J. Physiol. Biochem., **58** (1), 45-56, 2002

Improvement of DNA transfection with cationic liposomes

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(Received on January 16, 2002)

A. ROCHA, S. RUIZ and J. M. COLL. *Improvement of DNA transfection with cationic liposomes* (minireview). J. Physiol. Biochem., **58** (1), 45-56, 2002.

The increasing use of cationic liposomes as vectors for DNA transfection of eukaryotic cells is due to its high efficiency and reproducibility. After the interaction of the DNA cationic-liposome complexes (DNA-CLC) with the plasma membrane, the entry into the cells delivers the DNA-CLC to the endosome-lysosome pathway where some of the DNA-CLC are degraded. The non-degraded DNA that escapes to the cytoplasm, still has to transverse the nuclear membrane to be transcribed and then translated. To improve the efficiency of the whole process, we can manipulate the DNA (sequences, promoters, enhancers, nuclear localisation signals, etc), the DNA-CLC (lipids) or the plasmatic, endosomal and/or nuclear cellular membranes (ultrasound, electroporation, Ca^{++}, pH of the endosomes, mitosis, fusogenic peptides, nuclear localisation signals, etc). Most of these methods have been generally used individually but in combination, may greatly improve the efficiency and reproducibility of *in vitro* transfection. While much of this work remains yet to be done and present results further explored, the application of these efforts is essential to the future development of new gene therapy strategies.

Key words: Transfection, Cationic liposomes, Endosomes, Mitosis, pH, Ca^{++}, NLS, Peptides, Transgenes.

In some *in vitro* conditions the eukaryotic cells might take up a small percentage of DNA to the nucleus to be transcribed and expressed. This phenomena has been used to obtain transient expression of foreign genes (transgenes) in eukaryotic cells.

Stable integration for permanent expression of transgenes is one of the major objectives of *in vivo* permanent gene therapy (90). Despite much effort, however, no good correlation results have

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yet been obtained between *in vitro* and *in vivo* in this area (16). Nevertheless the study of *in vitro* transient transfection may be essential to our understanding of the technical difficulties to obtain permanent gene therapy *in vivo* and explain some of the obtained results. Because transgene delivery and expression are fundamental steps in many forms of gene therapy and at least part of the mechanisms of entry are the same whether they are carried out *in vivo* or *in vitro*, the study of *in vitro* transfection can also help to design some of the *in vivo* strategies. Therefore, the transient expression of transgenes *in vivo* remains one of the simplest models to study some of the variables implicated in permanent expression of transgenes *in vivo* (26, 27, 60).

Cellular pathway of the DNA-cationic liposome complexes (DNA-CLC)

To express a foreign gene or transgene in an eukaryotic cell, the first barrier to be overcome is the plasma membrane (Fig. 1). After the addition of doubly labelled DNA-CLC to the cells, the DNA and the lipids (Table I) co-localise in the cell surface due to adsorption and/or electrostatic interactions (76). The delivery of DNA to the cytoplasm might then be by endocytosis (endosome-lysosome pathway) or either with the help of mechanical methods or by membrane fusion. The labelled DNA-CLC are later detected in vesicles distributed throughout the cytoplasm, where there is a second endosome/lysosome membrane barrier. Before the DNA is degraded in the lysosome, the DNA must escape free from the liposome (64, 84) and get to the nucleus where the RNA polymerases are located (with the exception of systems that use cytoplasmic polymerases like T7) (76).

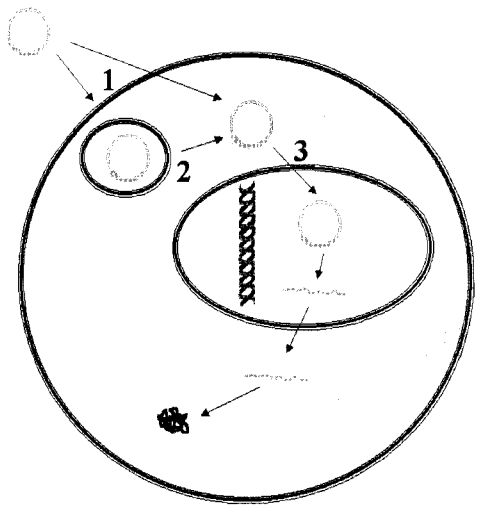

Fig. 1. *Cellular pathway of a DNA-CLC.* First, a DNA plasmid encoding the transgene, promoters, introns, terminators and/or other control elements for eukaryotic cells is designed. The DNA is then complexed with the cationic liposomes (DNA-CLC) and added to the cells. The DNA-CLC penetrates the plasma membrane either by the endosome/lysosome pathway (1, 2) or by fusion (1) and finally the nuclear membrane (3). Only inside the nucleus, can the RNA polymerases make the messenger RNA that would be then translated in the cytoplasm. In this work, we refer to the efficiency of expression or transfection (ε) as the percentage of cells treated with DNA-CLC which express the transgene (number of cells expressing the transgene/total number of cells treated x 100).

In this work, we refer to the efficiency of expression or transfection (ε) as the percentage of cells treated which express the transgene (number of cells expressing the transgene / total number of cells treated x 100). The best ε of CLC mediated transgene expression is ≤ 50%, however, the lack of control of some of the variables involved makes an ε of ~ 20% the most reproducible. The overall ε of the transfected DNA will be low, if maximal efficiencies are not obtained in each of the steps referred above. Since most of the

Table I. *Some of the lipids used to make DNA-cationic liposomes complexes (DNA-CLC).*

Name	Function	Formula
DOTAP	cationic lipid	1,2 dioleoyl–3–trimetylammonium propane
DOGS	cationic lipid	di-octadecylamidoglycil-espermine
Tfx	cationic lipid	tetrametylhydroxiethyl,2,3dioleyl,1,4 butanediammonium iodide
DOPE	fusogenic	1,2 dioleoyl–3–phosphatidylethanolamine
DPC	structural lipid	1,2 dioleoyl–3–phosphatidylcholine
Cholesterol	structural lipid	cholesterol

The cationic lipids have a positively charged head to interact with the negatively charged phosphates of DNA and an hydrophobic part constituted by fatty acids. Oleic acid (C18:1) is the fatty acid most commonly used because of its flexibility and lower fusion temperature.

DNA-CLC cationic liposomes penetrate the cells (94), it is not clear which is the limiting step.

Methods to improve the ε of the DNA-CLC mediated transfection have focused on modifying the DNA, the liposome and/or the cells (Table II). Most of these methods have been used singly and only a few reports describe the combination of these methods to improve ε.

Table II. *Some of the cellular methods to improve the efficiency of transfection ε of DNA-CLC mediated transfection.*

Membrane	Method	Reference
Plasma	Ultrasound	(52)
	Electroporation	(16)
	Chimeric peptides	(71)
Endosomal	pH	(62)
	Ca^{++}	(49)
	Chimeric peptides	(39)
Nuclear	Mitosis, meiosis	(94)
	NLS	(3)
	Chimeric peptides	(77)

The possible cellular methods to improve the ε of a DNA-CLC mediated gene transfer (25) have been classified in methods to transverse the plasma, endosomal and nuclear membranes. While these methods have been generally used singly, their use in combination might improve the ε of *in vitro* transfection and its reproducibility. Much of these possibilities remain to be explored.

Modifications in the DNA backbone for optimal transgene delivery

The DNA used for transient transfection are plasmids (circular double strain DNA) containing an expression cassette with the transgene and ~ 3 Kb of bacterial sequences to manipulate the construct in bacterias (24). Modifications in the plasmid backbone have been directed to increase transcription by promoter selection (5, 41, 68) or to increase plasmid replication inside the cells by including replication origins such as the SV40 origin. Improvements of 2-1000- fold in the ε have been described when the SV40 early promoter and enhancer were present in the plasmids (19). This effect seems to be due to the presence in the enhancer of binding targets for proteins with nuclear localisation signals. An increased ε has also been obtained by eliminating bacterial sequences to produce miniplasmids (17). On the other hand, it remains to be explored the possible use of plasmids containing vertebrate transposons like "sleeping beauty" for transient and permanent expression of transgenes (43).

Open plasmids (digested by a unique restriction enzyme) or linear DNA have been generally used for best integration into the genome for permanent expres-

sion, however, recent reports also used linear DNA with covalently closed ends for transient transfections. This new type of DNA molecules (MIDGE plasmids) increased both their resistance to DNAses and *in vivo* expression (83, 86). Degradation of the DNA must be carefully avoided during plasmid purification, since degraded DNA acts as an inducer of cell apoptosis (85).

Use of cationic liposome complexes (CLC) for DNA transfection

Due to the size and negative charges of DNA and to the number of membrane and enzymatic cellular barriers, the entrance and nuclear transcription of foreign naked DNA is not too efficient. To increase ε, early methods used calcium phosphate, cationic polymers to condense DNA (DEAE, polylisine), retrovirus, microinjection, electroporation, etc. (30, 63), however, these methods had problems of cellular toxicity, low reproducibility, complex execution or low ε. Cationic lipids are now widely used for *in vitro* gene transfer due to their high and reproducible ε (25) when compared to earlier methods (51, 91).

The cationic lipids used for liposome formation are amphipathic molecules (positively charged hydrophilic heads linked to hydrophobic long chain fatty acids). The derived liposomes are made by mixing several lipids in organic solvents, generally the cationic lipid (Table I) with phosphatidylcholine and cholesterol to increase stability (44). Once the organic solvent is eliminated, an aqueous buffer is added and the mixture is sonicated to obtain liposomes of several sizes which can be extruded to ~ 200-400 nm (93). All the manipulations should be made in the presence of nitrogen or vacuum to avoid the oxidation of the double bounds of fatty acids (oleic acid is the most commonly used). Liposomes made with cationic lipids spontaneously form complexes with DNA which are resistant to DNAses and actively introduced into the cells (53, 78). Although the first DNA-CLC made with cationic lipids were inhibited by the presence of serum, recently designed DNA-CLC work in its presence provided the complexes are formed in its absence. Although the DNA-CLC can be conserved at 4 °C, some of them can be lyophilised and then reconstituted with water before use (2). The charge ratio between the DNA and the lipids is an important variable for the performance of the complexes; to avoid its precipitation it should be near to neutrality. The structure of the complexes seems to be multilamelar with alternating lipid bilayers and DNA monolayers, as studied by X-ray diffraction (58, 79).

Use of ultrasound to transfer DNA

The first report to use ultrasound (~ 20 kHz) to deliver a plasmid codifying the gene of thymidine quinase in fibroblasts was described in 1985 (47). By using 50 μg/ml of DNA only 0.0001% of the cells were transfected. Although 1% efficiencies were later obtained, the DNA concentrations required by this method are still high (> 1 μg/ml).

The advantages of sonication to transfer DNA are: a) is fast, b) is simple and cheap, c) it can be used with many cell types and d) it could be applied locally *in vivo* for gene therapy. To be useful for *in vivo* treatments, however, much higher ε should be obtained *in vitro* along with a better understanding of the effects of sonication. The first cellular effect observed after sonication is heating (22). By increasing the intensities/frequencies a widening of the intercellular space and pore forma-

tion in the membranes are obtained (28, 65). Penetration of DNA into the cells follows proportionally to the degree of cavitation (29, 36, 59, 101). However, the comparative study of different reports are difficult due to the high number of variables involved (frequency, intensity, number of pulses, time of recovery between pulses, moment of addition of DNA, time, geometry, etc.) and to the few studies of the effects of all the variables (101).

Some studies report that the combination of sonication with liposomes increases ε about 7-fold (52, 95), but no studies explored the use of liposomes to reduce the high amount of DNA required for sonication to be effective. Although the plasmids seem to be only slightly altered by sonication (26, 101), they were totally protected from degradation after complexing with liposomes (98).

At the level of whole animals, therapeutic ultrasound (~ 2.2 W cm^2 to 3.000 kHz during 30 to 90 s) produced a widening in the intercellular space and breaking of the desmosomes that connect cells (28) and it has also been used to immunise trout against virus by DNA vaccination (26). Thus, by using 0.4-0.6 W cm^2 to 40 kHz during 24 s for 6 cm trout, the expression, optimal immunisation and protection were obtained at 10 μg/ml of DNA, a concentration too high to make it practical.

Electric pulses or electroporation

The physical variables in the electric pulse-mediated transfer of DNA into cells (40, 46) are the duration of the electric pulse T (5-20 msec) and the intensity of the field. Duration depends on the resistance of the medium (Ω) and the potency (μF) of the capacitor ($T = \Omega \cdot \mu F$). The Ω inversely proportional to its ionic concentration also depends on the volume and the width of the cuvette used. The intensity of the field (100-1000 V/cm) influences ε and cell survival. The time of the electric pulse and field intensity for optimal ε vary for each cell type. The ε in function of the intensity is very critical since the optimal ε coincides with 20-80% of cell mortality (12). The electric pulse use the discharge of a capacitor of 1-1000 μF, that could produce ~ 6.000 V/cm in cuvettes of 0.4 cm with low resistances (~ 20 Ω) to transfect cells in cell culture medium.

Cells in suspension or in monolayers can be transfected by electroporation. The cell type, the duration of its cell cycle and the growth phase distribution of the cellular population are important variables (16). For instance, fast growing cells (short cell cycle) are better transfected than slow growing cells. The increment of ε when the cells are incubated with colchicine before electroporation reflects the fact that the absence of nuclear membrane during the mitosis metaphase is the best moment for transfection (1). Due to the toxicity of colchicine *in vivo*, it is doubtful that this method could be applied *in vivo*, however other less toxic compounds and/or local application might be used to that purpose.

The medium used for electroporation affects the cells (21): a) its resistance influences T which in turn influences cell survival, b) its osmolarity affects cell survival, c) the concentration of divalent ions affects membrane stability and d) since the electric pulse induces a permeabilization of the plasma membrane which remains at low temperature during several hours, components in the electroporation medium can go inside the cells whereas some cellular components can exit the cells causing low cell survivals (81).

The ε of electroporation increases with the DNA concentration between 1-40

μg/ml. The temperature can be held at 4 °C to improve cell survival, but the electroporation can be also performed at room temperature (12). A hypotonic medium (31), the presence of ethanol (32) and of DMSO (66), the number and the duration of pulses increased ε. Only when pulses of ~ 5 ms were applied was permeabilization to large molecules such as βgalactosidase or plasmid DNA detectable (81).

To our knowledge, the effect of electroporation on the transfection of DNA-CLC has not yet been completely studied.

Bombardement

The physical bombardement with gold particles coated with DNA, has been used for gene delivery but mainly for *in vivo* surface tissues (33, 34). The bombardement with DNA-CLC has not been studied because it is difficult or impossible to perform by using this technique.

Control of the pH in the endosome/lysosome pathway: lysosomotropic agents

DNA-CLC endocytosis results in the complexes being processed by the endosomal-lysosomal pathway, normally leading to low pH dependent degradation of most of the DNA in the lysosome. Promoting fast endosomal release and cytoplasm delivery of the DNA is therefore necessary for efficient gene transfer to occur. Thus, it has been shown that the addition of compounds which inhibit the lowering of lysosomal pH (lysosomotropic agents) increased the ε (69). One of the most used lysosomotropic agents is chloroquine. Treatments with 20-100μM chloroquine during and after transfection increased ~ 2-fold the ε after transfection of DNA-CLC (62). Similarly, chloroquine also increases the expression of RNA-peptides and RNA-CLC (6). Because exposure to chloroquine during > 4 h causes cell death, sucrose (5-500 mM), ammonium chloride (10-40 mM) or polyvinilpirrolidone (0.01 –1 mg/ml) have also been used as alternative lysosomotropic agents (13).

To use liposomes with a higher ability to escape lysosomes, low-pH dependent fusogenic lipids (48, 89) and lipids with low-pH labile disulphide bonds (92) have been also designed. Thus, low pH-dependent liposomes (liposomes containing DOPE) labelled with double fluorescence, were endositosed and freed its DNA content to the cytoplasm as soon as the endosome was acidified (89).

Improvement of expression by the addition of calcium

Calcium seems to have two effects on transfection: to condense the DNA when added before transfection and to increase ε when added post-transfection. The capacity to form fine precipitates of DNA-Ca^{++} was used in the first reports of cellular DNA transfection. Later on it was observed that the presence of < 10 mM Ca^{++} during and after transfection (to avoid toxicity) increased the ε 3-20-fold. This effect is specifically inhibited by EGTA and it is not due solely to charges. This effect has been demonstrated in 5 cellular lines by using 6 different DNA-CLC (49).

The presence of 2 mM Ca^{++} is capable to counteract the inhibition of transfection by serum when the DNA is already inside the cell (37). This effect may be explained because the serum seems to inhibit the escape of the DNA from the endosomal DNA-CLC rather than the translocation through the plasma membrane (37). It is possible that this post-

transfectional activity of Ca^{++} could be due to its lysosomotropic capacity (38).

Absence of nuclear membranes: mitosis and meiosis

Fast growing cells are transfected much better than slow growing or quiescent cells. On the other hand, the cell density (% of confluence) for optimal ε varies for different cell types as a result of different growth rates and its dependence on cell density. Because most of the cells in these states take up DNA-CLC but only 20-50% express the transgene (84 2113), the differences in ε may be related to the cell cycle distribution of each of the cells in the population of transfected cells.

By using a plasmid with GFP, analysis of expression by FACS, cells synchronised by double thymidine block (2.5 mM) and studying the time course of ε, a 3-10-fold increase in the expression of plasmid delivered by CLC was obtained right after cells passed the first mitosis (94). After the second mitosis, the total level of expression remains about the same but the level of expression per cell (ε) decreases slightly because the newly expressed proteins are divided between the progeny cells. By using cell populations in different cell cycle stages isolated by centrifugation and transfected with DNA-CLC, it was also demonstrated that the cells with the highest mitotic activity have 30-500-fold more ε (10). Similar results have been confirmed by transfecting cells permeabilized with digitonin (23) or during mitosis (73). The opposite results (20-fold lower ε) were obtained with cells arrested in G1 with aphidicoline compared with asynchronous cells. In all these cases it is supposed that the increase in nuclear permeability during mitosis (absence of membranes) must contribute to a higher penetration of the DNA into the nucleus leading to a higher transcription and therefore translation of the transgene. The lack of nuclear membrane during meiosis has been also used to increase ε of transgenes in oocytes to produce transgenic cattle (11). However, not all the observed expression is due to the penetration of the DNA in the nucleus during mitosis, since there are premitotic cells that also express the transgene and the maximal ε obtained with synchronous cells is of 40% rather than 100% (94).

Use of nuclear localisation signals in peptides and in DNA

As discussed above, one of the limiting steps in DNA transfection resides in the low percentage of DNA translocated from the cytoplasm to the nucleus in resting cells. An improvement of this translocation has been demonstrated by using peptides and DNA specific sequences.

The most efficacious method described has been the use of nuclear localisation signals (NLS) of the antigen T of SV40 (CGGPKKKRKVG) (3). The formation by ionic interactions of complexes between DNA and NLS peptides increased the incorporation of the DNA to the nucleus. Thus, cytoplasmic injection of only 10 molecules of DNA-NLS (0.06 fg of plasmid) were required to produce transgenic zebrafish (57) and the use of NLS *in vitro* or *in vivo* allowed for a ~ 10-fold reduction in the amount of DNA required to obtain similar ε than in the absence of NLS (15, 57). The non-covalent combination of DNA-NLS increased ε in cells permeabilized with digitonin (88), whereas the covalent linkage between NLS and DNA did not.

The complexes between melittin-polyetylenimine and DNA, also improved (~ 4-fold) the nuclear access of DNA, most probably due to the presence of a

sequence (KRKR) similar to the NLS sequence from SV40 (77).

The translocation of RNA to the nucleus is mediated by NLS signals found in the nucleotide sequences in retroviruses. Similar signals do exist in DNA but are much less known. The first reported NLS found in DNA sequences have been described in SV40 (18). Thus by using digitonin permeabilized cells and isolated nuclei as models, it has been demonstrated that the DNA translocation to the nucleus requires not only cytoplasmic proteins but specific DNA sequences (18, 100). The specific DNA sequences consisted of 72 bp repeats localised in the enhancer region (a region that binds transcriptional factors) of the SV40 early promoter (20, 56). The presence of these sequence repeats increases transgene expression in several non dividing cellular types by using several methods of transfection (cationic liposomes, peptides, injection) (56, 96). Similar results were obtained by using combination of DNA-NLS and DNA-CLC with an increase of 2-1000-fold depending on the vector (19), but these promising results should be studied further.

Use of chimeric peptides

Chimeric peptides used for DNA transfection combine short peptides to bind DNA, condense DNA, target DNA to specific cells and/or release DNA from the endosome to the cytoplasm or to the nucleus (61, 70). The designed chimeric peptides might be: a) a single peptide (individual peptides covalently bound and separated by short amino acid sequences for best activity) or b) several individual non covalently auto-assembled peptides.

Viral low-pH dependent fusogenic peptides have been complexed to DNA. Fusogenic peptides are amphypathic peptides that change conformation with low pH, disrupting the phospholipids in the membranes (Table III). An important variation in this scheme is the use of peptides capable of fusion with the plasmatic membrane at physiological pH such as the fusion peptide from the gp41 of HIV (71).

Table III. *Examples of peptides with DNA condensing, fusion, pore-forming activities or NLS sequences.*

Sequence (amino-carboxyl)	Origin	Reference
GIGAVLKVLTTGLPALISWIKRKRQQ	melittin	(77)
GLFEAIAGFIENGWEGMIDGGGC	INF influenza HA	(39)
KFTIVFPHNQGHWKNVPSNYHYCP	pG VSV	(87)
GALFLGFLGAAGSTMGAWSQPKSKRKV	gp41 HIV	(71)
WEAALAEALAEALAEHLAEALAEALEALAA	GALA	(39)
GLFEALLELLESLWLLEA	covalent: JTS1	(35)
HHHHHWYG	covalent	(67)
WEAKLAKALAKALAKHLAKALAKALKACEA	covalent	(102)
$(LARL)_6$ KLLKLLLKLWLKLLKLLL	covalent	(75)
$(K)_{19}$VAYISRGGVSTYYSDTVKGRFTRQKYNKRA	anti-DNA Ab	(4)
palmitoyl-SPKRSPKRSPKR + INF	non-covalent	(99)
YKAKKKKKKKKWK + JTS1	non-covalent	(35)

The peptides might be monofunctional or chimeric (covalently or non covalently associated peptides with different activities). The functions that are chosen for the design of the chimeric peptides are DNA condensation, membrane fusion, membrane destabilisation, pore-forming activities or nuclear localisation signals.

Chimeric peptides made by binding viral fusion peptides (promote endosomal release) with NLS and complexing with DNA, have used the fusion peptides from the HA of influenza virus (39), the pG of VSV (87) and the gp41 of HIV (71, 72) (Table III). Other chimeric peptides have been designed by binding cationic peptides to condense DNA with NLS (67, 75, 102). Thus, several cationic polyamines of different sizes and structures such as polylysine, poly-arginine, espermidine, espermine, temporines (80), dendrimers, protamine, poly-ethyleneimine (55), etc, have been used to condense DNA to form particles of 100-200 nm. Some of these cationic polymers also possess intrinsic endosomolytic ability because its protonation at lower pH leads to endosomal swelling and bursting with subsequent enhancement of ε. To increase both endocytosis and targeting to specific cells by receptor-mediated mechanism, polylisine can be bound to RGD to interact with the cells having receptors to integrins (14, 42), to galactose or transferrin to interact with hepatocites, anti-CD3 or CD5 to interact with lymphocytes T, (97), palmitic acid or polytilenglycol for many cellular types (9), etc. The glycosylation of the amines of polylysine introduce hydrophilic groups to decrease its cytotoxicity *in vivo* (7, 8). Other strategies to target the DNA coupled polylysine to a DNA-binding peptide derived from an anti-DNA antibody (4) or to antibodies anti-cytoskeleton (45).

An hybrid molecule between dioleoyl phosphatidylethanolamine and melittin (a fusogenic peptide from venom bee) has also been reported (50, 77). This reagent transfected DNA in the presence of serum forming 50-250 nm particles with a net positive charge. Complexed with DNA in a 10/1 ratio, the dioleoyl-melittin/DNA efficiently transfected a large number of cell types (54).

Peptides non-covalently associated by electrostatic and/or hydrophobic interactions have also been used such as the influenza peptide (INF) with a palmitoylated condenser peptide (99), or a pore-forming peptide (JTS1) with a condenser peptide (35). Peptides and proteins have been complexed with biotinylated DNA by using streptavidin as a bridge. The complex was internalised by following the endosome/lysosome pathway (82), however, the relative concentrations among these non-covalently associated peptides must be controlled very carefully because of possible competition problems between the different activities of the peptides.

Concluding remarks

The DNA-CLC spontaneously enters the majority of the cells treated (94). This first translocation could also be forced by mechanical methods (ultrasound and electroporation) (16, 52). In order to increase ε from 20-50% to 80-100%, however, translocation of DNA through the endosome/lysosome and nuclear membranes is also required. Release of the DNA from the endosome to the cytoplasm before acidification to avoid DNA degradation can be facilitated with the addition of lysosomotropic agents such as ammonium chloride, chloroquine, sucrose or Ca^{++} (13, 49, 62). Low pH-dependent fusogenic lipids or peptides have been also used to increase cytoplasmic DNA delivery (48, 89). Electroporation (1), DNA-CLC (10, 73, 94), injection (11) or penetration of permeabilized cells (23) by foreign DNA is enhanced in the absence of nuclear membranes such as during mitosis or meiosis due to the requirement of nuclear RNA polymerase to transcribe RNA. The

use of NLS sequences in peptides (57) and in DNA (19), can also increase ε in resting cells (3). The individual use of these methods (as in most reports) or in combination (very seldomly reported with the exception of chimeric peptides) might improve the ε of *in vitro* transfection and its reproducibility.

Acknowledgements

Thanks are due to J.P. Coll by typing. This work was supported by Research Grants CT98-4003 and CT98-4398 from the FAIR Program of the European Economic Community, from INIA project SC00046, FEDER IFD97-467 and from CICYT project ACUO1003.

A. ROCHA, S. RUIZ Y J. M. COLL. *Mejora de la transfección de DNA con liposomas catiónicos* (minirrevisión). J. Physiol. Biochem., **58** (1), 45-56, 2002.

El aumento del uso de los liposomas catiónicos como vectores para la transfección de DNA se debe a su alta eficiencia y reproducibilidad. Después de la interacción de los complejos de DNA-liposomas catiónicos (DNA-CLC) con la membrana plasmática, la entrada en la célula pasa el DNA-CLC a los endosomas-lisosomas donde parte del DNA se degrada. El DNA no degradado que escapa al citoplasma, todavía tiene que atravesar la membrana nuclear para poder transcribirse y luego traducirse. Para mejorar la eficiencia del proceso total, podemos manipular el DNA (secuencias, promotores, amplificadores, señales de localización nuclear, etc.), el DNA-CLC (lípidos), o las membranas plasmáticas, endosomal y/o nuclear (ultrasonido, electroporación, Ca^{++}, pH de los endosomas, mitosis, péptidos fusogénicos, señales de localización nuclear, etc.). La mayoría de estos métodos se han utilizado individualmente pero en combinación pueden aumentar grandemente la eficiencia y reproducibilidad de las transfecciones *in vitro*. Mientras que todavía hace falta mucho trabajo en estos temas, la aplicación de estas tecnologías es esencial para el desarrollo de nuevas estrategias en terapia génica.

Palabras clave: Transfección, Liposomas catiónicos, Endosomas, Mitosis, pH, Ca^{++}, NLS, Péptidos, Transgenes.

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