mammalian oocytes and preimplantation embryos (37,128), ES cells (37,139), cultured fibroblasts (32), airway epithelial cells (57,83) and blood stem cells (35). While this approach is tedious, it is highly efficient and directly delivers the exogenous DNA to the nucleus. It is not only possible to control the site of DNA delivery but also to control the amount of DNA that enters the nucleus. Thus, microinjection provides a mechanism for minimizing nuclease degradation of the extracellular DNA in intracellular vesicles and enhancing transfection efficiency. Microinjection also has the potential for delivering a wide variety of materials, including DNA, RNA, proteins and nucleoprotein complexes, and has been effectively used to study gene expression, homologous recombination, oncogenesis and human disease. Furthermore, this approach can be used to identify the in vivo function of given gene products by inhibiting activity with antibodies or antisense RNA and for identifying mechanisms whereby exogenous DNA randomly or specifically integrates into the host cell genome.

The success of DNA microinjection depends on a number of factors including the manufacture of pipets (19), their tip bevel siliconization, DNA concentration and viscosity and the target cell. The ease of the procedure also depends on the injection equipment and the injection target. It is obviously easier to microinject a frog oocyte than a mouse oocyte that is 1000 times smaller. It is also easier to microinject DNA into the cytoplasm than the nucleus. Fortunately, these technical obstacles can be overcome. However, while this technique has the advantage of circumventing exposure to intracellular compartments that will degrade the DNA, it cannot be readily used to introduce DNA into large numbers of cells. It is therefore most useful for generating cell lines that carry transgenes of interest.

Viral Delivery Systems

Viral vector systems were developed to facilitate efficient delivery of DNA expression vectors into cells. The gene of interest is delivered in the form of a defective virus encapsulated within a virus envelope that increases the efficiency of gene transfer. The recipient cells are, in fact, infected with these defective viruses. The viruses have been modified so that they are no longer able to replicate autonomously and require passage through a helper cell line that contains the genetic instructions for coating or packaging the recombinant viral vector. Since each viral delivery system has advantages and drawbacks that depend on the characteristics of the virus, the choice of a given viral delivery system will depend on the endpoints desired. There are now numerous recombinant viral delivery systems, and these are listed in Table 1 (21,38, 60,80,92,101,114,117). Stable, long-term expression is possible with most of the vectors because they either integrate into the cell genome or are maintained as episomes for extended times when introduced into cells. Transient, short-term expression is also possible.

Transient expression. Viral systems such as baculovirus, alphavirus and vaccinia virus are particularly useful for generating large quantities of protein from a given gene. These virus systems have the advantage over bacterial amplification and expression systems because they are eukaryotic and can post-translationally modify the protein product. Their main limitation is host cell death a few days after infection that results in transient gene expression. The baculovirus system generally requires an insect cell for expression. This can be limiting when evaluating mammalian proteins because the glycosylation pathway in insect cells appears to be distinct from that in mammalian cells. Thus, modification of the resultant protein may not accurately reflect that of the mammalian system. Recent studies

have shown that the baculovirus system can be modified to introduce and express foreign genes in primary human (69,70) and rat hepatocytes (15), as well as in primary muscle, HeLa, COS7 and CV-1 cells (7,130).

Factors that appear to affect expression in mammalian cells include the promoter used to express the transgene, the multiplicity of infection, viral growth rate and viral exposure conditions. More persistent expression, albeit still transient, in mammalian cells has been achieved using hybrid systems composed of a baculovirus backbone containing elements from adeno-associated virus (105). However, in vivo studies have shown that baculoviral vectors are inhibited by the immune complement system, and thus have limited usefulness for direct delivery into animals or as a genetic therapy (70).

Alphaviruses have an efficient RNA replication system that results in high-expression of different proteins in a broad range of host cells. Because of the cytopathopathic nature of the virus, it is primarily employed for transient gene expression (92).

Recombinant vaccinia viruses have been used in vitro and in vivo for gene transfer (21,100,106). This system replicates in the cytoplasm and can be engineered to transcribe there as well. Replication of the virus can be modified by UV irradiation to mitigate the cytotoxic effects of the virus and optimize the expression of the transgene (21).

Stable expression. Stable viral delivery systems that provide long-term, mid-level production of protein or gene expression include retroviruses, adenoviruses and adeno-associated viruses. Retroviral-based systems readily integrate into the genome of infected cells, and their viral protein envelopes can be adapted to a wide variety of host cells (40). The efficiency of the infection depends largely on the virus titer and the proliferative state of the cells. If titer is high enough, it is possible to infect 100% of the cells. However, unless the cells are actively proliferating, the efficiency of infection will be minimal. In the case of retrovirus infection, it has been possible to enhance the efficacy of infection by growing the host cells in the presence of the cells that package the virus. Because the reverse-transcribed provirus integrates into the genome, it is maintained within the cells for multiple generations. These viruses are best used for the development of stably transfected cell lines and for the study of gene product function, although numerous in vivo and gene therapy studies have been carried out using retroviruses (51).

The adenoviruses and the adeno-associated viruses have been used in vitro and in vivo (10,18,91,117,121), as well as in CF gene therapy trials (30,48,50, 65,152). Because these viruses have a tropism for the airways, it was postulated that they would be effective for delivering genes specifically into the airways. However, there have been complications caused by a host immune response that have made the adenoviruses less effective than first anticipated (30,152). The adeno-associated virus is more versatile and will transduce a broader range of cell types in a different number of mammalian species, without the same immune complications associated with the adenovirus systems. Long-term adeno-associated virus transgene expression of both marker and therapeutic genes has been reported in several in vivo studies (48,91,111,117). However, adeno-associated virus is limited by the size of the gene that can be inserted in the recombinant virus (39). Furthermore, while the wild-type virus appears to preferentially integrate into a specific site on chromosome 19, the recombinant adeno-associated virus has lost this specificity and will integrate randomly. Recent modifications to the adeno-associated virus vectors have been able to restore some site-specific integration of the recombinant vector (109).

CONCLUSIONS

The application of the mammalian expression vector systems and gene transfer methodologies have played an important role in understanding gene regulation as well as protein structure and function. The gene transfer and gene expression approaches discussed here represent some of the most commonly used expression vector systems and transfection protocols. The mode of DNA transfer into cells depends on numerous factors: *(i)* the DNA transfer efficiency required, *(ii)* whether the transfection is in vitro or in vivo and *(iii)* whether expression is to be stable or transient. Numerous approaches have been used to facilitate the transfer of DNA into cells. While the most efficient appear to be recombinant viral vectors, nonviral approaches have also proven effective; the most commonly used are described in this review. At present, the most versatile and promising nonviral vehicles, in terms of in vitro or in vivo transfection, are the liposome/cationic polymers/polylysine vehicles. The use of any one strategy depends on the goals of the experiment and study. Once the endpoints are defined, it is possible to design the most effective strategy to achieve a desired outcome.

The strategies described here are an introduction to the tools of mammalian gene expression and reflect concepts and considerations important for gene analysis and for development of genetic therapy regimens. These concepts can be applied to various mammalian cell

systems and have been used by us to study genes associated with respiratory function. Mammalian expression strategies are essential to our understanding of genes and their relationship to mammalian physiology. As is evident from the above discussion, the approaches to transgene delivery are not only used for the study of gene function but can also be expanded for the development of genetic therapy regimens. The features and considerations of vector construction and transgene delivery discussed above will be critical for designing gene therapy protocols for specific disease states.

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REFERENCES

1. **Aiyar, A., C. Tyree and B. Sudgen.** 1998. The plasmid replicon of EBV consists of multiple *cis*-acting elements that facilitate DNA synthesis by cell and a viral maintenance element. EMBO J. *17*:6394-6403.
2. **Alam, J. and J.L. Cook.** 1990. Reporter genes: application to the study of mammalian gene transcription. Anal. Biochem. *188*:245-254.
3. **Andreason, G.L. and G.A. Evans.** 1988. Introduction and expression of DNA molecules in eukaryotic cells by electroporation. BioTechniques *6*:650-660.
4. **Ariyama, Y., T. Sakabe, T. Shinomiya, T. Mori, Y. Fukuda and J. Inazawa.** 1998. Identification of amplified DNA sequences on double minute chromosomes in a leukemic cell line KY821 by means of spectral karyotyping and comparative genomic hybridization. J. Hum. Genet. *43*:187-190.
5. **Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl.** 1997. Current Protocols in Molecular Biology. John Wiley & Sons, New York.
6. **Baker, A., M. Saltik, H. Lehrmann, I. Killisch, V. Mautner, G. Lamm, G. Christofori and M. Cotten.** 1997. Polyethylenimine (PEI) is a simple, inexpensive and effective reagent for condensing and linking plasmid DNA to adenovirus for gene delivery. Gene Ther. *4*:773-782.
7. **Barsoum, J., R. Brown, M. McKee and F.M. Boyce.** 1997. Efficient transduction of mammalian cells by a recombinant baculovirus having the vesicular stomatitis virus G glycoprotein. Hum. Gene Ther. *8*:2011-2018.
8. **Baum, C., P. Forster, S. Hegewisch-Becker and K. Harbers.** 1994. An optimized electroporation protocol applicable to a wide range of cell lines. BioTechniques *17*:1058-1062.
9. **Belt, P.B., H. Groeneveld, W.J. Teubel, P. van de Putte and C. Backendorf.** 1989. Construction and properties of an Epstein-Barr-virus-derived cDNA expression vector for human cells. Gene *84*:407-417.
10. **Berkner, K.L.** 1988. Development of adenovirus vectors for the expression of heterologous genes. BioTechniques *6*:616-629.
11. **Blaese, R.M., K.W. Culver, L. Chang, W.F. Anderson, C. Mullen, A. Niehuis, C.S. Carter, C. Dunbar et al.** 1993. Treatment of severe combined immunodeficiency disease (SCID) due to adenosine. Hum. Gene Ther. *4*:521-527.
12. **Blom van Assendelft, G., O. Hanscombe, F. Grosveld and D.R. Greaves.** 1989. The β-globin dominant control region activates homologous and heterologous promoters in a tissue-specific manner. Cell *56*:969-977.
13. **Boucher, R.C.** 1999. Status of gene therapy for cystic fibrosis lung disease. J. Clin. Invest. *103*:441-445.
14. **Boussif, O., F. Lezoualc'h, M.A. Zanta, M. Mergny, D. Scherman, B. Demeinex and J.P. Behr.** 1995. A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. Proc. Natl. Acad. Sci. USA *92*:7297-7301.
15. **Boyce, F.M. and N.L. Bucher.** 1996. Baculovirus-mediated gene transfer into mammalian cells. Proc. Natl. Acad. Sci. USA *93*:2348-2352.
16. **Brash, D.F., R.R. Reddel, M. Quanrud, K. Yang, M.P. Farrell and C.C. Harris.** 1987. Strontium phosphate transfection of human cells in primary culture: stable expression of the simian virus 40 large T-antigen in primary human bronchial epithelial cells. Mol. Cell. Biol. *7*:2031-2034.
17. **Brinster, R.L., H.-Y. Chen, M.E. Trumbauer, R. Senear, R. Warren and R.D. Palmiter.** 1985. Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. Proc. Natl. Acad. Sci. USA *82*:4438-4442.
18. **Brody, S.L. and R.G. Crystal.** 1994. Adenovirus-mediated *in vivo* gene transfer. Ann. NY Acad. Sci. *716*:90-101.
19. **Brown, K.T. and D.G. Fleming.** 1986. Advanced Micropipette Techniques for Cell Physiology. John Wiley & Sons, New York.
20. **Caplan, N.J., E.W.F.W. Alton, P.G. Middleton, J.R. Dorin, B.J. Stevenson, X. Gao, S.R. Durham, P.K. Jeffery et al.** 1995. Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. Nat. Med. *1*:39-46.
21. **Carroll, M.W. and B. Moss.** 1997. Poxviruses as expression vectors. Curr. Opin. Biotechnol. *8*:573-577.
22. **Chan, C.K. and D.A. Jans.** 1999. Enhancement of polylysine-mediated transferrinfection by nuclear localization sequences: polylysine does not function as a nuclear localization sequence. Hum. Gene Ther. *10*: 1695-1702.
23. **Chang, D.C.** 1992. Design of protocols for electroporation and electrofusion: selection of electrical parameters. *In* D.C. Chang, J.A. Saunders, A.E. Sowers (Eds.), Guide to Electroporation and Electrofusion. Academic Press, San Diego, CA.
24. **Chen, C.A. and H. Okayama.** 1988. Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. BioTechniques *6*:632-638.
25. **Colosimo, A., F. Sangiuolo, S. Di Sario, P. Amicucci, A.L. Serafino, A. Novelli, M. Sabani, M. Castro et al.** 1998. Current status of cationic liposome-mediated gene therapy in cystic fibrosis. Acta Pharm. *4*:221-303.
26. **Colosimo, A., A.L. Serafino, F. Sangiuolo, S. Di Sario, E. Bruscia, P. Amicucci, G. Novelli, B. Dallapiccola and G. Mossa.** 1999. Gene transfection efficiency of tracheal epithelial cells by Dc-Chol-DOPE/DNA complexes. Biochim. Biophys. Acta *1419*:186-194.
27. **Cozens, A.L., M.J. Yezzi, L. Chin, W.E. Finkbeiner, J.A. Wagner and D.C. Gruenert.** 1992. Characterization of immortal cystic fibrosis tracheobronchial gland epithelial cells. Proc. Natl. Acad. Sci. USA *89*:5171-5175.
28. **Cozens, A.L., M.J. Yezzi, K. Kunzelmann, T. Ohrui, K. Eng, W.E. Finkbeiner, J.H. Widdicombe and D.C. Gruenert.** 1994. CFTR expression and ion transport in polarized immortal humanbronchial epithelial cells. Am. J. Respir. Cell Mol. Biol. *10*:38-47.
29. **Cozens, A.L., M.J. Yezzi, M. Yamaya, J.A. Wagner, D. Steiger, S.S. Garber, L. Chin, E.M. Simon et al.** 1992. A transformed human epithelial cell line that retains tight junctions post crisis. In Vitro Cell. Dev. Biol. *28A*:735-744.
30. **Crystal, R.G., N.G. McEvaney, M.A. Rosenfeld, C.-S. Chu, A. Mastrangeli, J.G. Hay, S.L. Brody, H.A. Jaffe et al.** 1994. Administration of an adenovirus containing the human CFTR cDNA to the respiratory tract of individuals with cystic fibrosis. Nat. Genet. *8*:42-51.
31. **Curiel, D.T.** 1994. High-efficiency gene transfer mediated by adenovirus-polylysine-DNA complexes. Ann. NY Acad. Sci. *716*: 36-56.
32. **Daicumakos, E.G.** 1973. Methods for micromanipulation of human somatic cells in culture, p. 287-311. *In* D.M. Prescott (Ed.), Methods in Cell Biology. Academic Press, New York.
33. **Darquet, A.M., B. Cameron, P. Wils, D. Scherman and J. Crouzet.** 1997. A new DNA vehicle for nonviral gene delivery: su-

percoiled minicircle. Gene Ther. *4*:1341-1349.
34. **Darquet, A.M., R. Rangara, P. Kreiss, B. Schwartz, S. Naimi, P. Delaere and J. Crouzet.** 1999. Minicircle: an improved DNA molecule for *in vitro* and *in vivo* gene transfer. Gene Ther. *6*:209-218.
35. **Davis, B.R., J. Yannariello-Brown, N.L. Prokopishyn, Z. Lou, M.R. Smith, J. Wang, N.D.V. Carsrud and D.B. Brown.** 2000. Glass needle-mediated microinjection of macromolecules and trnasgenes into primary human blood stem/progenitor cels. Blood *95*:437-444.
36. **Debs, R., E. Brunette, E. Lin, M. Shaw, A. Harkin and A. Montgomery**. 1990. Biodistribution tissue reaction and lung retention of pentamidine aerosolized as three different salts. Am. Rev. Respir. Dis. *142*:1164-1167.
37. **DePamphilis, M.L., S.A. Herman, E. Martinez-Salas, L. E. Chalifour, D.O. Wirak, D.Y. Cupo and M. Miranda.** 1988. Microinjecting DNA into mouse ova to study DNA replication and gene expression and to produce transgenic animals. BioTechniques *6*:662-679.
38. **Drumm, M.L., H.A. Pope, W.H. Cliff, J.M. Rommens, S.A. Marvin, L.C. Tsui, F.S. Collins, R.A. Frizzell and J.M. Wilson.** 1990. Correction of the cystic fibrosis defect *in vitro* by retrovirus-mediated gene transfer. Cell *62*:1227-1233.
39. **Egan, M., T. Flotte, S. Afione, R. Solow, P.L. Zeitlin, B.J. Carter and W.B. Guggino.** 1992. Correction of defective PKA regulation of outwardly rectifying chloride channels by insertion of cystic fibrosis transmembrane regulator into CF airway epithelial cells. Nature *358*:581-584.
40. **Eglitis, M.A. and W.F. Anderson.** 1988. Retroviral vectors for introduction of genes into mammalian cells. BioTechniques *6*:608-614.
41. **Fajac, I., P. Briand, M. Monsigny and P. Midoux.** 1999. Sugar-mediated uptake of glycosylated polylysines and gene transfer into normal and cystic fibrosis airway epithelial cells. Hum. Gene Ther. *10*:395-406.
42. **Farhood, H., R. Bottega, R.M. Epand and L. Huang.** 1992. Effect of cationic cholesterol derivatives on gene transfer and protein kinase C activity. Biochim. Biophys. Acta *1111*:239-246.
43. **Fasbender, A., J. Zabner, J. Chillon, T.O. Moninger, A.P. Puga, B.L. Davidson and M.J. Welsh.** 1997. Complexes of adenovirus with polycationic polymers and cationic lipids increase the efficiency of gene transfer *in vitro* and *in vivo*. J. Bioch. Chem. *272*: 6479-6489.
44. **Fehse, B., Z. Li, U.M. Schade, A. Uhde and A.R. Zander.** 1998. Impact of a new generation of gene transfer markers on gene therapy. Gene Ther. *5*:429-430.
45. **Felgner, P.L.** 1997. Nonviral strategies for gene therapy. Sci. Am. *276*:102-106.
46. **Ferkol, T., J.C. Perales, E. Eckman, C.S. Kaetzel, R.W. Hanson and P.B. Davis.** 1995. Gene transfer into the airway epithelium of animals by targeting the polymeric immunoglobulin receptor. J. Clin. Invest. *95*:493-502.
47. **Fisher, K.J. and J.M. Wilson.** 1997. The transmembrane domain of diphtheria toxin improves molecular conjugate gene transfer. Biochem. J. *321*:49-58.
48. **Flotte, T., B. Carter, C. Conrad, W. Guggino, T. Reynolds, B. Tosenstein, G. Taylor, S. Walden and R. Wetzel.** 1996. A phase I study of an adeno-associated virus-CFTR gene vector in adult CF patients with mild lung disease. Hum. Gene Ther. *7*:1145-1159.
49. **Flotte, T.R.** 1999. Gene therapy for cystic fibrosis. Curr. Opin. Mol. Ther. *1*:510-516.
50. **Flotte, T.R. and B.J. Carter.** 1995. Adeno-associated virus vectors for gene therapy. Gene Ther. *2*:357-362.
51. **Friedmann, T.** 1989. Progress toward human gene therapy. Science *244*:1275-1281.
52. **Friend, D.S., D. Papahadjopoulus and R.J. Debs.** 1996. Endocytosis and intracellular processing accompanying transfection mediated by cationic liposomes. Biochim. Biophys. Acta *1278*:41-50.
53. **Furth, P.A. and C.C. Baker.** 1991. An element in the bovine papillomavirus late 3′ untranslated region reduces polyadenylated cytoplasmic RNA levels. J. Virol. *65*:5806-5812.
54. **Gao, X. and L. Huang.** 1995. Cationic liposome-mediated gene transfer. Gene Ther. *2*:710-722.
55. **Gill, D.R., K.W. Southern, K.A. Mofford, T. Seddon, L. Huang, F. Sorgi, A. Thomson, L.J. MacVinish et al.** 1997. A placebo-controlled study of liposome-mediated gene transfer to the nasal epithelium of patients with cystic fibrosis. Gene Ther. *4*:199-209.
56. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell *23*:175-182.
57. **Goncz, K.K. and D.C. Gruenert.** 1999. Site-directed alteration of DNA by small fragment homologous replacement (SFHR), p. 85-99. *In* E.B. Kmiec (Ed.), Gene Targeting Vector Protocols. Humana Press, Totowa, NJ.
58. **Gorman, C.M., B.H. Howard and R. Reeves.** 1983. Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. Nucleic Acids Res. *11*: 7631-7648.
59. **Grosveld, F., G. Blom van Assendelft, D.R. Greaves and G. Kollias.** 1987. Position-independent high-level expression of the human β-globin gene in transgenic mice. Cell *51*:975-985.
60. **Gruber, H.E., K.D. Finley, R.M. Hershberg, S.S. Katzman, P.K. Laikind, J.E. Seegmiller, T. Friedmann, J.K. Yee and D.J. Jolly.** 1985. Retroviral vector-mediated gene transfer into human hematopoietic progenitor cells. Science *230*:1057-1061.
61. **Gruenert, D.C.** 1987. Differentiated properties of human epithelial cells transformed *in vitro*. BioTechniques *5*:740-749.
62. **Gruenert, D.C., C.B. Basbaum, M.J. Welsh, M. Li, W.E. Finkbeiner and J.A. Nadel.** 1988. Characterization of human tracheal epithelial cells transformed by an origin-defective simian virus 40. Proc. Natl. Acad. Sci. USA *85*:5951-5955.
63. **Gruenert, D.C., M.J. Yezzi, L. Chin and A.L. Cozens.** 1991. Immortalized cell cultures of CF-tissues as a tool to study ion transport properties, p. 52-64. *In* G. Döring, R. Greger, D. Kaiser and J. Schmidtke (Eds.), Mukoviszidose 1991: Ergebnisse aus Forschung und Klink. Forschungsgemeinschaft Mukoviszidose in Zusammenarbeit mit der Kali-Chemie Pharma, GmbH, Hannover, Germany.
64. **Haensler, J. and F.C. Szoka.** 1993. Polyamidoamine cascade polymers and their use for efficient transfection of cells in culture. Bioconjug. Chem. *4*:85-93.
65. **Hallek, M. and C.M. Wendtner.** 1996. Recombinant adeno-associated virus (rAAV) vectors for somatic gene therapy: recent advances and potential clinical applications. Cytokines Mol. Ther. *2*:69-79.
66. **Heiser, W.C.** 1994. Gene transfer into mammalian cells by particle bombardment. Anal. Biochem. *217*:185-196.
67. **Higgs, D.R.** 1998. Do LCRs open chromatin domains? Cell *95*:299-302.
68. **Hodgson, C.P. and F. Solaiman.** 1996. Virosomes: cationic liposomes enhance retroviral transduction. Nat. Biotechnol. *14*:339-342.
69. **Hofmann, C., V. Sandig, G. Jennings, M. Rudolph, P. Schlag and M. Strauss.** 1995. Efficient gene transfer into human hepatocytes by baculovirus vectors. Proc. Natl. Acad. Sci. USA *92*:10099-10103.
70. **Hofmann, C. and M. Strauss.** 1998. Baculovirus-mediated gene transfer in the presence of human serum or blood facilitated by inhibition of the complement system. Gene Ther. *5*:531-536.
71. **Holmes, A.R., A.F. Dohrman, A.R. Ellison, K.K. Goncz and D.C. Gruenert.** 1999. Intracellular compartmentalization of DNA fragments in cultured airway epithelial cells mediated by cationic lipids. Pharm. Res. *16*:1020-1025.
72. **Iannuzzi, M.C., J.L. Weber, J. Yankaskas, R. Boucher and F.S. Collins.** 1988. The introduction of biologically active foreign genes into human respiratory epithelial cells using electroporation. Am. Rev. Respir. Dis. *138*:965-968.
73. **Jaffe, A., A. Bush, D.M. Geddes and E.W.F.W. Alton.** 1999. Prospects for gene therapy in cystic fibrosis. Arch. Dis. Child. *3*:286-289.
74. **Karin, M., A. Haslinger, H. Holtgreve, G. Cathala, E. Slater and J.D. Baxter.** 1984. Activation of a heterologous promoter in response to dexamethasone and cadmium by metallothionein gene 5′-flanking DNA. Cell *36*:371-379.
75. **Kasahara, N., A.M. Dozy and Y.W. Kan.** 1994. Tissue-specific targeting of retroviral vectors through ligand-receptor interactions. Science *266*:1373-1376.
76. **Kioussis, D. and R. Festenstein.** 1997. Locus control regions: overcoming heterochromatin-induced gene activation in mammals. Curr. Opin. Genet. Dev. *7*:614-619.
77. **Klein, T.M. and S. Fitzpatrick-McElligott.** 1993. Particle bombardment:a universal approach for gene transfer to cells and tissues. Curr. Opin. Biotechnol. *4*:583-590.
78. **Ko, M.S., H. Nakauchi and N. Takahashi.** 1990. The dose dependence of glucocoti-

coid-inducible gene expression results from changes in the number of transcriptionally active templates. EMBO J. *9*:2835-2842.
79. **Kollen, W.J., F.M. Schembri, G.J. Gerwig, J.F. Vliegenthart, M.C. Glick and T.F. Scanlin.** 1999. Enhanced efficiency of lactosylated poly-L-lysine-mediated gene transfer into cystic fibrosis airway epithelial cells. Am. J. Respir. Cell. Mol. Biol. *20*:1081-1086.
80. **Kovesdi, I., D.E. Brough, J.T. Bruder and J. Wickham.** 1997. Adenoviral vectors for gene transfer. Curr. Opin. Biotechnol. *8*:583-589.
81. **Kriegler, M.** 1990. Gene Transfer and Gene Expression: A Laboratory Manual. Stockton Press, New York.
82. **Kukowska-Latallo, J.F., A.U. Bielinska, J. Johnson, R. Spindler, D.A. Tomalia and J.R. Baker.** 1996. Efficient transfer of genetic material into mammalian cells using Starbust polyamidoamine dendrimers. Proc. Natl. Acad. Sci. USA *93*:4897-4902.
83. **Kunzelmann, K., J.Y. Legendre, D.L. Knoell, L.C. Escobar, Z. Xu and D.C. Gruenert.** 1996. Gene targeting of CFTR DNA in CF epithelial cells. Gene Ther. *3*:859-867.
84. **Lasic, D.D. and N.S. Templeton.** 1996. Liposomes in gene therapy. Adv. Drug Deliv. Rev. *20*:221-266.
85. **Lee, N.H., J. Earle-Hughes and C.M. Fraser.** 1994. Agonist-mediated destabilization of m1 muscarinic acetylcholine receptor mRNA. Elements involved in mRNA stability are localized in the 3′-untranslated region. J. Biol. Chem. *269*:4291-4298.
86. **Lee, R.J. and L. Huang.** 1997. Lipidic vector system for gene transfer. Crit. Rev. Ther. Drug Carrier Syst. *14*:173-206.
87. **Legendre, J.Y. and F.C. Szoka, Jr.** 1992. Delivery of plasmid DNA into mammalian cell lines using pH-sensitive liposomes: comparison with cationic liposomes. Pharm. Res. *9*:1235-1242.
88. **Lei, D.C., K. Kunzelmann, T. Koslowsky, M.J. Yezzi, L.C. Escobar, Z.D. Xu, A.R. Ellison, J.M. Rommens et al.** 1996. Episomal expression of wild-type CFTR corrects cAMP-dependent chloride transport in respiratory epithelial cells. Gene Ther. *3*:427-436.
89. **Li, J.M., J.S. Han, Y. Huang, P.K. Tain, S.M. Qu, M. Yao, H.Q. Jiang, D.F. Wan, J.C. Luo, C.X. Gu and J.R. Gu.** 1999. A novel gene delivery system targeting cells expressing VEGF receptors. Cell Res. *9*:11-25.
90. **Lopata, M.A., D.W. Cleveland and B. Sollner-Webb.** 1984. High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment. Nucleic Acids Res. *12*:5707-5017.
91. **Lubovy, M., S. McCune, J.Y. Dong, J.F. Prchal, T.M. Townes and J.T. Prchal.** 1996. Stable transduction of recombinant adeno-associated virus into hematopoietic stem cells from normal and sickle cell patients. Biol. Blood Marrow Transplant *2*:24-30.
92. **Lundstrom, K.** 1997. Alphaviruses as expression vectors. Curr. Opin. Biotechnol. *8*: 578-582.
93. **Luthman, H. and G. Magnusson.** 1983. High efficiency polyoma DNA transfection of chloroquine treated cells. Nucleic Acids Res. *11*:1295-1308.
94. **Majors, J. and H.E. Varmus.** 1983. A small region of the mouse mammary tumor virus long terminal repeat confers glucocorticoid hormone regulation on a linked heterologous gene. Proc. Natl. Acad. Sci. USA *80*:5866-5870.
95. **Malone, R.W., P.L. Felgner and I.M. Verma.** 1989. Lipofectin-mediated RNA transfection. Proc. Natl. Acad. Sci. USA *86*:6077-6081.
96. **Matsui, H., L.G. Johnson, S.H. Randell and R.C. Boucher.** 1997. Loss of binding and entry of liposome-DNA complexes decreases transfection efficiency in differentiated airway epithelial cells. J. Biol. Chem. *272*:1117-1126.
97. **McNally, M.A., J.S. Lebkowski, T.B. Okarma and L.B. Lerch.** 1988. Optimizing electroporation parameters for a variety of human hematopoietic cell lines. BioTechniques *6*:882-885.
98. **Midoux, P., C. Mendes, A. Legrand, J. Raimond, R. Mayer, M. Monsigny and A.C. Roche.** 1993. Specific gene transfer mediated by lactosylated poly-L-lysine into hepatoma cells. Nucleic Acids Res. *21*:871-878.
99. **Midoux, P. and M. Monsigny.** 1999. Efficient gene transfer by histidylated polylysine/pDNA complexes. Bioconjug. Chem. *10*:406-411.
100. **Moss, B.** 1996. Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. Proc. Natl. Acad. Sci. USA *93*:11341-11348.
101. **Mullen, C.A., M. Kilstrup and R.M. Blaese.** 1992. Transfer of the bacterial gene for cytosine deaminase to mammalian cells confers lethal sensitivity to 5-fluorocytosine: a negative selection system. Proc. Natl. Acad. Sci. USA *89*:33-37.
102. **Nabel, E.G., D. Gordon, Z.Y. Yang, L. Xu, H. San, G.E. Plautz, B.Y. Wu, X. Gao et al.** 1992. Gene transfer *in vivo* with DNA-liposome complexes: lack of autoimmunity and gonadal localization. Hum. Gene Ther. *3*:649-656.
103. **Nabel, G.J., E.G. Nabel, Z.Y. Yang, B.A. Fox, G.E. Plautz, X. Gao, L. Huang, S. Shu, D. Gordon and A.E. Chang.** 1993. Direct gene transfer with DNA-liposome complexes in melanoma: expression, biologic activity, and lack of toxicity in humans. Proc. Natl. Acad. Sci. USA *90*:11307-11311.
104. **Nevaldine, B.H., R. Rizwana and P.J. Hahn.** 1999. Differential sensitivity of double minute chromosomes to hydroxyurea treatment in cultured methotrexate-resistant mouse cells. Mutat. Res. *406*:55-62.
105. **Palombo, F., A. Monciotti, A. Recchia, R. Cortese, G. Ciliberto and N. La Monica.** 1998. Site-specific integration in mammalian cells mediated by a new hybrid baculovirus-adeno-associated virus vector. J. Virol. *72*:5025-5034.
106. **Paoletti, E**. 1996. Applications of pox virus vectors to vaccination: an update. Proc. Natl. Acad. Sci. USA *93*:11349-11353.
107. **Peppel, K., J.M. Vinci and C. Baglioni.** 1991. The AU-rich sequences in the 3′ untranslated region mediate the increased turnover of interferon mRNA induced by glucocorticoids. J. Exp. Med. *173*:349-355.
108. **Perales, J.C., T. Ferkol, H. Beegen, O.D. Ratnoff and R.W. Hanson.** 1994. Gene transfer *in vivo*: sustained expression and regulation of genes introduced into the liver by receptor-targeted uptake. Proc. Natl. Acad. Sci. USA *91*:4086-4090.
109. **Pieroni, L., C. Fipaldini, A. Monciotti, D. Cimini, A. Sgura, E. Fattori, O. Epifano, R. Cortese et al.** 1998. Targeted integration of adeno-associated virus-derived plasmids in transfected human cells. Virology *249*: 249-259.
110. **Pollard, H., J.S. Remy, G. Loussouarn, S. Demolombe, J.P. Behr and D. Escande.** 1998. Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. J. Bio. Chem. *273*:7507-7011.
111. **Ponnazhagan, S., M.C. Yoder and A. Srivastava.** 1997. Adeno-associated virus type 2-mediated transduction of murine hematopoietic cells with long-term repopulating ability and sustained expression of a human globin gene *in vivo*. J. Virol. *71*: 3098-3104.
112. **Ponta, H., N. Kennedy, P. Skroch, N.E. Hynes and B. Groner.** 1985. Hormonal response region in the mouse mammary tumor virus long terminal repeat can be dissociated from proviral promoter and has enhancer properties. Proc. Natl. Acad. Sci. USA *82*: 1020-1024.
113. **Porteous, D.J., J.R. Dorin, G. McLachlan, H. Davidson-Smith, H. Davidson, B.J. Stevenson, A.D. Carothers, W.A. Wallace et al.** 1997. Evidence for safety and efficacy of DOTAP cationic liposome mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. Gene Ther. *4*:210-218.
114. **Possee, R.D.** 1997. Baculoviruses as expression vectors. Curr. Opin. Biotechnol. *8*:569-572.
115. **Potter, H.** 1993. Application of electroporation in recombinant DNA technology. Methods Enzymol. *217*:461-478.
116. **Potter, H., L. Weir and P. Leder.** 1984. Enhancer-dependent expression of human k immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation. Proc. Natl. Acad. Sci. USA *81*:7161-7165.
117. **Rabinowitz, J.E. and J. Samulski.** 1998. Adeno-associated virus expression systems for gene transfer. Curr. Opin. Biotechnol. *9*:470-475.
118. **Raptis, L., H.L. Brownell, K.L. Firth and L.W. Mackenzie.** 1994. A novel technique for the study of intercellular, junctional communication: electroporation of adherent cells on a partly conductive slide. DNA Cell Biol. *13*:963-975.
119. **Reddel, R.R., Y. Ke, B.I. Gerwin, M.G. McMenamin, J.F. Lechner, R.T. Su, D.E. Brash, J.B. Park et al.** 1988. Transforma-

tion of human bronchial epithelial cells by infection with SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. Cancer Res. *48*:1904-1909.
120. **Rossi, F.M. and H.M. Blau.** 1998. Recent advances in inducible gene expression systems. Curr. Opin. Biotechnol. *9*:451-456.
121. **Russell, D.W. and R.K. Hirata.** 1998. Human gene targeting by viral vectors. Nat. Genet. *18*:325-330.
122. **Saez, E., D. No, A. West and R.M. Evans.** 1997. Inducible gene expression in mammalian cells and transgenic mice. Curr. Opin. Biotechnol. *8*:608-616.
123. **Sambrook, J., E.F. Fritsch and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual. CSH Laboratory Press, Cold Spring Harbor, NY.
124. **Sanford, J.C., F.D. Smith and J.A. Russell.** 1993. Optimizing the biolistic process for different biological applications, p. 217. *In* R. Wu (Ed.), Methods in Enzymology. Academic Press, San Diego, CA.
125. **Schreier, H. and S.M. Sawyer.** 1996. Liposomal DNA vectors for cystic fibrosis gene therapy. Current applications, limitations, and future directions. Adv. Drug Deliv. Rev. *19*:73-87.
126. **Scotto, L. and R.K. Assoian.** 1993. A GC-rich domain with bifunctional effects on mRNA and protein levels: implications for control of transforming growth factor beta 1 expression. Mol. Cell. Biol. *13*:3588-3597.
127. **Sebestyen, M.G., J.J. Ludtke, M.C. Bassik, G. Zhang, V. Budker, E.A. Lukhtanov, J.E. Hagstrom and J.A. Wolff.** 1998. DNA vector chemistry: the covalent attachment of signal peptides to plasmid DNA. Nat. Biotechnol. *16*:80-85.
128. **Shen, Y.-M., R.R. Hirschhorn, W.E. Mercer, E. Surmacz, Y. Tsutsui, K.J. Soprano and R. Baserga.** 1982. Gene transfer: DNA microinjection compared with DNA transfection with a very high efficiency. Mol. Cell Biol. *2*:1145-1154.
129. **Shigekawa, K. and W.J. Dower.** 1988. Electroporation of eukaryotes and prokaryotes: a general approach to the introduction of macromolecules into cells. BioTechniques *6*:742-751.
130. **Shoji, I., H. Aizaki, H. Tani, K. Ishii, T. Chiba, I. Saito, T. Miyamura and Y. Matsuura.** 1997. Efficient gene transfer into various mammalian cells, including non-hepatic cells, by baculovirus vectors. J. Gen. Virol. *78*:2657-2664.
131. **Small, M.B., Y. Gluzman and H.L. Ozer.** 1982. Enhanced transformation of human fibroblasts by origin-defective SV40. Nature *296*:671-675.
132. **Sorscher, E.J., J.J. Logan, R.A. Frizzell, R.K. Lyrene, Z. Bebok, J.Y. Dong, M.D. Duvall, P.L. Felgner et al.** 1994. Gene therapy for cystic fibrosis using cationic liposome mediated gene transfer: a phase I trial of safety and efficacy in the nasal airway. Hum. Gene Ther. *5*:1259-1277.
133. **Strydom, S., P. Van Jaarsveld, E. Van Helden, M. Ariatti and A. Hawtrey.** 1993. Studies on the transfer of DNA into cells through use of avidin-polylysine conjugates complexed to biotinylated transferrin and DNA. J. Drug Target. *1*:165-174.
134. **Sudgen, B., K. Marsh and J. Yates.** 1985. A vector that replicates as a plasmid and can be efficiently selected in B-lymphocytes transformed by Epstein-Barr virus. Mol. Cell. Biol. *5*:410-413.
135. **Takagi, S. and M. Kimura.** 1997. Low-voltage electric discharge biolistic device. BioTechniques *23*:650-652.
136. **Tang, M.X., C.T. Redemann and F.C. Szoka, Jr.** 1996. *In vitro* gene delivery by degraded polyamidoamine dendrimers. Bioconjug. Chem. *7*:703-714.
137. **Tang, M.X. and F.C. Szoka.** 1998. Structure of polycation-DNA complexes and theories of DNA compactions, p. 27-51. *In* A.V. Kabanov, P.L. Felgner, L.W. Seymour (Eds.), Self-assembling complexes for gene delivery: from laboratory to clinical trial. John Wiley & Sons, New York.
138. **Templeton, N.S., D.D. Lasic, P.M. Frederik, H.H. Strey, D.D. Roberts and G.N. Pavlakis.** 1997. Improved DNA:liposome complexes for increased systemic delivery and gene expression. Nat. Biotechnol. *15*:647-652.
139. **Thomas, K.R., K.R. Folger and M.R. Capecchi.** 1986. High frequency targeting of genes to specific sites in the mammalian genome. Cell *44*:419-428.
140. **Thompson, T.A., M.N. Gould, J.K. Burkholder and N.-S. Yang.** 1993. Transient promoter activity in primary rat mammary epithelial cells evaluated using particle bombardment gene transfer. In Vitro Cell. Dev. Biol. *29A*:165-170.
141. **Toncheva, V., M.A. Wolfert, P.R. Dash, D. Oupicky, K. Ulbrich, L.W. Seymour and E.H. Schacht.** 1998. Novel vectors for gene delivery formed by self-assembly of DNA with poly(L-lysine) grafted with hydrophilic polymers. Biochim. Biophys. Acta *1380*: 354-368.
142. **Ueno, S., Y. Kotani, K. Kondoh, A. Sano, Y. Kakimoto and A.T. Campagnoni.** 1994. The 3′-untranslated region of mouse myelin basic protein gene increases the amount of mRNA in immortalized mouse oligodendrocytes. Biochem. Biophys. Res. Commun. *204*:1352-1357.
143. **Wagner, E., C. Plank, K. Zatloukal, M. Cotten and M.L. Birnstiel.** 1992. Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle. Proc. Natl. Acad. Sci. USA *89*:7934-7938.
144. **Weber, M., K. Moller, M. Welzeck and J. Schorr.** 1995. Effects of lipopolysaccharide on transfection efficiency in eukaryotic cells. BioTechniques *19*:930-940.
145. **Wils, P., V. Escriou, A. Warnery, F. Lacroix, D. Lagreaux, M. Ollivier, J. Crouzet, J.F. Mayaux and D. Scherman.** 1997. Efficient purification of plasmid DNA for gene transfer using triple-helix affinity chromatography. Gene Ther. *4*:323-330.
146. **Wilson, S.P. and A.L. Smith.** 1997. Addition of glycerol during DNA exposure enhances calcium phospate transfection. Anal. Biochem. *246*:148-150.
147. **Yang, N.S., J.K. Burkholder, B. Roberts, B. Martinell and D. McCabe.** 1990. *In vivo* and *in vitro* gene transfer to mammalian somatic cells by particle bombardment. Proc. Natl. Acad. Sci. USA *87*:9568-9572.
148. **Yang, N.S. and W.H. Sun.** 1995. Gene gun and other non-viral approaches for cancer gene therapy. Nat. Med. *1*:481-483.
149. **Yang, T., W.C. Heiser and J.M. Sedivy.** 1995. Efficient *in situ* electroporation of mammalian cells grown on microporous membranes. Nucleic Acids Res. *23*:2803-2810.
150. **Yates, J.L., N. Warren and B. Sudgen.** 1985. Stable replication of a plasmid derived from Epstein-Barr virus in various mammalian cells. Nature *313*:812-815.
151. **Yonemitsu, Y., Y. Kaneda, A. Muraishi, T. Yoshizuni, K. Sugimachi and K. Sueishi.** 1997. HVJ (Sendai virus)-cationic liposomes: a novel and potentially effective liposome-mediated technique for gene transfer to the airway epithelium. Gene Ther. *4*:631-638.
152. **Zabner, J., L.A. Coulture, R.J. Gregory, S.M. Graham, A.E. Smith and M.J. Welsh.** 1993. Adenovirus-mediated gene transfer transiently corrects the chloride transport defect in nasal epithelia of patients with cystic fibrosis. Cell *75*:207-216.
153. **Zabner, J., A.J. Fasbender, T. Moninger, K.A. Poellinger and M.J. Welsh.** 1995. Cellular and molecular barriers to gene transfer by a cationic lipid. J. Biol. Chem. *270*:18997-19007.
154. **Zelenin, A.V., A.A. Alimov, I.A. Zelenina, M.L. Semenova, M.A. Rodova, B.K. Chernov and V.A. Kolesnikov.** 1993. Transfer of foreign DNA into the cells of developing mouse embryos by microprojectile bombardment. FEBS Lett. *315*:29-32.
155. **Zelenin, A.V., A.V. Titomirov and V.A. Kolesnikov.** 1989. Genetic transformation of mouse cultured cells with the help of high-velocity mechanical DNA injection. FEBS Lett. *244*:65-67.
156. **Zelphati, O. and F.C. Szoka, Jr.** 1996. Intracellular distribution and mechanism of delivery of oligonucleotides mediated by cationic lipids. Pharm. Res. *13*:1367-1372.
157. **Zheng, Q.A. and D.C. Chang.** 1991. High-efficiency gene transfection by *in situ* electroporation of cultured cells. Biochim. Biophys. Acta *1088*:104-110.

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