Review

Nonviral Approaches Satisfying Various Requirements for Effective *in Vivo* Gene Therapy

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Development of an efficient method of gene introduction to target cells is the key issue in treating genetic and acquired diseases by *in vivo* gene therapy. Although various nonviral approaches have been developed, any method needs to be optimized in terms of the target disease and transgene product. The most important information required is (i) target cell-specificity of gene transfer, (ii) efficiency, (iii) duration of transgene expression, and (iv) the number of transfected cells following *in vivo* application of a vector. These characteristics are determined by the properties of the vector used, as well as the route of its administration, biodistribution, interaction with biological components and the nature of the target cells. Cell-specific gene transfer can be achieved by controlling the tissue disposition of plasmid DNA (pDNA), although the interaction of the pDNA complex with biological components might limit the specificity. Various approaches have been reported to increase the efficiency of transgene expression, from cationic lipids/polymers to physical stimuli, but some of those are ineffective under *in vivo* conditions. The duration of transgene expression is a complex function involving variables including the cell type, transfection method, and plasmid construct. Immune response often reduces the level and duration of transgene expression. In addition, the number of transfected cells is important, especially in cases in which the therapeutic protein localizes within the target cells. Successful clinical application of nonviral gene delivery methods rely on the development of such methods optimized for a particular target disease.

Key words nonviral vector; targeted delivery; large-volume injection; endosomal release; plasmid construct

The *in vivo* gene transfer profile required for effective gene therapy depends on the target disease. Possible important features include: (i) target cell-specificity of gene transfer, (ii) the efficiency, (iii) duration of transgene expression, and (iv) the number of transfected cells, following *in vivo* application of a vector (carrier) system (Fig. 1). These features are determined not only by the properties of the vector used, but also by the nature of the target cells, route and method of administration, and the biodistribution of the vector. Therefore, increased *in vitro* transfection efficiency does not always lead to improved *in vivo* gene transfer.

Although nonviral vectors are believed to be less effective in gene transfer than viral ones, some approaches were associated with sufficiently high levels of transgene expression to allow treatment of certain diseases.[1,2] Since nonviral vectors possess several advantages in that they are less toxic, less immunogenic, and easier to prepare, they could be ideal methods for *in vivo* gene therapy. So far, a variety of nonviral delivery methods have been developed[3] and some of them are presently undergoing clinical trials.[4,5] In addition, the importance of plasmid constructs for transgene expression has been realized and some disadvantages of nonviral vectors, such as the short duration of transgene expression, can be overcome by optimizing the construct structure. Therefore, to achieve efficient *in vivo* gene therapy, one should optimize the overall properties of any gene delivery and transfer approach and this may include (i) the solute for administration, (ii) administration route, (iii) design of plasmid construct, and (iv) selection of target cells. In this review, we discuss all the factors that are important for determining the efficacy of *in vivo* gene transfer in gene replacement therapy for inherent genetic diseases.

TRANSGENE EXPRESSION PROFILES REQUIRED FOR A SPECIFIC DISEASE

For an effective *in vivo* gene therapy, the major properties of a vector are persistent and high-level transgene expression with minimal toxic and immunological side-effects. Although such characteristics are generally required for a vector system, they depend on the target diseases and transgene products. Therefore, it is not likely that there will be a single vector system suitable for all applications.

Localization of transgene products, *i.e.*, inside or outside transfected cells, is one of the most important factors in any consideration of these characteristics. Table 1 summarizes the required characteristics of *in vivo* gene transfer. If a transgene product is a protein secreted into the circulating blood where it exhibits biological activity, various cells could be used as platforms for its synthesis. This is the case for blood coagulation factors XIII and XI, and erythropoietin. In actual fact, in the gene therapy approaches for hemophilia, not only hepatocytes[6] that produce the coagulation factors in healthy subjects, but also other cells such as fibroblasts[7] and muscle cells[8,9] have been investigated as target cells producing those factors. Further studies are needed to identify the type of cells most appropriate for *in vivo* gene transfer for a specific disease whose key protein is one that is secreted. The properties of cells, such as their location, life span, and blood flow rate, will determine the efficacy of gene transfer.

In the case of secreted proteins, major concerns are the level and persistence of transgene expression. Although the number of transfected cells is also considered an important factor for guaranteeing less inter-individual variation, this is not so critical. For hemophilia B, only 1—2% of the normal level of factor IX is believed to be effective for severe hemophiliacs to exhibit a substantial improvement in clinical phenotype.[10]

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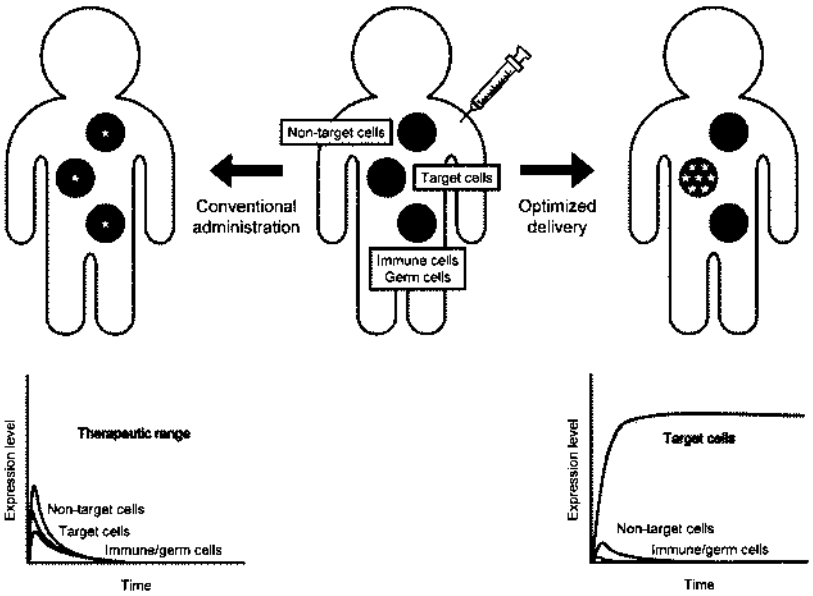

Fig. 1. The Goal of *in Vivo* Gene Therapy

Following *in vivo* administration of a vector, gene transfer (indicated as stars) should occur in many target cells without gene transfer into non-target, immune or germ cells (upper panel). Optimized delivery will be required. The level of transgene expression in the target cells should be in the therapeutic range of the transgene product, and the expression should be prolonged in the cells (lower panel).

Table 1. Characteristics Required for *in Vivo* Gene Transfer

	Localization of transgene product	
	Central (blood) circulation (Extracellular space)	Intracellular space
Target tissue/cell	Possibly altered to other tissues/cells (Muscle cells, *etc.*)	Strictly restricted
Number of transfected cells	Marginally important	As many as possible (Functional recovery at cellular level)
Level of transgene expression	Dependent on target disease/protein	Dependent on target disease/protein

Table 2. Target Cell (Tissue)-Specific *in Vivo* Gene Transfer Approaches

Target	Vector	Method	References
Skeletal muscle	Naked pDNA	Local injection	12
	Naked pDNA	Intravascular injection	13—15
Heart muscle	Naked pDNA	Local injection	16
Liver (hepatocytes,	Naked pDNA	Local injection	17
liver nonparenchymal	Naked pDNA	Intraportal injection	18
cells)	Glycosylated polyplex	Intravenous injection	19—23
	Glycosylated lipoplex	Intraportal (or intravenous) injection	24, 25
Brain	Naked pDNA	Local injection	26, 27
Skin	Naked pDNA	Local injection	28
Urological organs	Naked pDNA	Local injection	29
Thyroid	Naked pDNA	Local injection	30
Tumor	Naked pDNA	Local injection	31—33
	Transferrin-conjugated polyplex	Local (or systemic) administration	34
Lung (airway cells)	Lipoplex	Intratracheal administration	35, 36
	Antibody-conjugated polyplex	Intratracheal administration	37
Lung (vascular endothelial cells)	Lipoplex	Intravenous injection	38—40
	Antibody-conjugated polyplex	Intravenous injection	41

On the other hand, intracellular proteins, such as dystrophin and cystic fibrosis transmembrane conductance regulator protein, need to be synthesized within target cells where they are needed to maintain cellular functions. In such cases, in addition to the level and persistence of transgene expression, the number of transfected cells is important as far as obtaining therapeutic benefit is concerned. Uniform transgene expression might be required for gene therapy in patients with dystrophin-deficiency, *i.e.*, Duchenne muscular dystrophy.[11)]

TARGET CELL-SPECIFIC GENE TRANSFER

Target cell-specific gene transfer is important for various aspects of *in vivo* gene therapy. Transgene expression in non-target cells could lead to side-effects. In particular, the uptake of pDNA by immune cells is a major obstacle to nonviral gene therapy approaches, because the uptake sometimes triggers a severe immune reaction which reduces the level and duration of transgene expression in target cells. To achieve target cell-specific (selective) gene transfer, a variety of approaches have been examined from the selection of the administration route of pDNA to the use of tissue-specific promoters (Table 2).

Selection of Administration Route The route of administration is a key issue for targeted delivery of pharmaceuticals. Pharmacokinetic considerations clearly show that drug

delivery to a target is always greater with the intraarterial route than the intravenous one.[42] Topical administration of drug into the skin, muscle, trachea, and oral cavity is sometimes used to obtain local or systemic effects, although it is difficult to retain the drug around the injection site because of its absorption into the systemic circulation.

Local Administration Compared with conventional, low-molecular weight drugs, pDNA is a huge molecule with a molecular weight of at least 2000 kDa. This greatly restricts its diffusion within the tissue where pDNA is injected, because the diffusion as well as the absorption of an injectant into the circulation is largely governed by its molecular weight.[43] Furthermore, the complex formation of pDNA with cationic liposomes limits the diffusion within tissues due to the increased size and net charge.[33,44] Therefore, pDNA locally injected into tissues, such as muscle and skin, may only transfect cells around the injection site, which makes *in vivo* gene transfer tissue-specific.

In 1990, Wolff *et al.*[12] reported that transgene expression in skeletal muscle can be achieved by a simple intramuscular injection of naked pDNA. Since then, other tissues, including the heart muscle,[16] liver,[17] brain,[26,27] skin,[28] urological organs,[29] thyroid[30] and tumors,[31—33] have been transfected by direct injection of pDNA into the interstitial space of the corresponding tissue. The disposition of locally injected pDNA depends on the structure of the tissue injected, the blood flow rate, and other factors, which are still little understood. The volume of injectant has been suggested to be one of the factors that affect gene transfer following local administration.

Local administration into a cavity is another route for tissue-specific gene transfer. For gene transfer into the airway cells in the lung, the intratracheal route is often attempted using naked pDNA or pDNA/cationic liposome complexes.[35,36] Intestinal epithelial cells are attractive targets because of their easy accessibility by oral or rectal administration of pDNA, although gene transfer into these cells is quite difficult with nonviral vectors.[45]

Intravascular Administration Intraarterial administration of pDNA ensures its initial encounter with a target tissue, which may result in target-selective *in vivo* gene transfer. Intravascular delivery of pDNA to target tissues has been reported in various tissues with a range of nonviral vectors. Intraportal injection of naked pDNA in a large-volume, hypertonic, solution efficiently transfects about 1% of hepatocytes throughout the entire liver with a few nonparenchymal cells in mice.[18] A similar approach has been applied to skeletal muscle of the mouse,[14] rat[13] and rhesus monkey.[15] In these studies, transfected cells were spread within the target muscle.

pDNA complex with a cell-specific vector can be injected into vessels leading to the target tissue to ensure cell-specific delivery. Kawakami *et al.*[24] reported that intraportal injection of pDNA/galactosylated cationic liposome complexes results in the highest transgene expression in the liver, among the tissues examined. When injected intravenously, the same complex showed the highest expression in the lung.

A major barrier to this approach is the transendothelial transport of pDNA, if the target cells are behind the endothelial cells and basement membranes of capillaries. The structure of capillary walls varies depending on the tissue involved and can be divided into three general types: continuous, fenestrated, and discontinuous endothelium.[46,47] pDNA can only pass through a vascular wall composed of discontinuous endothelial cells under normal conditions. Discontinuous endothelium only exists in the liver, spleen and bone marrow, and has gaps of 30—500 nm between the endothelial cells, and there is little or no basement membrane. Only a relatively small pDNA complex can pass through the blood vessels and directly interact with parenchymal cells. There are several approaches to improve the transport of molecules across blood vessels by increasing the vascular permeability. The continuous-type of endothelial cells in the brain (blood–brain barrier) severely limits the permeability of water-soluble compounds. Therefore, large-volume, hypertonic solutions are often used in this route of administration to open the tight junctions of the endothelial cells.[48] The alteration of vascular permeability using a vasodilator has also been examined.[49,50] Such modification of vascular permeability is a requirement for *in vivo* gene transfer into tissue parenchyma following intravascular administration of pDNA.

On the other hand, there is no need for pDNA to extravasate if target cells can be accessed by intravascular pDNA. Intravascular delivery of a pDNA complex mostly results in transgene expression in endothelial cells.[38] The endothelial cells in the lung are easily transfected by an intravenous cationic pDNA complex and, therefore, this approach is sometimes used as a lung-specific gene transfer method.[39] However, the biodistribution of pDNA/cationic liposome complex is influenced by the composition of the cationic liposomes, resulting in large variations in gene transfer *in vivo*.[40,51,52]

Targeted Delivery by Nonviral Vectors The tissue disposition of a compound is determined by its interaction with blood and tissue components, which depends on the physicochemical properties of the compound and the anatomical and physiological characteristics of tissues.[53,54] pDNA itself is a huge macromolecule with a strong negative charge. The uptake by Kupffer cells *via* a scavenger receptor-like mechanism largely determines its biodistribution following intravascular administration.[55—57] Therefore, to control the biodistribution of pDNA, its physicochemical properties also need to be controlled.

Complex formation with positively charged molecules is an easy way to reduce the negative charge of pDNA. A net positive charge on the pDNA complex facilitates the interaction of the complex with cells, resulting in gene transfer into the cells. Although such cationic charge-mediated gene transfer could be used for tissue-selective gene transfer into the lung, cellular uptake of such a cationic pDNA complex is a nonspecific process.

In an attempt to improve the cell-specificity of gene transfer, homing devices have been introduced onto vectors. They include: asialoglycoproteins,[19] carbohydrates,[20,21,25,58,59] transferrin,[34,60] antibodies,[37,41,61] and lung surfactant proteins.[62] These ligands offer increased affinity of the pDNA complex with target cells, but do not guarantee target cell-specific gene transfer. Barriers in the biodistribution processes of pDNA reduce the ratio of transgene expression in target and non-target cells.[3] Therefore, control of the overall characteristics of a pDNA complex is required for selective delivery of pDNA to target cells. Figure 2 summarizes the steps for *in vivo* cell-specific gene transfer encountered by a pDNA com-

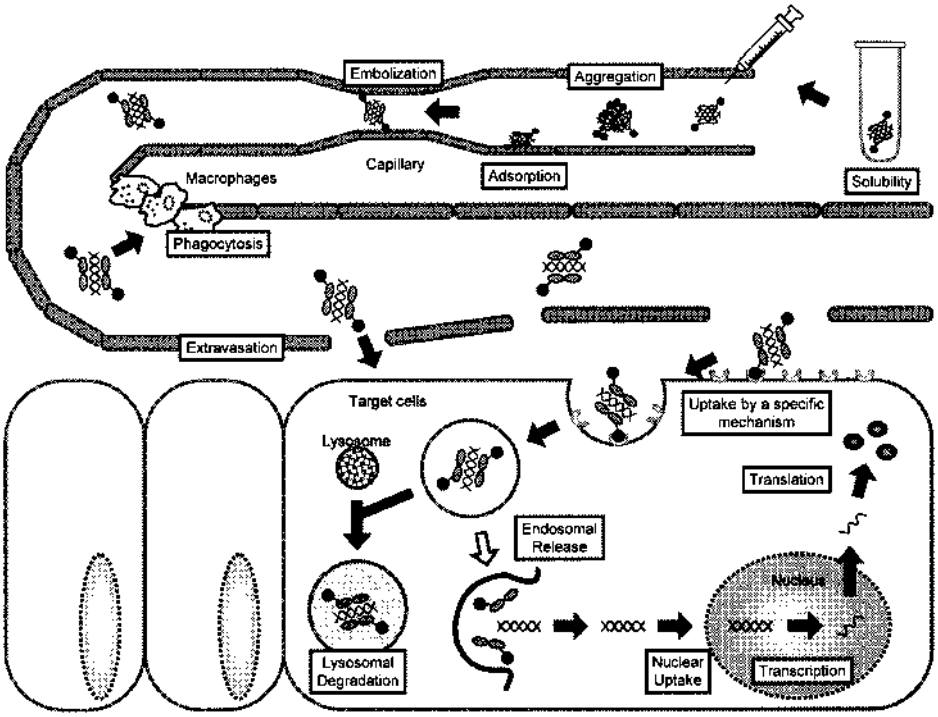

Fig. 2. Fate of Targeted Delivery of a pDNA/Nonviral Carrier Complex

The complex should be soluble in the injection solution, avoid aggregation, non-specific binding to tissues, embolization, and phagocytosis, extravasate (when target cells are tissue parenchymal cells), be recognized by the specific molecule, be internalized, escape from endosomal/lysosomal degradation, be transported into the nucleus, and be transcribed and translated.

plex with a homing device following intravascular administration.

To achieve cell-specific gene transfer to hepatocytes or liver nonparenchymal cells, we synthesized glycosylated poly(L-lysine) (PLL) derivatives and prepared a pDNA complex for targeted delivery to cells possessing carbohydrate receptors.[21,22] Well-designed pDNA/galactosylated PLL (Gal-PLL) complexes were delivered to the liver in up to 80% of the injected dose. Separation of the liver cells revealed that such complexes were preferentially taken up by hepatocytes, the cells possessing asialoglycoprotein receptors. When the transfection efficiency of the pDNA/galactosylated polymer complex was boosted by the use of a fusogenic peptide (an acid-sensitive peptide designed based on the amino-terminal of influenza virus hemagglutinin subunit HA-2 or mHA2), the amount of transgene product in the hepatocytes accounted for over 95% of the total amount in all the tissues examined,[23] indicating the success of this target cell-specific gene transfer *in vivo*. Figure 3 summarizes the tissue disposition of pDNA and transgene expression following intravenous injection of pDNA complexed with a hepatocyte-targeted polymeric carrier in mice.[23]

Tissue (Cell)-Specific Promoters Use of a tissue-specific promoter is another strategy for achieving target cell-specific gene transfer. Some promoters are active only in a specific type of cells, which offers high specificity of transgene expression following *in vivo* administration of pDNA. However, a major drawback of tissue-specific promoters is the weakness of their transduction efficiency. Therefore, tissue-specific promoters have scarcely been used for nonviral approaches whose transfection efficiency is generally much less than that of viral vectors. Herweijer *et al.*[63] have reported that a liver-specific albumin promoter is expressed at much lower levels of transgene product than viral ones, such as cytomegalovirus (CMV) promoter.

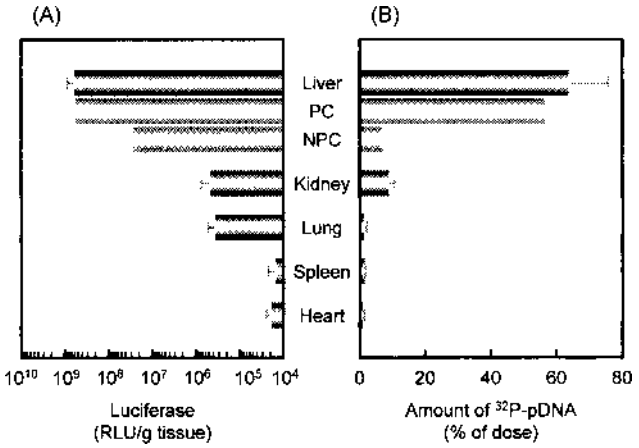

Fig. 3. (A) Transgene Expression (Luciferase) in Mouse Organs Following Intravenous Injection of pDNA/Galactosylated Poly(L-ornithine)-mHA2 Complex

At 6 h after injection, mice were killed and the luciferase activity of tissue extracts was analyzed.

(B) Tissue Distribution of ^{32}P-Radioactivity at 30 min Following Intravenous Injection of ^{32}P-pDNA/Galactosylated Poly(L-ornithine)-mHA2 Complex in Mice

Hepatocytes (PC) and liver nonparenchymal cells (NPC), such as Kupffer and endothelial cells, were separated by differential centrifugation. Contribution of each cell type to the total liver values was calculated based on the activities in both cell types and their numbers in the liver (1.25×10^8 cells/g liver for hepatocytes and 0.65×10^8 for NPC).

LEVEL OF TRANSGENE EXPRESSION

The level of transgene expression in target cells is directly correlated with the efficacy of an *in vivo* gene transfer approach for a particular disease, which depends mostly on the strength of the promoter and the amount of pDNA delivered into the nucleus of the target cells. Generally, the level of transgene expression is determined by the number of transfected cells and the number of copies of pDNA taken up into each cell.[64] In most cases of gene replacement therapy, nonviral approaches hardly achieve sufficient transgene expression to obtain any therapeutic effects. Although the level of transgene products is generally less than that required for treatment, too high an expression may induce disorders that result from excessive production. Recently, Zhu *et al.*[65] reported that overexpression of γ-sarcoglycan induced severe muscular dystrophy in normal mice, suggesting that the level of transgene expression should be carefully controlled in replacement gene therapy to ensure safety during human clinical trials.

Intramuscular injection of naked pDNA, one of most thoroughly studied nonviral gene transfer methods, results in target-selective, prolonged, but very weak transgene expression, even with very powerful viral promoters such as CMV. Therefore, a major challenge to increase the level of transgene expression relies largely on improving the delivery of pDNA to the nucleus. Endosomal release, stabilization of pDNA within the cytoplasm,[66—68] and nuclear transport[69—71] are the major processes governing the efficiency of gene transfer.

Increasing the pDNA in the Cytoplasm/Nucleus. Molecules Altering the Intracellular Disposition of pDNA After endocytosis, the pDNA complex is largely retained in perinuclear endosomes/lysosomes, which limits its transport into the cytoplasm/nucleus and is a major barrier for eventual transfection. Therefore, endosomal release of pDNA is a target for efficient gene transfer (Fig. 2).

One of the strategies involves using fusogenic lipids or peptides to disrupt the endosomal membrane. Dioleoylphosphatidylethanolamine (DOPE) is sometimes employed as a fusogenic helper lipid in pDNA/cationic liposome complexes. When cationic lipids bind to anionic lipids in the cellular membrane, phase separation, which initiates the inverted hexagonal phase formation, and membrane destabilization may occur. On the other hand, fusogenic peptides derived from fusion-active viruses can form pores in the lipid membrane[72] but some peptides can only do this at an acidic pH. They destabilize the endosomal membrane by the reduction in pH followed by cytoplasmic release of endosomal pDNA complex. The transfection efficiency into mouse liver was enhanced by the covalent binding of a fusogenic peptide to a hepatocyte–specific pDNA complex.[23]

Another approach involves the use of a vector with a high buffering capacity and the ability to swell when protonated. Such a system, *e.g.* polyethyleneimine (PEI),[73] reduces the acidification of the endosome, induces a large inflow of ions and water, subsequently leading to rupture of the endosomal membrane. Histidine-[74,75] or imidazole-[76] containing polymers allow efficient release of the pDNA complex from endocytotic vesicles into the cytoplasm.

Anionic lipids can displace pDNA in a pDNA/cationic liposome complex.[77] It has been hypothesized that anionic lipids, which are normally found in the endosomal membrane, efficiently dissociate the cationic lipids from the complex and release pDNA into the cytoplasm. Sakurai *et al.*[78] reported that a highly cationic complex of pDNA and *N*-(1-2,3-dioleyloxypropyl)-*N*,*N*,*N*-trimethylammonium (DOTMA)/DOPE liposomes had difficulty in being released from endocytotic vesicles compared with a weakly cationic one, resulting in less transgene expression in cultured cells.

Nuclear localization signal (NLS) peptide has been used in an attempt to deliver pDNA into the nucleus, *via* electrostatic binding of pDNA to cationic NLS-containing molecules. This active transport of pDNA into the nucleus is important for the efficiency of gene transfer since pDNA is unstable in the cytoplasm. Lechardeur *et al.*[68] reported that microinjected pDNA is rapidly degraded in the cytoplasm with an apparent half-life of 50—90 min. Cytoplasmic nuclease might be responsible for the degradation.

Physical and Electrical Approaches pDNA can be shot into target tissues or cells by a gene gun, which uses gold particles coated with pDNA.[79] This approach allows pDNA to directly penetrate through cell membranes into the cytoplasm or even nuclei, and to bypass the endosomes/lysosomes, thus avoiding enzymatic degradation. Skin, liver and skeletal muscle have been successfully transfected after surgical exposure of the tissue.[79—81] If shallow penetration of bombarded pDNA into tissues is a problem, jet-injection of pDNA may be the solution.[82,83]

The application of short and intense electrical pulses can produce reversible permeability of cell membranes.[84,85] Extracellular molecules can enter the cell *via* the pores created by the electrical pulses. Electrophoretic and electroosmotic transport under the influence of an electrical field may also facilitate the transport of charged molecules, like pDNA, across the membrane.[86] After initial permeation, the pores close and pDNA is trapped within the cell. Therefore, electroporation following a local injection of pDNA increases the chance of pDNA uptake by cells adjacent to the injection site. *In vivo* electroporation generally increases transgene expression up to 1000-fold compared with injection of naked pDNA without electroporation, in tissues such as skin,[87] liver,[88] melanoma,[89] and muscle.[90] The application of ultrasound has also been investigated in an attempt to improve *in vivo* transgene expression, and this facilitated non-endocytotic uptake of pDNA into cells.[91]

Pressure produced by a large volume of solution also facilitates cellular uptake of pDNA, probably *via* a non-endocytotic process. Liu *et al.*[92] and Zhang *et al.*[93] reported that a rapid injection of a large volume of naked pDNA solution (for example, 1.6 ml saline solution for a 20 g mouse, which is almost equivalent to the total blood volume of the animal) *via* the mouse tail vein can induce efficient gene transfer in internal organs including the lung, spleen, heart, kidney and liver, with the highest level observed in the liver. The mechanisms of gene transfer by this method are not fully understood, but the hydrostatic pressure seems to force pDNA into the liver.[92] Kobayashi *et al.*[94] showed that not only pDNA but also proteins and other macromolecules can be delivered to liver cells by the same procedure, supporting a nonspecific mechanism for the cellular uptake of pDNA by this approach. A large-volume injection of naked pDNA has also

been applied to tissue-selective gene transfer by creating a closed loop of blood vessels into hepatocytes[18] and skeletal muscle.[13—15]

Promoter Strength The level of transgene expression is, of course, highly dependent on the strength of the promoters. In most nonviral approaches, strong viral promoters, such as simian virus 40 (SV40) early or late promoter and CMV immediate early promoter, have been used to compensate for the weaker potential of nonviral vectors compared with viral ones as far as transfection activity is concerned. To further increase the efficiency of transgene expression, transcriptional regulatory elements of pDNA have been examined.[95]

DURATION OF TRANSGENE EXPRESSION

Nonviral delivery of pDNA usually does not undergo chromosomal integration, which would be a factor determining the duration of transgene expression. Therefore, the degradation of pDNA and/or the transcriptional inactivation of the promoter are the major causes for such loss. One approach to prolong transgene expression is to continuously supply pDNA to the target cells by controlled release of pDNA. Improved stability of pDNA might also prolong the expression. The plasmid construct needs to be optimized because silencing promoters and/or the CpG motif-mediated immune reaction would limit transgene expression. In addition, the life span of cells, especially transfected ones, is another factor governing the persistence of expression.

Controlled Release and Stabilization of pDNA Controlled release of bioactive pDNA can be achieved by encapsulating it into biodegradable matrices. To ensure sustained release and expression, pDNA should be protected from degradation before and after its release from matrices. A low concentration of pDNA continuously released from a formulation could be readily degraded by nucleases. Then the pDNA needs to find a way to the nucleus of the target cell.

Several controlled gene delivery systems have been developed using various polymers, such as gelatin,[96] atelocollagen,[97] polylactic-polyglycolic acid (PLGA) polymers.[98,99] Ochiya *et al.*[97] prepared pDNA in a cylindrical formulation composed of atelocollagen (Minipellet) and succeeded in obtaining prolonged release of active pDNA. When administered intramuscularly into mice, pDNA in the pellet formulation exhibited an increasing pharmacological activity up to 60 d, which was much longer than that obtained following administration of naked pDNA. Instead of naked pDNA, its complex with cationic carriers is sometimes used to enhance the stability of pDNA during the preparation of formulations and to protect pDNA from degradation by nucleases.[98]

Another factor determining the duration is the loss of pDNA from the transfected cells since DNA is not stable in the cytoplasm. Lechardeur *et al.*[68] have reported that microinjected pDNA is rapidly degraded in the cytoplasm with an apparent half-life of 50—90 min. Cytoplasmic nuclease might be responsible for this degradation so that microinjection of free pDNA directly into the nucleus bypasses the cytoplasmic degradation and results in a much higher level of gene expression than microinjection of pDNA into the cytoplasm.[66,67]

Plasmid Construct Transcriptional regulatory elements determine various parameters, such as cell-specificity, efficiency and duration of transgene expression. So far, promoter/enhancer elements have been most extensively studied for their effects on gene transfer.[63,100] For nonviral gene delivery approaches, very strong promoter/enhancers have been widely used to obtain high levels of transgene expression, such as CMV immediate-early promoter and SV40 early promoter, but these promoters can be easily attenuated. In addition, differences in the properties of pDNA produced in bacteria from mammalian DNA can induce immune reactions, which often reduce the duration as well as the level of transgene expression.

CpG Motif Compared with DNA of eukaryotic cells (frequency of *ca.* 1 : 64), bacterial genomic DNA contains a higher frequency of the dinucleotide sequence CpG (1 : 16).[101] Prokaryotic DNA is relatively unmethylated compared with the eukaryotic form, in which approximately 80% of the cytosines are methylated, a modification known to eliminate immunostimulation. These differences allow the mammalian immune system to recognize and respond to foreign DNA of bacterial origin,[102] such as pDNA derived from bacterial sources. Although such stimulation of the immune system is desirable for cancer immunotherapy or vaccination, it can be unfavorable for many gene therapy indications.[103] In addition to the immunostimulatory CpG motifs, neutralizing CpG sequences that can neutralize the immune activating properties of the stimulatory motifs has been reported.[104]

The inflammatory response after the injection of naked pDNA into skeletal muscle is actually related to the CpG motifs in the pDNA.[105] The immune reaction against pDNA is amplified by the use of cationic liposome and high levels of cytokines, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α, have been detected after their intratracheal instillation or intravenous injection.[39,106,107] These cytokines are not only toxic to the treated animals but also inhibit transgene expression. The presence of stimulatory CpG motifs in pDNA seems to be directly correlated with cytokine production.[108] Therefore, this property of CpG is a significant obstacle to replacement gene therapy.

Yew *et al.*[108] eliminated 270 of 526 CpG dinucleotides in a reporter pDNA, either by eliminating nonessential regions within the plasmid backbone or by site-directed mutagenesis. A CpG-reduced pDNA was then found to be significantly less immunostimulatory than the original pDNA. Tan *et al.*[109] reported that an intraperitoneal injection of dexamethasone suppressed cationic lipid–protamine–pDNA complex-induced cytokine production and led to significantly higher and prolonged transgene expression. Recently, a PCR amplified fragment has been examined with a view to avoiding immune responses against CpG motifs of all nonessential regions within the plasmid backbone.[110]

Promoter Inactivation The transcriptional activity of CMV promoter is very powerful, but likely to be inactivated over time in cells such as hepatocytes.[63,100,111] Such inactivation of the CMV promoter has also been observed in transgenic mice.[112] Cytokines, such as TNF-α and IFN-γ, are involved in the inactivation of viral promoters.[113] Yew *et al.*[114] reported that a hybrid promoter consisting of CMV enhancer and human *UBB* (encoding ubiquitin B) promoter can prolong transgene expression up to three months in the lung and 42 d in the liver. Herweijer *et al.*[63] showed that mouse albumin promoter prolonged transgene expression in the liver,

Table 3. Characteristics of Typical Target Cells for *in Vivo* Gene Transfer

Type of cell	Intestinal epithelial cells	Hepatocytes	Myotubes
Life span	<1 week	>5 months	Extremely long
Accessibility	Easy (Oral)	Difficult	Easy (Topical)
Blood flow[a)]	Abundant (36 ml/h/g tissue)	Abundant (51 ml/h/g tissue)	Moderate (3 ml/h/g tissue)
Specific properties	Thick mucus layer	Specific receptors	Developed extracellular matrix

a) Values are calculated based on reported values.[115)]

compared with viral promoters like CMV.

Lifespan of (Transfected) Cells If gene transfer occurs in differentiated cells, such as the lung epithelial or endothelial cells, hepatocytes, or skeletal myotubes, which is the most common for *in vivo* gene transfer by nonviral methods, the life-span of transfected cells limits sustained transgene expression. The lifespan of cells varies greatly depending on the type of cells: from less than 1 week for intestinal epithelial cells to life-long for nerve cells and skeletal and cardiac muscle cells. Table 3 compares the characteristics of potential target cells for *in vivo* gene transfer: intestinal epithelial cells, hepatocytes and myotubes.

Transgene expression in muscle cells can persist for several months after intramuscular injection,[116,117)] indicating that a considerable fraction of the transfected cells survive for a long period. However, administration by this procedure may induce cell death. A large-volume injection of naked pDNA has resulted in an extremely high, but short, transgene expression,[92,93)] and the procedure induced hepatocellular damage.[63)] The loss of gene-expressing cells through an apoptotic process has been reported to occur in the lung following systemic administration of a cationic lipid–protamine–pDNA complex.[39)]

NUMBER OF TRANSFECTED CELLS

As discussed in Table 1, the number of transfected cells is very important for cases in which transgene products localize within transfected cells *e.g.* dystrophin.[11)] Although an intramuscular injection of naked pDNA results in relatively efficient transgene expression, transfected cells localize near the injection site and the efficiency is approximately 1% that of muscle fibers.[12,116)] The disposition of transfected cells is limited to the region around the injection site, about 10 mm in diameter in dog muscle.[118)] Direct injection into the liver also resulted in transgene expression over an area of 5 mm around the injection sites.[17)] Limited disposition of pDNA injected locally is a major reason for this highly localized gene transfer (Fig. 4). When complexed with pDNA, cationic liposomes reduced the spread of pDNA in tumors following direct injection into the tissues.[44)] Although the disposition of locally injected pDNA can be partially improved by using polyvinyl pyrrolidone,[119)] hyaluronidase,[64,120)] or electroporation,[90,121)] these effects are limited to areas adjacent to the injection site.

Several approaches have been examined to overcome this hurdle. Injection of a sucrose solution prior to pDNA injection has been shown to force the generation of spaces between muscle fibers, thereby improving the disposition of pDNA throughout the muscle.[122)] Digestion of extracellular matrix by proteases, such as collagenase and hyaluronidase, also increases gene transfer by naked pDNA or AAV vector,[64,120)] and so could improve the disposition of those vectors within the muscle tissue injected. Molecules inducing muscle regeneration, such as bupivacaine, are also effective.[123,124)]

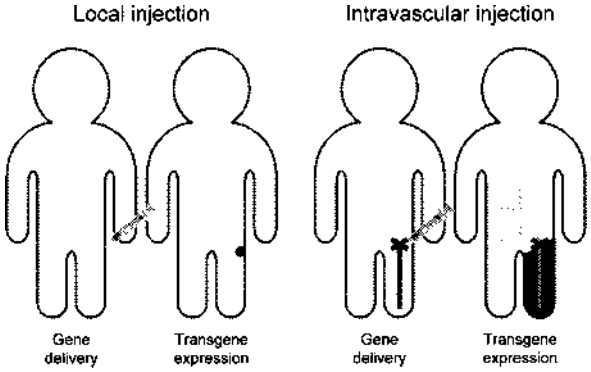

Fig. 4. Disposition and Gene Transfer of Locally or Intravascularly Injected pDNA

The disposition of pDNA injected into tissues is normally limited and, therefore, gene transfer will occur in cells very close to the injection site. On the other hand, intravascularly injected pDNA can distribute throughout tissues and have the opportunity to achieve gene transfer in a large number of cells. Pale lines represent blood vessels and solid areas represent cells expressing transgene products.

Another strategy for *in vivo* gene transfer to a large number of cells is intravascular delivery of pDNA. Due to the well-developed vasculature within tissues, such as internal organs, skeletal muscle and brain, pDNA can be delivered to the vicinity of a number of parenchymal cells of these tissues. Rapid injection of a large volume of naked pDNA solution resulted in very high transgene expression in the liver. Transfected cells were spread throughout the liver and approximately 40% of them expressed a transgene product.[92)] A similar approach to skeletal muscle was also effective in inducing transgene expression in many myotubes in various species.[13—15)] In monkeys, an average of 6.9% of myofibers were transfected in both leg and arm muscles.

Liu *et al.*[125)] succeeded in gene transfer into the diaphragm muscle of the *mdx* mouse, a model of Duchenne muscular dystrophy (DMD). Significant gene transfer was also found after intravenous injection of naked pDNA followed by a brief occlusion of blood flow at the vena cava. Approximately 40% of muscle fibers of the diaphragm were positive for dystrophin in *mdx* mice injected with pDNA encoding the full-length *Dmd* cDNA.

CONCLUSION

Successful *in vivo* gene therapy requires the development of a rational gene transfer approach that fulfills various requirements for each target disease. Development of target cell-specific delivery and controlled release technologies, the combined use of a variety of approaches, and the optimization of administration methods are needed to achieve effective *in vivo* gene therapy. Further basic and clinical studies in this field should allow *in vivo* gene therapy to become a realistic medical option in the near future.

REFERENCES

1) Miao C. H., Thompson A. R., Loeb K., Ye X., *Mol. Ther*., **3**, 947—957 (2001).
2) Fewell J. G., MacLaughlin F., Mehta V., Gondo M., Nicol F., Wilson E., Smith L. C., *Mol. Ther*., **3**, 574—583 (2001).
3) Nishikawa M., Huang L., *Hum. Gene Ther*., **12**, 861—870 (2001).
4) Nabel G. J., Nabel E. G., Yang Z. Y., Fox B. A., Plautz G. E., Gao X., Huang L., Shu S., Gordon D., Chang A. E., *Proc. Natl. Acad. Sci. U.S.A*., **90**, 11307—11311 (1993).
5) Caplen N. J., Alton E. W., Middleton P. G., Dorin J. R., Stevenson B. J., Gao X., Durham S. R., Jeffery P. K., Hodson M. E., Coutelle C., Huang L., Porteous D. J., Williamson R., Geddes D. M., *Nat. Med*., **1**, 39—46 (1995).
6) Snyder R. O., Miao C., Meuse L., Tubb J., Donahue B. A., Lin H. F., Stafford D. W., Patel S., Thompson A. R., Nichols T., Read M. S., Bellinger D. A., Brinkhous K. M., Kay M. A., *Nat. Med*., **5**, 64—70 (1999).
7) Roth D. A., Tawa N. E., Jr., O'Brien J. M., Treco D. A., Selden R. F., *N. Engl. J. Med*., **344**, 1735—1742 (2001).
8) Herzog R. W., Yang E. Y., Couto L. B., Hagstrom J. N., Elwell D., Fields P. A., Burton M., Bellinger D. A., Read M. S., Brinkhous K. M., Podsakoff G. M., Nichols T. C., Kurtzman G. J., High K. A., *Nat. Med*., **5**, 56—63 (1999).
9) Kay M. A., Manno C. S., Ragni M. V., Larson P. J., Couto L. B., McClelland A., Glader B., Chew A. J., Tai S. J., Herzog R. W., Arruda V., Johnson F., Scallan C., Skarsgard E., Flake A. W., High K. A., *Nat. Genet*., **24**, 257—261 (2000).
10) Kay M. A., High K., *Proc. Natl. Acad. Sci. U.S.A*., **96**, 9973—9975 (1999).
11) Phelps S. F., Hauser M. A., Cole N. M., Rafael J. A., Hinkle R. T., Faulkner J. A., Chamberlain J. S., *Hum. Mol. Genet*., **4**, 1251—1258 (1995).
12) Wolff J. A., Malone R. W., Williams P., Chong W., Acsadi G., Jani A., Felgner P. L., *Science*, **247**, 1465—1468 (1990).
13) Budker V., Zhang G., Danko I., Williams P., Wolff J. A., *Gene Ther*., **5**, 272—276 (1998).
14) Liang K., Nishikawa M., Huang L., *Mol. Ther*., **1**, S226 (2000).
15) Zhang G., Budker V., Williams P., Subbotin V., Wolff J. A., *Hum. Gene Ther*., **12**, 427—438 (2001).
16) Lin H., Parmacek M. S., Morle G., Bolling S., Leiden J. M., *Circulation*, **82**, 2217—2221 (1990).
17) Hickman M. A., Malone R. W., Lehmann-Buinsma K., Sih T. R., Knoell D., Szoka F. C., Walzem R., Carlson D. M., Powell J. S., *Hum. Gene Ther*., **5**, 1477—1483 (1994).
18) Budker V., Zhang G., Knechtle S., Wolff J. A., *Gene Ther*., **3**, 593—598 (1996).
19) Wu G. Y., Wu C. H., *J. Biol. Chem*., **263**, 14621—14624 (1991).
20) Perales J. C., Ferkol T., Beegen H., Ratnoff O. D., Hanson R. W., *Proc. Natl. Acad. Sci. U.S.A*., **91**, 4086—4090 (1994).
21) Nishikawa M., Takemura S., Takakura Y., Hashida M., *J. Pharmacol. Exp. Ther*., **287**, 408—415 (1998).
22) Nishikawa M., Takemura S., Yamashita F., Takakura Y., Meijer D. K., Hashida M., Swart P. J., *J. Drug Targeting*, **8**, 29—38 (2000).
23) Nishikawa M., Yamauchi M., Morimoto K., Ishida E., Takakura Y., Hashida M., *Gene Ther*., **7**, 548—555 (2000).
24) Kawakami S., Fumoto S., Nishikawa M., Yamashita F., Hashida M., *Pharm. Res*., **17**, 306—313 (2000).
25) Kawakami S., Sato A., Nishikawa M., Yamashita F., Hashida M., *Gene Ther*., **7**, 292—299 (2001).
26) Ono T., Fujino Y., Tsuchiya T., Tsuda M., *Neurosci. Lett*., **117**, 259—263 (1990).
27) Schwartz B., Benoist C., Abdallah B., Rangara R., Hassan A., Scherman D., Demeneix B. A., *Gene Ther*., **3**, 405—411 (1996).
28) Raz E., Carson D. A., Parker S. E., Parr T. B., Abai A. M., Aichinger G., Gromkowski S. H., Singh M., Lew D., Yankauckas M. A., Baird A. M., Rhodes G. H., *Proc. Natl. Acad. Sci. U.S.A*., **91**, 9519—9523 (1994).
29) Yoo J. J., Soker S., Lin L. F., Mehegan K., Guthrie P. D., Atala A., *J. Urol*., **162**, 1115—1118 (1999).
30) Sikes M. L., O'malley B. W., Jr., Finegold M. J., Ledley F. D., *Hum. Gene Ther*., **5**, 837—844 (1994).
31) Plautz G. E., Yang Z. Y., Wu B. Y., Gao Z., Huang L., Nabel G. J., *Proc. Natl. Acad. Sci. U.S.A*., **90**, 4645—4649 (1993).
32) Son K., Huang L., *Gene Ther*., **10**, 343—345 (1996).
33) Nomura T., Yasuda K., Yamada T., Okamoto S., Mahato R. I., Watanabe Y., Takakura Y., Hashida M., *Gene Ther*., **6**, 121—129 (1999).
34) Kircheis R., Schuller S., Brunner S., Ogris M., Heider K. H., Zauner W., Wagner E., *J. Gene Med*., **1**, 111—120 (1999).
35) Yoshimura K., Rosenfeld M. A., Nakamura H., Scherer E. M., Pavirani A., Lecocq J. P., Crystal R. G., *Nucleic Acids Res*., **20**, 3233—3240 (1992).
36) McCluskie M. J., Chu Y., Xia J. L., Jessee J., Gebyehu G., Davis H. L., *Antisense Nucleic Acid Drug Devel*., **8**, 401—414 (1998).
37) Ferkol T., Perales J. C., Eckman E., Kaetzel C. S., Hanson R. W., Davis P. B., *J. Clin. Invest*., **95**, 493—502 (1995).
38) Liu Y., Mounkes L. C., Liggitt H. D., Brown C. S., Solodin I., Heath T. D., Debs R. J., *Nat. Biotechnol*., **15**, 167—173 (1997).
39) Li S., Wu S. P., Whitmore M., Loeffert E. J., Wang L., Watkins S. C., Pitt B. R., Huang L., *Am. J. Physiol*., **276**, L796—L804 (1999).
40) Sakurai F., Nishioka T., Saito H., Baba T., Okuda A., Matsumoto O., Taga T., Yamashita F., Takakura Y., Hashida M., *Gene Ther*., **8**, 677—686 (2001).
41) Li S., Tan Y., Viroonchatapan E., Pitt B. R., Huang L., *Am. J. Physiol*., **278**, L504—L511 (2000).
42) Hunt, C. A., MacGregor, R. D., Siegel, R. A., *Pharm. Res*., **3**, 333—344 (1986).
43) Nara E., Masegi M., Hatono T., Hashida M., *Pharm. Res*., **9**, 161—168 (1992).
44) Nomura T., Nakajima S., Kawabata K., Yamashita F., Takakura Y., Hashida M., *Cancer Res*., **57**, 2681—2686 (1997).
45) Sandberg J. W., Lau C., Jacomino M., Finegold M., Henning S. J., *Hum. Gene Ther*., **5**, 323—329 (1994).
46) Bennett H. S., Luft J. H., Hampton J. C., *Am. J. Physiol*., **196**, 381—390 (1959).
47) Simionescu N., *Physiol. Rev*., **63**, 1536—1579 (1983).
48) Ziylan Y. Z., Robinson P. J., Rapoport S. I., *Am. J. Physiol*., **247**, R634—R638 (1984).
49) Rainov N. G., Ikeda K., Qureshi N. H., Grover S., Herrlinger U., Pechan P., Chiocca E. A., Breakefield X. O., Barnett F. H., *Hum. Gene Ther*., **10**, 311—318 (1999).
50) Greelish J. P., Su L. T., Lankford E. B., Burkman J. M., Chen H., Konig S. K., Mercier I. M., Desjardins P. R., Mitchell M. A., Zheng X. G., Leferovich J., Gao G. P., Balice-Gordon R. J., Wilson J. M., Stedman H. H., *Nat. Med*., **5**, 439—443 (1999).
51) Mahato R. I., Kawabata K., Takakura Y., Hashida M., *J. Drug Targeting*, **3**, 149—157 (1995).
52) Mahato R. I., Kawabata K., Nomura T., Takakura Y., Hashida M., *J. Pharm. Sci*., **84**, 1267—1271 (1995).
53) Takakura Y., Takagi A., Hashida M., Sezaki H., *Pharm. Res*., **4**: 293—300 (1987).
54) Takakura Y., Fujita T., Hashida M., Sezaki H., *Pharm. Res*., **7**, 339—346 (1990).
55) Kawabata K., Takakura Y., Hashida M., *Pharm. Res*., **12**, 825—830 (1995).
56) Yoshida M., Mahato R. I., Kawabata K., Takakura Y., Hashida M., *Pharm. Res*., **13**, 599—603 (1996).
57) Takakura Y., Takagi T., Hashiguchi M., Nishikawa M., Yamashita F., Doi T., Imanishi T., Suzuki H., Kodama T., Hashida M., *Pharm. Res*., **16**, 503—508 (1999).
58) Midoux P., Mendes C., Legrand A., Raimond J., Mayer R., Monsigny M., Roche A. C., *Nucl. Acids Res*., **21**, 871—878 (1993).
59) Diebold S. S., Kursa M., Wagner E., Cotten M., Zenke M., *J. Biol. Chem*., **274**, 19087—19094 (1999).
60) Wagner E., Zenke M., Cotten M., Beug H., Birnstiel M. L., *Proc. Natl. Acad. Sci. U.S.A*., **87**, 3410—3414 (1990).
61) Ferkol T., Kaetzel C. S., Davis P. B., *J. Clin. Invest*., **92**, 2394—2400 (1993).
62) Ross G. F., Morris R. E., Ciraolo G., Huelsman K., Bruno M., Whitsett J. A., Baatz J. E., Korfhagen T. R., *Hum. Gene Ther*., **6**, 31—40 (1995).
63) Herweijer H., Zhang G., Subbotin V. M., Budker V., Williams P., Wolff J. A., *J. Gene Med*., **3**, 280—291 (2001).
64) McMahon J. M., Signori E., Wells K. E., Fazio V. M., Wells D. J., *Gene Ther*., **8**, 1264—1270 (2001).
65) Zhu X., Hadhazy M., Groh M. E., Wheeler M. T., Wollmann R., McNally E. M., *J. Biol. Chem*., **276**, 21785—21790 (2001).

66) Capecchi M. R., *Cell*, **22**, 39—46 (1980).
67) Zabner J., Fasbender A. J., Moninger T., Poellinger K. A., Welsh M. J., *J. Biol. Chem*., **270**, 18997—19007 (1995).
68) Lechardeur D., Sohn K. J., Haardt M., Joshi P. B., Monck M., Graham R. W., Beatty B., Squire J., O'brodovich H., Lukacs G. L., *Gene Ther*., **6**, 482—497 (1999).
69) Kaneda Y., Iwai K., Uchida T., *J. Biol. Chem*., **264**, 12126—12129 (1989).
70) Remy J.-S., Kichler A., Mordvinov V., Schuber F., Behr J.-P., *Proc. Natl. Acad. Sci. U.S.A*., **92**, 1744—1748 (1995).
71) Zanta M. A., Belguise-Valladier P., Behr J.-P., *Proc. Natl. Acad. Sci. U.S.A*., **96**, 91—96 (1999).
72) Plank C., Zauner W., Wagner E., *Adv. Drug Delivery Rev*., **34**, 21—35 (1998).
73) Boussif O., Lezoualch F., Zanta M. A., Mergny M. D., Scherman D., Demeneix B., Behr J. P., *Proc. Natl. Acad. Sci. U.S.A*., **92**, 7297—7301 (1995).
74) Midoux P., Monsigny M., *Bioconjugate Chem*., **10**, 406—441 (1999).
75) Benns J. M., Choi J. S., Mahato R. I., Park J. S., Kim S. W., *Bioconjugate Chem*., **11**, 637—645 (2000).
76) Putnam D., Gentry C. A., Pack D. W., Langer R., *Proc. Natl. Acad. Sci. U.S.A*., **98**, 1200—1205 (2001).
77) Xu Y., Szoka F. C., Jr., *Biochemistry*, **35**, 5616—5623 (1996).
78) Sakurai F., Inoue R., Nishino Y., Okuda A., Matsumoto O., Taga T., Yamashita F., Takakura Y., Hashida M., *J. Controlled Release*, **66**, 255—269 (2000).
79) Yang N. S., Burkholder J., Roberts B., Martinell B., McCabe D., *Proc. Natl. Acad. Sci. U.S.A*., **87**, 9568—9572 (1990).
80) Williams R. S., Johnston S. A., Riedy M., Devit M. J., Mcelligott S. G., Sanford J. C., *Proc. Natl. Acad. Sci. U.S.A*., **88**, 2726—2730 (1991).
81) Zelenin A. V., Kolesnikov V. A., Tarasenko O. A., Shafei R. A., Zelenina I. A., Mikhailov V. V., Semenova M. L., Kovalenko D. V., Artemyeva O. V., Ivaschnko T. E., Evgrafov O. V., Dickson G., Baranovand V. S., *FEBS Lett*., **414**, 319—322 (1997).
82) Furth P. A., Shamay A., Wall R. J., Henninghausen L., *Anal. Biochem*., **205**, 365—368 (1992).
83) Walther W., Stein U., Fichtner I., Malcherek L., Lemm M., Schlag P. M., *Gene Ther*., **8**, 173—180 (2001).
84) Mir L. M., Banoun H., Paoletti C., *Exp. Cell Res*., **175**, 15—25 (1988).
85) Somiari S., Glasspool-Malone J., Drabick J. J., Gilbert R. A., Heller R., Jaroszeski M. J., Malone R. W., *Mol. Ther*., **2**, 178—187 (2000).
86) Johnson P. G., Gallo S. A., Hui S. W., Oseroff A. R., *J. Invest. Dermatol*., **111**, 457—463 (1998).
87) Titomirov A. V., Sukharev S., Kistanova E., *Biochim. Biophys. Acta*, **1088**, 131—134 (1991).
88) Heller R., Jaroszeski M., Atkin A., Moradpour D., Gilbert R., Wands J., Nicolau C., *FEBS Lett*., **389**, 225—228 (1996).
89) Rols M. P., Delteil C., Golzio M., Dumond P., Cros S., Teissie J., *Nat. Biotechnol*., **16**, 168—171 (1998).
90) Aihara H., Miyazaki J., *Nat. Biotechnol*., **16**, 867—870 (1998).
91) Huber P. E., Pfisterer P., *Gene Ther*., **7**, 1516—1525 (2000).
92) Liu F., Song Y. K., Liu D., *Gene Ther*., **6**, 1258—1266 (1999).
93) Zhang G., Budker V., Wolff J. A., *Hum. Gene Ther*., **10**, 1735—1737 (1999).
94) Kobayashi N., Kuramoto T., Yamaoka K., Hashida M., Takakura Y., *J. Pharmacol. Exp. Ther*., **297**, 853—860 (2001).
95) Xu Z. L., Mizuguchi H., Ishii-Watabe A., Uchida E., Mayumi T., Hayakawa T., *Gene*, **272**, 149—156 (2001).
96) Truong-Le V. L., August J. T., Leong K. W., *Hum. Gene Ther*., **9**, 1709—1717 (1998).
97) Ochiya T., Takahama Y., Nagahara S., Sumita Y., Hisada A., Itoh H., Nagai Y., Terada M., *Nat. Med*., **5**, 707—710 (1999).
98) Capan Y., Woo B. H., Gebrekidan S., Ahmed S., DeLuca P. P., *Pharm. Res*., **16**, 509—513 (1999).
99) Klugherz B. D., Jones P. L., Cui X., Chen W., Meneveau N. F., DeFelice S., Connolly J., Wilensky R. L., Levy R. J., *Nat. Biotechnol*., **18**, 1181—1184 (2000).
100) Guo Z. S., Wang L. H., Eisensmith R. C., Woo S. L. C., *Gene Ther*., **3**, 802—810 (1996).
101) Bird A. P., *Trends Genet*., **3**, 342—347 (1987).
102) Krieg A. M., Yi A. K., Matson S., Waldschmidt T. J., Bishop G. A., Teasdale R., Koretzky G. A., Klinman D. M., *Nature* (London), **374**, 546—549 (1995).
103) Paillard F., *Hum. Gene Ther*., **10**, 2089—2090 (1999).
104) Krieg A. M., Wu T., Weeratna R., Efler S. M., Love-Homan L., Yang L., Yi A.-K., Short D., Davis H. L., *Proc. Natl. Acad. Sci. U.S.A*., **95**, 12631—12636 (1998).
105) McMahon J. M., Wells K. E., Bamfo J. E., Cartwright M. A., Wells D. J., *Gene Ther*., **5**, 1283—1290 (1998).
106) Yew N. S., Wang K. X., Przybylaka M., Bagley R. G., Stedman M., Marshall J., Scheule R. K., Cheng S. H., *Hum. Gene Ther*., **10**, 223—234 (1999).
107) Tousignant J. D., Gates A. L., Ingram L. A., Johnson C. L., Nietupski J. B., Cheng S. H., Eastman S. J., Scheule R. K., *Hum. Gene Ther*., **11**, 2493—2513 (2000).
108) Yew N. S., Zhao H., Wu I.-H., Song A., Tousignant J. D., Przybylska M., Cheng S. H., *Mol. Ther*., **1**, 255—262 (2000).
109) Tan Y., Li S., Pitt B. R., Huang L., *Hum. Gene Ther*., **10**, 2153—2161 (1999).
110) Hofman C. R., Dileo J. P., Li Z., Li S., Huang L., *Gene Ther*., **8**, 71—74 (2001).
111) Yew N. S., Wysokenski D. M., Wang K. X., Ziegler R. J., Marshall J., McNeilly D., Cherry M., Osburn W., Cheng S. H., *Hum. Gene Ther*., **8**, 575—584 (1997).
112) Loser P., Jennings G. S., Strauss M., Sandig V., *J. Virol*., **72**, 180—190 (1998).
113) Qin L., Ding Y., Pahud D. R., Chang E., Imperiale M. J., Bromberg J. S., *Hum. Gene Ther*., **8**, 2019—2029 (1997).
114) Yew N. S., Przybylska M., Ziegler R. J., Liu D., Cheng S. H., *Mol. Ther*., **4**, 75—82 (2001).
115) Bischoff K. B., Dedrick R. L., Zaharko D. S., Longstreth J. A., *J. Pharm. Sci*., **60**, 1128—1133 (1971).
116) Acsadi G., Dickson G., Love D. R., Jani A., Walsh F. S., Gurusinghe A., Wolff J. A., Davies K. E., *Nature* (London), **352**, 815—818 (1991).
117) Manthorpe M., Cornefert-Jensen F., Hartikka J., Felgner J., Rundell A., Margalith M., Dwarki V., *Hum. Gene Ther*., **4**, 419—431 (1993).
118) O'hara A. J., Howell J. M., Taplin R. H., Fletcher S., Lloyd F., Kakulas B., Lochmuller H., Karpati G., *Muscle Nerve*, **24**, 488—495 (2001).
119) Mumper R. J., Duguid J. G., Anwer K., Barron M. K., Nitta H., Rolland A. P., *Pharm. Res*., **13**, 701—709 (1996).
120) Favre D., Cherel Y., Provost N., Blouin V., Ferry N., Moullier P., Salvetti A., *Gene Ther*., **7**, 1417—1420 (2000).
121) Mathiesen I., *Gene Ther*., **6**, 508—514 (1999).
122) Davies H. L., Whalen R. G., Demeneix B. A., *Hum. Gene Ther*., **4**, 151—159 (1993).
123) Wells D. J., *FEBS Lett*., **332**, 179—182 (1993).
124) Danko I., Fritz J. D., Jiao S., Hogan K., Latendresse J. S., Wolff J. A., *Gene Ther*., **1**, 114—121 (1994).
125) Liu F., Nishikawa M., Clemens P. R., Huang L., *Mol. Ther*., **4**, 45—51 (2001).