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Peptide-mediated RNA delivery: a novel approach for enhanced transfection of primary and post-mitotic cells

Thierry Bettinger*, Robert C. Carlisle, Martin L. Read, Manfred Ogris and Leonard W. Seymour

CRC Institute for Cancer Studies, University of Birmingham, Birmingham B15 2TA, UK

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ABSTRACT

Synthetic vectors were evaluated for their ability to mediate efficient mRNA transfection. Initial results indicated that lipoplexes, but not polyplexes based on polyethylenimine (PEI, 25 and 22 kDa), poly(L-lysine) (PLL, 54 kDa) or dendrimers, mediated efficient translation of mRNA in B16-F10 cells. Significant mRNA transfection was achieved by lipoplex delivery in quiescent (passage 0) human umbilical vein endothelial cells (HUVEC), and by passage 4, 10.7% of HUVEC were transfected compared to 0.84% with DNA. Lack of expression with PEI 25 kDa/mRNA or PLL 54 kDa/mRNA in a cell-free translation assay and following cytoplasmic injection into Rat1 cells indicated that these polyplexes were too stable to release mRNA. In contrast, polyplexes formed using smaller PEI 2 kDa and PLL 3.4 kDa gave 5-fold greater expression in B16-F10 cells compared to DOTAP, but were dependent on chloroquine for transfection activity. Endosomolytic activity was incorporated by conjugating PEI 2 kDa to melittin and resulting PEI 2 kDa–melittin/mRNA polyplexes mediated high transfection levels in HeLa cells (31.1 ± 4.1%) and HUVEC (58.5 ± 2.9%) in the absence of chloroquine, that was potentiated to 52.2 ± 2.7 and 71.6 ± 1.7%, respectively, in the presence of chloroquine. These results demonstrate that mRNA polyplexes based on peptide-modified low molecular weight polycations can possess versatile properties including endosomolysis that should enable efficient non-viral mRNA transfection of quiescent and post-mitotic cells.

INTRODUCTION

Since the pioneering work of Malone *et al.* (1) 12 years ago describing the successful use of cationic lipids to deliver mRNA, there have been surprisingly few examples of RNA used in the field of gene therapy. This is likely to be due to the relative difficulty of mRNA synthesis, compared with plasmid DNA, coupled with an inherent susceptibility to nuclease degradation and perceived difficulties in its handling. In fact, mRNA-based transfection has several important advantages over DNA, stemming mainly from its cytoplasmic site of expression that obviates the necessity for nuclear entry. Entry of DNA into the nucleus is well known to be a major factor restricting success of non-viral DNA delivery systems, particularly in quiescent and post-mitotic cells since there is no cell cycle-dependent breakdown of the nuclear envelope (2,3). Since non-cycling cells usually comprise the majority of target cells *in vivo*, including many human cancers, this represents an important limitation of conventional non-viral gene delivery.

The ability of mRNA to express therapeutic proteins within slowly dividing or non-cycling cells has been attracting significant attention recently, with the demonstration that dendritic cells, that are normally refractory to treatment with DNA, can be readily transfected with mRNA (4,5). This has enabled development of cancer vaccination strategies using mRNA isolated from tumour cells or biopsies to express tumour-associated peptides within autologous antigen-presenting dendritic cells *ex vivo* prior to their reintroduction into the host (reviewed in 6).

Several transfection agents have been evaluated for their usefulness in mRNA delivery (reviewed in 7). Most published reports make use of cationic lipids, usually found to achieve high levels of protein expression (8,9). In contrast, very few investigators have reported the use of polycations for mRNA delivery, despite their widespread use in DNA-based transfections. The reports that are available, using vectors such as DEAE-dextran (1), poly(L-lysine) (10) or dendrimers (5), show very low levels of mRNA expression, although no study has addressed the reason for the generally poor activity of polycations compared with cationic lipids.

A primary objective of this study was to identify the key requirements of synthetic vectors enabling efficient mRNA expression, in order to develop better-defined and more versatile vectors for delivery of mRNA. Accordingly, mRNA-mediated transfection was compared using a range of cationic lipids and polycations. We have verified the ability of mRNA to achieve expression within non-cycling cells, with DOTAP/mRNA lipoplexes showing significant levels of reporter gene expression in quiescent primary human umbilical vein endothelial cells (HUVEC), whereas DOTAP/DNA gave no measurable signal. We also found that the strength of electrostatic interaction between the mRNA and the transfection agent had a dramatic effect on the level of expression achieved, with thermodynamically stable polyplex vectors being less suitable for

*To whom correspondence should be addressed at present address: Bracco Research, Department of Formulation, 31 Route de la Galaise, CH-1228 Plan-les-Ouates, Geneva, Switzerland. Tel: +41 22 884 88 08; Fax: +41 22 884 88 85; Email: tbettinger@brg.bracco.com

mRNA translation. Decreasing the electrostatic interaction between the carrier and the mRNA by using shorter polycations gave major increases in expression, with mRNA polyplexes formed using low molecular weight PEI and PLL achieving 5-fold greater levels of luciferase expression than DOTAP/mRNA. However, the polyplexes formed using low molecular weight polycations lost their endosomolytic activity and required chloroquine to mediate mRNA expression. Endosomolysis was restored by conjugating low molecular weight PEI to the membrane-active peptide melittin, and high levels of mRNA expression were demonstrated in the absence of chloroquine. This study provides new insights for the delivery of mRNA, identifying the availability of free or loosely-bound mRNA in the cytoplasm as the key requirement for efficient expression, and providing the possibility of designing versatile and efficient vectors for mRNA delivery by incorporating peptides with a range of biological activities.

MATERIALS AND METHODS

Cell lines and primary cells

The mouse melanoma cell line B16-F10, the rat fibroblast line Rat1, and human cervical carcinoma cell line HeLa were grown in Dulbecco's modified Eagle's medium (DMEM) containing Glutamax (1 mM), glucose (1 g/l, Gibco BRL) and 10% foetal calf serum (FCS). HUVEC were obtained from umbilical cords at the Birmingham Women's Hospital and maintained in medium M199 containing 20% FCS supplemented with glutamine (2 mM), 5% antibiotic antimycotic solution (Sigma, Poole, UK) and fibroblast growth factor (10 ng/ml, Sigma).

Synthesis of mRNA transcripts

mRNA encoding luciferase was prepared by *in vitro* transcription of the luciferase SP6 plasmid [producing a transcript bearing a poly(A) tail (A_{30})] and luciferase T7 plasmid [no poly(A) tail] (Promega, Southampton, UK). Prior to transcription the luciferase SP6 plasmid was linearised using *Xmn*I and the luciferase T7 plasmid was linearised using *Sac*I. *In vitro* transcription of the luciferase SP6 DNA template by SP6 RNA polymerase was carried out using an SP6 RiboMAX™ kit as described by the manufacturer (Promega) with or without $m^7G(5')pppG(5')$ cap analog (Ambion, Oxfordshire, UK) at a ratio of 5:1 cap analog/GTP. When the cap analog was omitted, the GTP concentration was raised accordingly to 5 mM. The luciferase T7 DNA was transcribed in a similar fashion using a T7 RiboMAX™ kit (Promega). Products were characterised by gel electrophoresis and were all ~1800 bp in length.

The green fluorescent protein (GFP)-encoding plasmid pGEM4Z/GFP/A64 was a kind gift from Dr D. Boczkowski (Duke University Medical Center, NC). The construction of this plasmid is described elsewhere (11) and mRNA encoding GFP was generated using the T7 RiboMAX™ kit. This 894 bp mRNA contained a poly(A) tail (A_{64}) and product size was checked by electrophoresis.

Purification of mRNA was performed by RQ1 DNase I digestion, followed by extraction with phenol:chloroform:isoamyl and chloroform and precipitation by addition of sodium acetate (0.1 vol, 3 M, pH 5.5) and ethanol (2.5 vol, 100%). Precipitated RNA was washed with 70% ethanol, dissolved in water, quantified spectrophotometrically at 260 nm and examined by agarose gel electrophoresis after denaturation at 65°C for 15 min.

Synthesis of the polyethylenimine 2 kDa–melittin conjugate

Melittin peptide containing an N-terminal cysteine (CIGAVLKVLTTGLPALISWIKRKRQQ, 2896 g/mol, Alta Biosciences, Birmingham, UK) was conjugated with polyethylenimine 2 kDa (PEI 2 kDa; Aldrich, Gillingham, UK) using the heterobifunctional cross-linker succinimidyl 3-(2-pyridyldithio) propionate (SPDP, Sigma). PEI was modified with SPDP by adding 1.35 mg of SPDP (4.3 µmol) to 800 µl of a HEPES buffered saline solution (20 mM HEPES pH 7.8, 250 mM NaCl) containing 43 µl of PEI 2 kDa (100 mg/ml solution). The reaction was incubated at room temperature for 1 h. The derivatised PEI (PEI 2 kDa–SSPy) was resolved from excess SPDP by gel filtration on a Sephadex G15 column (15 × 250 mm) equilibrated with 20 mM HEPES buffer pH 7.4. The average number of pyridyldithio residues per molecule of PEI was determined by titration of amino groups of PEI by the TNBS assay (12), and by UV measurement of the release 2-thiopyridyl group at 343 nm following reduction with excess dithiothreitol (DTT) (13) (PEI 2 kDa/SSPy; 1/1.5). The conjugation reaction was initiated by mixing the PEI 2 kDa–SSPy conjugate with melittin at a molar ratio of 1:1.6, respectively, in HEPES buffer pH 7.4, 500 mM NaCl. The reaction was left for 2.5 h at room temperature. Unbound melittin was separated from the PEI 2 kDa–melittin conjugate by cationic exchange chromatography on a MonoS HR 5/10 column (Pharmacia; gradient elution 10–80% buffer A for 60 min; buffer A, 5 M NaCl/20 mM HEPES pH 7.4; buffer B, 20 mM HEPES pH 7.4). The conjugate eluted at 2–2.5 M NaCl as monitored at 240 and 280 nm. The main fractions were pooled and dialysed against 20 mM HEPES pH 7.4 for 2 days at 4°C using a Spectra/Por 3 membrane (MWcutoff = 3500; Spectrum, Houston, TX), then freeze-dried and resuspended in water. The amino content was determined by TNBS assay (12).

Lipoplex and polyplex-mediated mRNA transfection

A range of commercially available cationic lipids and cationic polymers were used for mRNA transfection at various cationic lipid or polycation nitrogen to RNA phosphate ratios (N/P). The cationic lipids used in these studies were DOTAP {*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium salts; Sigma} and DOGS (dioctadecylamidoglycyl spermine), that was kindly provided by Dr J.-S. Remy (Strasbourg, France). The cationic polymers used were branched polyethylenimine (PEI, 2 and 25 kDa; Aldrich), linear PEI (22 kDa; provided by Dr J.-S. Remy), poly(L-lysine) (PLL, 3.4 and 54 kDa; Sigma) and Superfect™ (polyamidoamine dendrimer 35 kDa; Qiagen, Crawley, UK).

For complexation of nucleic acid, the desired amount of polycation (9 µl of 10 mM aqueous amine nitrogen stock solution for N/P = 10, or 15 µl of Superfect™ for N/P = 8.5), or lipoplex (1.2 µl of a 13 mM aqueous amine nitrogen stock solution of DOTAP for N/P = 1.8, equivalent to a w/w ratio DOTAP/nucleic acid of 4, as recommended by the manufacturer; for DOGS, 9 µl of a 2 mM ethanolic solution for six charge equivalents) and 3 µg of heat-denatured RNA (10 min at 65°C) or DNA (pGL3-*luc* from Promega, or p*EGFP*-C1

from Clontech, UK) were diluted separately in 75 µl HEPES 10 mM pH 7.4, 150 mM NaCl. After 15 min, the polycation or cationic lipid was added to the RNA solution.

The mixture was gently vortexed and, after 15 min, the resulting lipoplexes or polyplexes were added directly to a 48-well plate (50 µl/well) containing 5×10^5 cells per well (cells were plated at least 24 h before transfection). Treated cells were incubated at 37°C in a 5% CO_2 humidified environment in 125 µl of DMEM without serum unless specified. Transfection studies with PEI 2 kDa, PLL 3.4 kDa and PEI 2 kDa–melittin were performed both in the presence and absence of chloroquine. After 4 h, the mixture containing complexes was discarded and 500 µl of fresh medium containing 10% FCS was added to each well. Cells were cultured for 6–48 h prior to analysis of reporter gene expression.

Assay of reporter genes

Luciferase expression following transfection was measured by a luminescence assay using cell lysates. The culture medium was discarded and cell lysates harvested after incubation of cells for 30 min at room temperature in 100 µl of lysis Reagent 1X (Promega). The lysate was gently vortexed and centrifuged for 5 min at 10 000 *g* at 4°C. Twenty microlitres of supernatant was diluted into 100 µl of luciferase reaction buffer (20 mM glycylglycine, 1 mM $MgCl_2$, 0.1 mM EDTA, 3.3 mM DTT, 0.5 mM ATP, 0.27 mM coenzyme A lithium salt) and the luminescence was integrated over 10 s on a Lumat LB 9507 (Berthold). Results were expressed as relative light units (RLU) per milligram of cell protein, and determined using the BCA assay (Pierce, Chester, UK). A standard curve measuring light units against the concentration of recombinant luciferase protein (Promega) in the lysis Reagent 1X was linear over the entire experimental range, with 1×10^7 RLU activity corresponding to 2 ng luciferase.

Analysis of GFP expression was carried out on a Coulter Epics XL flow cytometer. Cells were trypsinised at appropriate times after transfection, washed with phosphate-buffered saline (PBS) and then fixed in 2% paraformaldehyde. GFP was excited using the 488 nm line of an Argon laser, and emitted light collected at 520 nm (green fluorescence) and 575 nm (red fluorescence) to enable correction for autofluorescence by diagonal gating (14). Background fluorescence and autofluorescence were determined using mock treated cells. Cellular debris showing reduced side- and forward-scatter was excluded from analysis. The software programme WinMDI was used to analyse data and expressed as the percentage of GFP-positive cells.

In vitro translation of mRNA using rabbit reticulocyte lysate

In vitro translation was performed using a nuclease-treated rabbit reticulocyte lysate system (Promega), according to the manufacturer's recommendations. Briefly, cap-*luc*-A_{30} (0.5 µg in 12.5 µl, either free or condensed by cationic lipids or polycations) was added to the reticulocyte lysate (17.5 µl). Luciferase expression was measured after incubation (90 min, 37°C) as previously described. In some experiments, poly(aspartic acid) (pAsp) was added to the reticulocyte lysate at a final concentration of 100 µg/ml.

Microinjection studies

Microinjection studies were conducted using an Eppendorf transjector 5246, an Eppendorf micromanipulator 5171 and an inverted fluorescence microscope (Zeiss Axiovert 100). The Rat1 fibroblast cell line was chosen due to its well defined nucleus and large cytoplasm.

Rat1 cells were plated onto glass Cellocate grids (BDH/Merck) and grown in DMEM with 10% serum (24 h, 37°C). The grids were then washed with PBS and transferred into 2 ml DMEM containing HEPES (to improve the buffering capacity of the medium during microinjection) but no serum. Complexes were formed in HEPES buffer (20 mM, pH 7.4) at an N/P ratio of 10 for PEI 25 kDa, 5 for PEI 2 kDa and 1.8 for DOTAP, to achieve a final RNA concentration of 40 µg/ml in a volume of 50 µl. Prior to injection, the samples were centrifuged at 1×10^5 r.p.m. for 5 min to sediment large aggregates. Samples were back-loaded into Femptotip II microinjection needles (BDH/Merck) and cells injected using an injection pressure of 30–50 psi, a backpressure of 100 psi and injection duration of 0.5 s. Approximately 40 injections were made per sample in each experiment, and the grid co-ordinates of each recorded. The glass grid was then washed with PBS, transferred back into DMEM containing 10% serum, and incubated at 37°C. After 24 h the number of GFP-positive cells was counted, using UV illumination and a 490 nm emission filter. In certain experiments, pAsp was added following complex formation (final concentration 100 µg/ml) prior to microinjection.

Analysis of cell cycle

For cell cycle analysis, cells were harvested by trypsinisation, washed, resuspended in PBS and fixed by dropping into a solution of 80% ethanol. Fixed cells were re-equilibrated in PBS and treated (1 h at 37°C in the dark) with a solution of PBS containing Triton X-100 (0.005%), RNase A (10 µg/ml) and SYTOX Green (0.5 µM; Molecular Probes, Leiden, The Netherlands). Cells were then analysed on a Coulter Epics XL flow cytometer equipped with a 488 nm Argon laser. Fluorescence was detected using a 530/20 nm bandpass emission filter, and results were analysed using the programme WinMDI.

RESULTS

Production of luciferase- and GFP-encoding mRNA by *in vitro* transcription

To enable assessment of the influence of mRNA structure and transgene encoded on the reporter gene expression observed, four different mRNAs were produced by *in vitro* transcription using linearised plasmids (Fig. 1A). Three of the mRNAs encoded for luciferase and contained either a (5′) m^7GpppG cap (cap-*luc*), (3′) A_{30} tail (*luc*-A_{30}) or both (cap-*luc*-A_{30}). A mRNA encoding for GFP was prepared with both (5′) m^7GpppG cap and (3′) A_{64} tail. Gel analysis confirmed that each transcript had the predicted size, was intact and free of any contaminating DNA (Fig. 1B).

Determination of the time course of mRNA expression in B16-F10 cells

The time course of DOTAP-mediated expression of cap-*luc*-A_{30} and cap-*GFP*-A_{64} in B16-F10 cells was monitored over 48 and

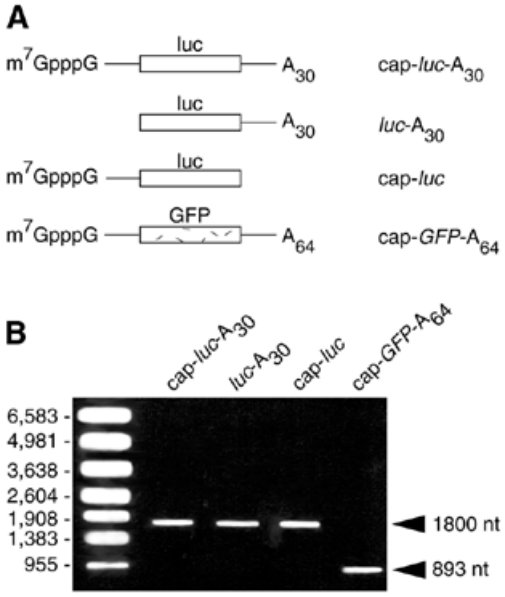

Figure 1. (**A**) Schematic representation of mRNAs used in this study. The *in vitro* transcripts were capped (m^7GpppG) or uncapped, with or without a poly(A) tail, and encode for either luciferase or GFP. (**B**) RNA transcribed *in vitro* was denaturated by heating at 65°C for 15 min and analysed on a ethidium bromide-stained 0.8% agarose gel (2 µg of purified RNA per lane) and visualised by UV translumination. No differences in migration of mRNA-*luc* transcripts with or without poly(A) tail were observed under these conditions.

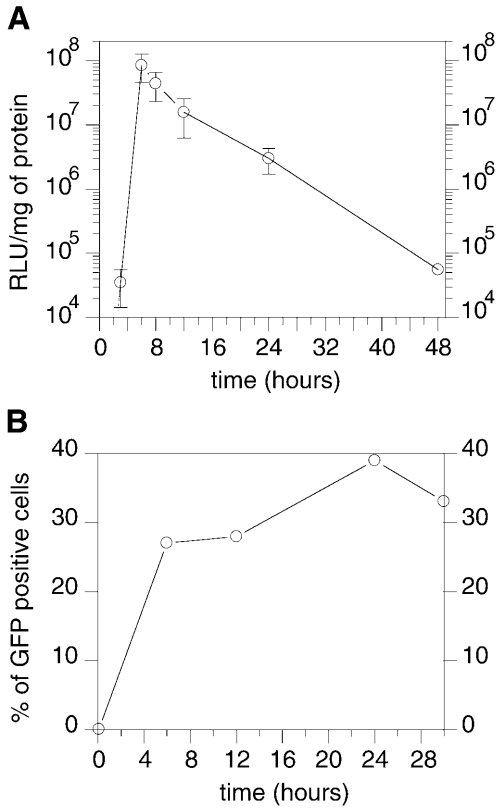

Figure 2. Time course of expression of mRNA transfection in B16-F10 cells. Complexes were prepared by mixing 1 µg of cap-*luc*-A_{30} with 5.4 nmol of DOTAP in HEPES buffer pH 7.4 as described in Materials and Methods. After 4 h post-transfection, 500 µl of fresh media containing 10% FCS were added to each well. Luciferase (**A**) or GFP (**B**) expression were assayed over 48 h and 30 h post-transfection, respectively.

30 h, respectively (Fig. 2). The maximum expression of luciferase was achieved 6 h post transfection, followed by a rapid decline (Fig. 2A). After 24 h, transgene expression amounted to only 4% of that observed at 6 h. The maximum expression of luciferase was ~25-fold higher when a 10-fold greater cell density (5×10^4 cells compared to 5×10^3 cells per well) was used (results not shown). This time-dependent profile of luciferase expression was found to be reproducible in B16-F10 cells, although the precise time course appears to differ between cell types, as others have described different kinetics. For example, the time course of luciferase expression in HOS cells using a cap-*luc*-A_{50} RNA reached a plateau at 4 h post-transfection and lasted up to 8 h (15). In the human breast carcinoma cell line, MDA MB-435 cells, the luciferase activity increased over the first 12 h and 80% of the maximum luciferase activity was still measured after 24 h, although the size of the poly(A) tail was not specified in that study (8). The kinetics of expression of GFP showed a different profile, with a peak of expression at 24 h (Fig. 2B). The different time courses of luciferase and GFP expression are likely to be related to both the mRNA stability, resulting mainly from different poly(A) tail lengths, and differential protein stability. Indeed in mammalian cells, luciferase protein has a half-life of only 3 h (16), whereas GFP is relatively stable, with a half-life of ~26 h (17).

Influence of mRNA structure and transfection agent on mRNA expression in B16-F10 cells

The structure of the mRNA construct had a significant effect on the efficiency of mRNA-mediated transfection in B16-F10 cells. In experiments using DOTAP, luciferase expression was 5-fold greater with *luc*-A_{30} than with the cap-*luc* construct, showing the importance of the poly(A) tail (results not shown). Similarly, transgene expression was 300-fold greater using cap-*luc*-A_{30} than using *luc*-A_{30}, demonstrating the necessity of having both a cap and poly(A) tail present. No luciferase expression was detectable using mRNA that lacked both poly(A) tail and cap (results not shown).

Several preparations of polyplexes and lipoplexes were tested for their ability to promote transfection using cap-*luc*-A_{30} in B16-F10 cells (Fig. 3). Polycations with different topology were used, namely linear PEI 22 kDa, PLL 54 kDa, branched PEI 25 kDa and fractured polyamidoamine dendrimer (35 kDa, Superfect™). These cationic polymers are among the best synthetic vectors available for DNA delivery, but their usefulness for mRNA transfection has not been critically evaluated before, nor compared with the activity of lipoplexes such as those formed using DOTAP and DOGS.

Interestingly none of the polycations examined, including linear PEI and Superfect™, were able to mediate detectable expression of cap-*luc*-A_{30}. In contrast, lipoplexes mediated substantial levels of mRNA-transfection: DOTAP achieved the highest level of 1.4×10^7 RLU/mg of protein, although this was 10-fold lower than that achieved with DOTAP/DNA.

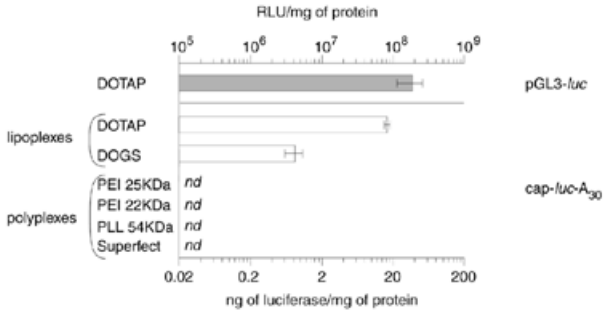

Figure 3. Lipoplexes or polyplexes/mRNA-mediated transfection of B16-F10 cells. cap-*luc*-A_{30} (1 µg) condensed by cationic lipids (DOTAP N/P = 1.8, DOGS six charge equivalent) or cationic polymers (PEI 25 kDa N/P = 10, PEI 22 kDa N/P = 5, PLL 54 kDa N/P = 2 and Superfect N/P = 8.5) were delivered to B16-F10 cells and luciferase gene expression evaluated 6 h post-transfection. The efficiency of transfection was compared to the pGL3/*luc*/DOTAP lipoplexes (luciferase expression measured 24 h post-transfection). Each experiment was performed in triplicate. *nd*, not detected.

DOGS/RNA also exhibited substantial transgene expression, albeit <5% that of DOTAP/RNA.

The transfection frequency mediated by DOTAP/RNA in B16-F10 cells was determined using mRNA encoding for GFP (cap-*GFP*-A_{64}). Flow cytometry analysis following transfection showed that 36.6 ± 0.9% of the cells were positive compared to 21.8 ± 1.1% obtained using the equivalent DNA lipoplexes (DOTAP/p*EGFP*) (results not shown).

Transfection of quiescent primary HUVEC with DOTAP/cap-*GFP*-A_{64} lipoplexes

One major advantage of using mRNA as the genetic material for modification of the cellular phenotype is its ability to undergo expression by translation in the cytoplasm, avoiding the nuclear import step that is obligatory for most DNA-based systems. This is likely to be particularly important in non-proliferating cells, where the nuclear membrane provides a consistent barrier to entry of nucleic acids into the nucleus. We therefore evaluated the ability of DOTAP/RNA complexes to mediate transfection of non-dividing cells, using HUVEC immediately following their isolation from the cord (p0). Cell cycle analysis by flow cytometry using SYTOX green showed that virtually all of the HUVEC were in G0/G1 phase of the cell cycle over the period 0–36 h post isolation (Fig. 4C). mRNA-based cationic lipoplexes showed good ability to transfect these cells; using cap-*GFP*-A_{64}, ~2.7 ± 0.1% of HUVEC were transfected (Fig. 4A). No GFP-expression could be detected using DNA in parallel experiments based on DOTAP/p*EGFP* complexes (Fig. 4B). The transfection activity of DOTAP/mRNA was also compared with DOTAP/DNA using HUVEC that had been allowed to proliferate, at passage 4 (Fig. 4F; 73.8% of the cells in G0/G1 phase, 12.5% in S phase and 13.7% in G2 phase). In these cells the frequency of mRNA-mediated transfection was increased by >3-fold, compared with p0 cells, and remained at least 10 times more efficient than parallel DNA-mediated transfections (Fig. 4D and E).

In vitro translation of mRNA encoding luciferase

The availability of mRNA for translation when formulated with transfection vectors was assessed *in vitro* using a cell-free translation system based on rabbit reticulocyte lysate (Fig. 5). The efficiency of translation of free mRNA (cap-*luc*-A_{30}) was compared with that of mRNA complexed with PEI 25 kDa or DOTAP. Translation of free mRNA produced 1.4×10^7 light units (LU), while DOTAP/RNA lipoplexes showed only 2×10^5 LU. This level of luciferase expression obtained with DOTAP/RNA was 5-fold and 100-fold greater than DOGS/RNA

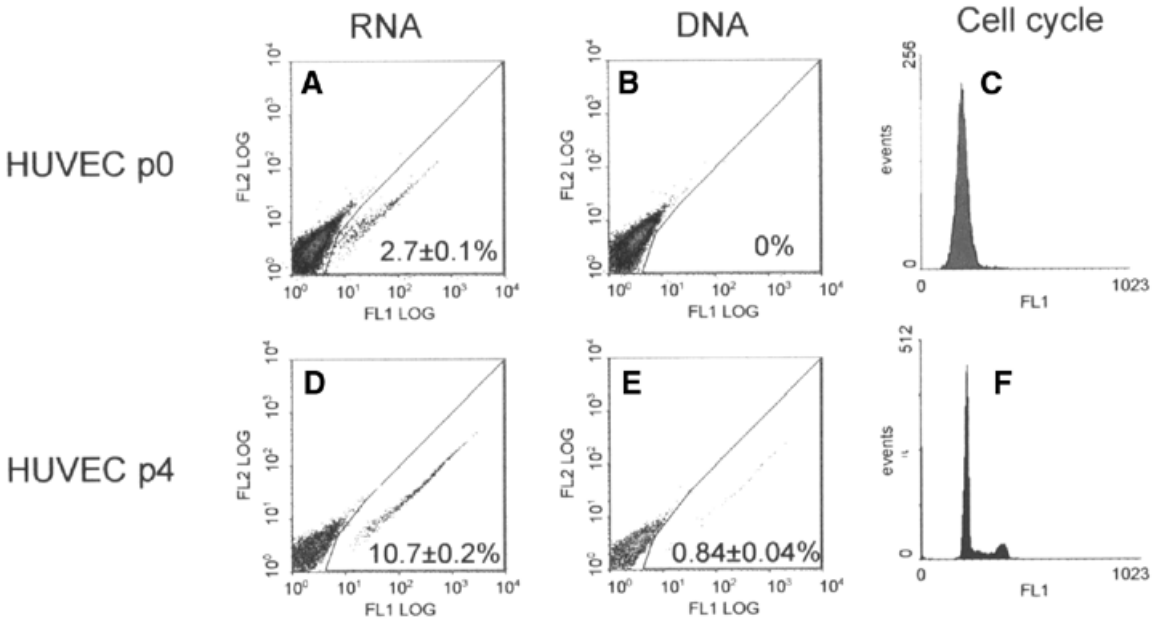

Figure 4. Relative efficiency of DNA and mRNA-mediated transfection in quiescent and slow dividing HUVEC. DOTAP/cap-*GFP*-A_{64} and DOTAP/p*EGFP* complexes were delivered, respectively, to p0 (**A** and **B**) and p4 (**D** and **E**) HUVEC. Cells were plated in 48-well plates coated with collagen, and GFP expression measured after 24 h by flow cytometry analysis as described in Materials and Methods. The cell cycle of HUVEC at passage 0 and 4 were determined by the use of SYTOX Green.

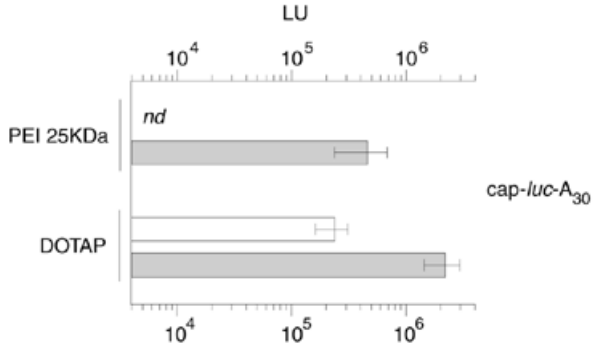

Figure 5. *In vitro* translation of mRNA using rabbit reticulocyte lysate. cap-*luc*-A_{30} (1 µg) was incubated free or condensed by PEI 25 kDa at N/P = 10, and DOTAP at N/P = 1.8 with nuclease-treated rabbit reticulocyte lysate and luciferase activity was measured after 90 min. Luciferase expression for free cap-*luc*-A_{30} is 1.4×10^7 LU. To determine whether the stability of lipoplexes and polyplexes influences the translatibility of cap-*luc*-A_{30}. 100 µg/ml of polyaspartic acid (pAsp) was added to the rabbit reticulocyte lysate prior to PEI 25 kDa and DOTAP/RNA complexes (white bars without pAsp, shaded bars with 100 µg/ml pAsp). *nd*, not detected.

Table 1. Microinjection of Rat1 cells

	Injected cells	GFP-positive cells	% GFP-positive cells
cap-*GFP*-A_{64}	113	100	88.4
cap-*GFP*-A_{64} + pAsp	31	29	93
PEI 25 kDa/cap-*GFP*-A_{64}	75	0	0
PEI 25 kDa/cap-*GFP*-A_{64} + pAsp	33	27	82
PEI 2 kDa/cap-*GFP*-A_{64}	86	9	10.5

Translation efficiency of cap-*GFP*-A_{64} free or condensed with PEI 25 kDa or PEI 2 kDa at N/P = 10 and 5, respectively. mRNA was microinjected in the cytoplasm of Rat1 cells at a concentration of 40 µg/ml (74 000 copies/cell). After 24 h the number of GFP-positive cells was counted by fluorescence microscopy. In some experiments, PEI 25 kDa/cap-*GFP*-A_{64} complexes were co-incubated with poly(aspartic acid) (pAsp, final concentration 100 µg/ml) prior to microinjection to assess whether the mRNA can be made available for translation by destabilisation of the polyplexes. The presence of a Texas Red-dextran marker within cells was used as an indicator of successful microinjection.

and PLL 54 kDa/RNA, respectively (data not shown), and no translation at all could be detected using PEI 25 kDa/RNA. These results suggest that the ribosome-based translation machinery is unable to recognise the mRNA template in PLL 54 kDa/RNA and PEI 25 kDa/RNA complexes, but that translation can occur to some extent using lipoplexes, notably DOTAP/RNA. El Ouahabi *et al.* (18) showed that complexation of RNA-*luc* by cationic lipids strongly reduces its accessibility to the translational machinery, with almost a complete loss of luciferase expression at N/P ratios over 3 in a cell-free translation system. To establish whether the absence of translation using PEI 25 kDa/RNA complexes was due to inaccessibility of the mRNA in the complex, rather than damage mediated to the RNA during the polyelectrolyte binding reaction, excess pAsp (100 µg/ml) was added to the cell-free translation system. It can be seen that pAsp treatment partially restored translation (4×10^5 LU), and similar treatment of DOTAP/RNA complexes also increased translation (2×10^6 LU). In these conditions it is likely that the cationic components of the complexes bind the pAsp, releasing at least some of the mRNA into the rabbit reticulocyte lysate and enabling translation.

Microinjection of mRNA-GFP into mammalian cells

The efficiency of translation of cap-*GFP*-A_{64} formulated with or without PEI 25 kDa was also assessed by direct injection of RNA-containing polyplexes into Rat1 fibroblasts. Table 1 summarises the data obtained after cytoplasmic injection of 74 000 copies per cell of cap-*GFP*-A_{64} either free or complexed with PEI 25 kDa at N/P = 10. Approximately 90% of Rat1 cells injected with free cap-*GFP*-A_{64} expressed amounts of GFP that could be detected by fluorescence microscopy. When mRNA was complexed with PEI 25 kDa prior to injection no GFP-positive cells were observed. To determine whether the inactivity of PEI 25 kDa/mRNA complexes could be reversed by dissociating the complexes, an excess of pAsp was added to PEI 25 kDa/cap-*GFP*-A_{64} polyplexes 30 min prior to microinjection, in order to destabilise polyplexes and thus release the nucleic acid. Under these conditions, 35 out of 40 cells expressed GFP, indicating that the mRNA could be made available for translation following disruption of the polyplexes with pAsp. DOTAP/RNA complexes were not microinjected into cells, as they tended to aggregate in the capillary causing obvious blockage.

Evaluation of mRNA polyplexes based on short polycations for transfection and microinjection

On the basis of these observations we reasoned that the inability of polycation/mRNA polyplexes to undergo *in vitro* translation or to mediate detectable expression in transfection assays might reflect their high number of positive charges, compared with cationic lipids. For example, whereas each molecule of DOTAP contains just one positive charge, and each molecule of DOGS contains 3 positive charges at physiological pH, a single molecule of PLL (54 kDa) contains over 400 positive charges and PEI (25 kDa) contains ~150 charged amino groups at pH 7. Ribosomal translation machinery may therefore be ineffective at displacing cationic charges to gain access to mRNA, and polyelectrolyte mRNA complexes too stable to undergo translation. To address this possibility we evaluated the usefulness of mRNA complexes formed using short polycations, namely PLL 3.4 kDa and PEI 2 kDa (approximately 16 and 12 positive charges, respectively). Figure 6 shows that both types of complexes were able to mediate significant levels of gene expression in B16-F10 cells. A 5- and 100-fold higher level of luciferase expression was observed compared to DOTAP/mRNA and DOGS/mRNA, respectively. Both of these agents were dependent on chloroquine to mediate their activity, with low transgene expression detected in the absence of chloroquine (~1×10^5 RLU/mg of protein). We also microinjected RNA complexes formed using low molecular weight PEI 2 kDa into Rat1 cells, and this led to 10.5% detectable GFP transfection (Table 1), suggesting that the short polycation PEI 2 kDa was more efficient than PEI 25 kDa at releasing RNA into the cytoplasm of Rat1 cells.

Melittin-mediated enhancement of expression of mRNA polyplexes based on short polycations

Although PEI 2 kDa mediated a high level of luciferase expression in B16-F10 cells in the presence of chloroquine, only very low

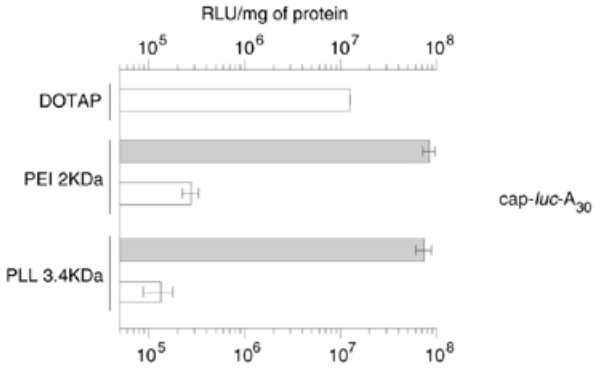

Figure 6. Transfection efficiency in B16-F10 cells of short polycation/mRNA complexes. B16-F10 cells were transfected with 1 µg of cap-*luc*-A_{30} condensed either by DOTAP at N/P = 1.8, or PEI 2 kDa at N/P = 5 or PLL 3.4 kDa at N/P = 7.5 in DMEM without serum in the absence (white bars) or presence (shaded bars) of 100 µM chloroquine. The cells were incubated with the complexes for 4 h and the medium discarded and replaced with fresh medium containing 10% FCS. Luciferase activity was measured after 6 h. Results are shown as a mean and standard deviation from triplicate samples.

activity was detected in HeLa cells and HUVEC (Fig. 7). In order to design a more powerful chloroquine-independent mRNA transfection system, we combined the ability of PEI 2 kDa to present mRNA in a form suitable for translation with a membrane-active peptide to mediate escape from the endosome. For this purpose we prepared a PEI 2 kDa–melittin conjugate linked through a disulfide group, and assessed its ability to mediate mRNA transfection in HeLa cells and p4 HUVEC (Fig. 7). It can be seen that PEI 2 kDa–melittin mediated a high level of transfection in HeLa cells (52.2 ± 2.7%) and HUVEC (71.6 ± 1.7%) in the presence of chloroquine and 31.1 ± 4.1% and 58.5 ± 2.9% in the absence of chloroquine. DOTAP was as efficient as PEI 2 kDa–melittin at transfecting HeLa cells, but 5-fold less GFP expression was measured in HUVEC. These results showed that the PEI 2 kDa–melittin conjugate is very effective in delivering mRNA in post-mitotic primary cells.

DISCUSSION

Most cells that are targets for gene therapy such as bronchiolar epithelia, hepatocytes, vascular endothelia or muscle cells are either post-mitotic or have a very low rate of cycling *in situ*. Even in cancers, the growth fraction of human tumour parenchymal cells is often low (19,20). In order for somatic gene therapy to be successful in treating these diseases, gene delivery systems must be capable of efficient transgene expression in non-cycling cells. The nuclear membrane in non-mitotic cells provides a significant barrier for non-viral gene therapy systems based on DNA, and some researchers have resorted to extreme measures (such as partial hepatectomy) to stimulate cycling of target cells (21). More sophisticated approaches are developing nuclear-homing systems that are capable of introducing the DNA into the nucleus through the nuclear pore complex (22), or using cytoplasmic DNA expression based on phage polymerase systems (23).

A simpler and more efficient alternative to these strategies is to make use of mRNA as the therapeutic genetic material, avoiding the requirement for nuclear entry and enabling efficient expression in the cytoplasm of non-cycling cells. Early concerns over the duration of transgene expression that can be achieved using mRNA appears largely unfounded, given the stability that can be achieved using mRNA bearing both 5′ cap and a substantial 3′ poly(A) tail. We found that up to 9.4×10^6 copies of GFP were expressed in B16-F10 cells when transfected with DOTAP/mRNA-GFP, whereas in the same conditions only 1.6×10^4 copies of luciferase were expressed when mRNA-*luc* was transferred into cells. This

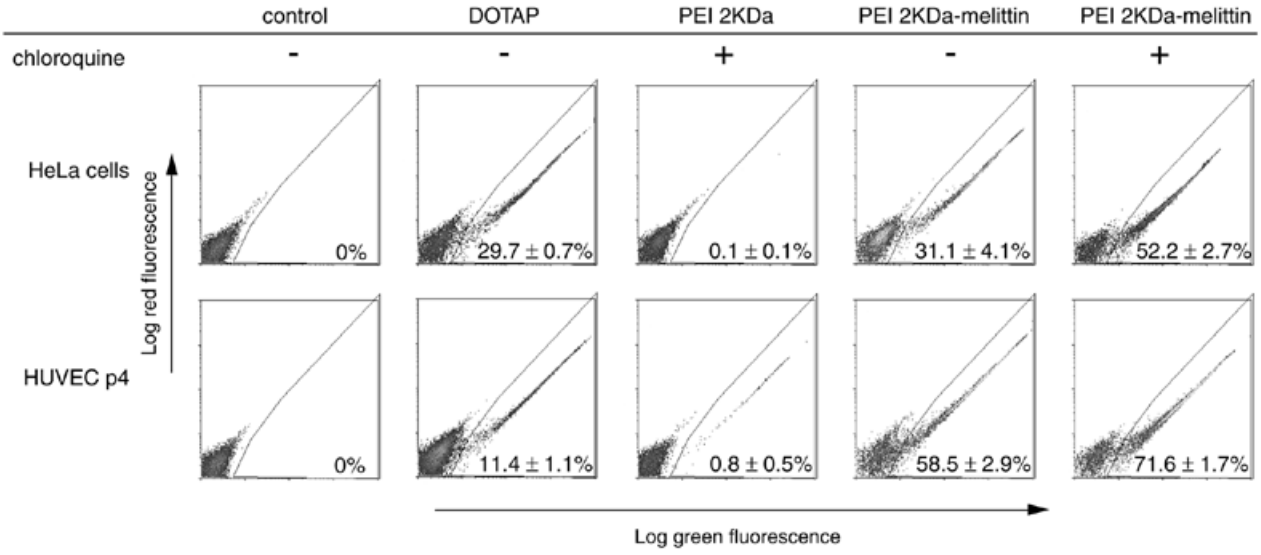

Figure 7. Transfection efficiency of PEI 2 kDa–melittin conjugate in HeLa cells and HUVEC. HeLa cells and passage 4 HUVEC were transfected with 1 µg of cap-*GFP*-A_{64} condensed either by DOTAP at N/P = 1.8, or PEI 2 kDa at N/P = 5 or PEI 2 kDa–melittin at N/P = 5 in DMEM with serum. In some cases, cells were treated with 100 µM chloroquine when transfected with PEI 2 kDa and with PEI 2 kDa–melittin. GFP expression was measured after 24 h by flow cytometry analysis as described in Materials and Methods.

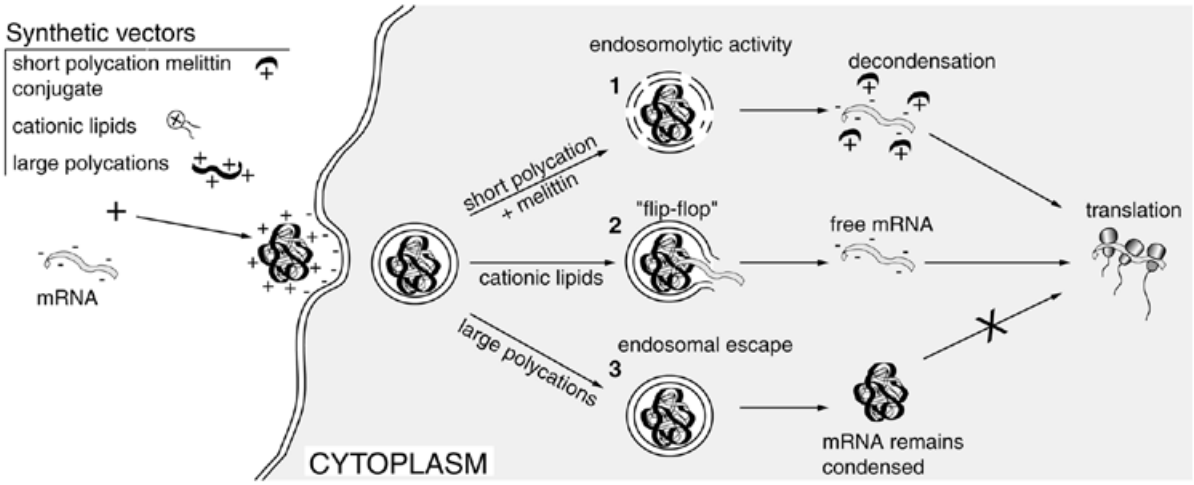

Figure 8. Proposed intracellular fate of delivered mRNA. The availability of mRNA to undergo translation in the cytoplasm is dependent on the type of synthetic vector used. (**1**) When delivered with short polycation containing the endosomolytic peptide melittin (PEI 2 kDa–melittin), complexes escape from the endosome and mRNA is efficiently released for translation to occur. In the case of unmodified short polycations (PEI 2 kDa, PLL 3.4 kDa), endosomal escape can be triggered by the buffering agent chloroquine. (**2**) When delivered with cationic lipids (DOTAP, DOGS), mRNA is released into the cytoplasm following a 'flip-flop' process between the cationic lipids and the anionic component of the endosomal membrane. (**3**) When larger polycations are used, polyplexes escape from the endosome either by release with chloroquine (PLL 54 kDa) or by the 'proton sponge' effect (PEI 25 kDa) (34). However, in the cytoplasm the polyplexes do not adequately release mRNA in a form suitable for translation to occur.

600-fold difference most likely reflects protein stability [the half-life for GFP is an estimated nine times longer than luciferase in mammalian cells (16,17)] as well as differential mRNA stability due to the longer poly(A) tail of GFP mRNA. Another possible drawback in using mRNA is its inherent instability, which can be overcome by the condensing agent. Indeed it has been shown that delivery of mRNA with cationic lipids can help to protect the mRNA from hydrolysis (18), which is in agreement with our *in vitro* transfection experiments where transfection efficiencies of both liposomal and polyelectrolyte preparations were not affected by the presence of 10% FCS (Fig. 7).

The activity of mRNA in quiescent primary cells was examined using HUVEC immediately following their isolation from the cord. Whereas DOTAP/mRNA achieved substantial expression of GFP, DOTAP/DNA encoding either GFP or luciferase (results not shown) gave no detectable signal at all. When the same cells were allowed to enter the cell cycle (26% S/G2) prior to transfection, mRNA achieved >10-fold greater frequency of transfection than equivalent procedures using plasmid DNA. Using rapidly proliferating B16-F10 cells (65% S/G2, data not shown), DOTAP/mRNA achieved high levels of luciferase expression, although DOTAP/DNA was ~10-fold better. It seems therefore that mRNA may be relatively more successful in quiescent or slowly proliferating cells, while DNA is comparable or superior in rapidly proliferating cells where nuclear entry is not significantly restricted. In most therapeutic applications *in vivo*, where cells are not cycling, there is therefore a strong case for the development of gene therapy systems based on delivery of mRNA.

The efficiency of mRNA delivery was dependent on the type of vector used. We propose, as shown schematically in Figure 8, that the intracellular fate of mRNA and availibility for translation is different with cationic lipids compared to polycations. Expression of mRNA can be achieved successfully in a broad range of cells when the mRNA is delivered using cationic lipid formulations such as DOTAP/RNA and DOGS/RNA, but not with polyplex mRNA formulations based for example on PLL 54 kDa and PEI 25 kDa which are ineffective. This difference in behaviour is most likely to be due to the relatively few positive charges that DOTAP and DOGS possess compared with large cationic polymers. Indeed DOTAP/mRNA was much more successful for cell-free translation using rabbit reticulocyte lysate than mRNA polyplexes based on PLL 54 kDa (data not shown) and PEI 25 kDa. This suggested that the translational machinery is discriminating in its recognition of mRNA, and can process mRNA only when it is either free or bound loosely to cationic molecules. If the mRNA is bound via too many electrostatic interactions simultaneously, such as in a polyplex based on a large polycation, ribosomal processing cannot occur and translation fails (Fig. 8).

To test this possibility we condensed mRNA with short polycations and examined its ability to undergo translation in cells following its introduction by intracytoplasmic microinjection and transfection procedures. In contrast to the behaviour of mRNA polyplexes formed using high molecular weight polycations that gave no expression, polyplexes formed using small polycations, namely PEI 2 kDa, produced >10% GFP-positive cells when injected directly into the cytoplasm. In transfection studies, although the polyplexes formed using low molecular weight polycations were dependent on chloroquine for endosomolysis, they achieved very high levels of reporter expression, producing even more luciferase than the corresponding cationic lipid procedures in B16-F10 cells. Given the relative instability of polyplexes based on low molecular weight polycations, particularly to the polyelectrolyte exchange reaction, it is feasible that cytoplasmic salts and polyanions such as endogenous mRNA may destabilise the complexes to yield free mRNA, while polyplexes based on larger polycations would be more resistant to destabilisation (Fig. 8) (24,25).

The possibility that polyelectrolyte complexes formed with larger polycations can be too stable to permit maximum expression of nucleic acids has been raised before, with DNA complexes based on short polylysine (3.9 kDa) showing dissociation in the cytoplasm and leading to 2-fold greater GFP expression than those based on longer polylysine (37 kDa) (26). Complexes of antisense oligonucleotides with PEI 25 kDa also showed unwanted cytoplasmic stability that may have decreased their ability to bind to their target RNA in T24 cells (27), and the delivery of RNA/DNA chimaeric oligonucleotides into primary hepatocytes using PEI 25 kDa has also been shown to be ineffective (28), possibly reflecting poor intracellular dissociation of the complexes.

There is a possibility that mRNA must be presented to the ribosome in a completely free state to enable translation to proceed. Xu and Szoka (29) hypothesised that some DNA is released free into the cytoplasm during entry of DOTAP/DNA complexes into cells, with cationic lipids remaining in the endosomal membrane. Assuming that DOTAP/mRNA follows an analogous pathway, mRNA could be released free into the cytoplasm, which might be a likely explanation for the high levels of expression observed using DOTAP/mRNA.

A major advantage of using polyelectrolyte systems, rather than lipids, for delivery of nucleic acids is the possibility for construction of well defined and discrete multicomponent vectors based on a self-assembling polyelectrolyte core. Apart from production of systems suitable for receptor-mediated targeting (30) or covalently stabilised for systemic delivery (31), peptides with specific biological functions can also be incorporated. To exemplify this using mRNA delivery we have synthesised a PEI 2 kDa–melittin conjugate and used it to condense mRNA. Melittin is a membrane-active peptide from the venom of the European honey bee that inserts into lipid membranes and promotes leakage and, at high concentrations, membrane disruption (32). Melittin has already been shown to enhance transgene expression by membrane disruption (33), and promote release of FITC-labelled PEI 25 kDa/DNA complexes from endosomes into the cytoplasm (M.Ogris, R.C.Carlisle, T.Bettinger and L.W.Seymour, submitted for publication). PEI 2 kDa–melittin/mRNA polyplexes formed here showed remarkable levels of reporter gene expression in cells known to be relatively resistant to transfection. In HUVEC the PEI 2 kDa–melittin/mRNA polyplex achieved 58% frequency of transfection, potentiated to >70% using chloroquine, compared with a maximum of 0.8% using PEI 2 kDa/mRNA and 11% using DOTAP.

There has previously been an attempt to deliver mRNA using a defined molecular conjugate, when Fisher and Wilson (10) formulated luciferase mRNA using a PLL 65 kDa–diphtheria toxin fusion protein conjugate. However, results were rather disappointing since levels of expression were 100-fold lower than DNA controls. One reason for this may be that the PLL 65 kDa contains too many positive charges for the mRNA to undergo ready translation following its entry into the cytoplasm (Fig. 8).

The use of mRNA offers many possibilities for the phenotypic modification of cells; however, the development of such approaches has been hampered by ineffective and poorly defined delivery systems. Although cationic lipid formulations can deliver mRNA successfully, applications of such vectors are relatively limited and rely on non-specific membrane activity as their means of cell entry. The elucidation in this study of the factors limiting RNA expression using polyelectrolyte systems, and the development of efficient peptide-mediated mRNA expression systems should pave the way for a broad range of sophisticated mRNA delivery vectors. These agents will certainly find important applications in targeted transfection of slowly proliferating or quiescent cells *in vitro* such as dendritic cells, and may also be suitable for transfection of post-mitotic cells *in vivo*, enabling a promising new approach to non-viral mRNA gene therapy.

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