Nuclear Localisation Sequence Templated Nonviral Gene Delivery Vectors: Investigation of Intracellular Trafficking Events of LMD and LD Vector Systems**

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The impact of a peptide that contains a nuclear localisation sequence (NLS) on intracellular DNA trafficking was studied. We used the adenoviral core peptide mu and an SV40 NLS peptide to condense plasmid DNA (pDNA) prior to formulation with 3β-[N-(N′, N′-dimethylaminoethane)carbamoyl]cholesterol/dioleoyl-L-α-phosphatidyl ethanolamine (DC-Chol/DOPE) liposomes to give LMD and LND vectors, respectively. Fluorescent-labelled lipid and peptides plus dye-labelled pDNA components were used to investigate gene delivery in dividing and S-phase growth-arrested cells. Confocal microscopic analyses reveal little difference in intracellular trafficking events. Strikingly, mu peptide associates with nuclei and nucleoli of cells within less than 15 mins incubation of LMD with cells, which suggests that mu peptide has an NLS function. These NLS properties were confirmed by cloning of a mu-β-galactosidase fusion protein that localises in the nuclei of cells after cytosolic translation. In dividing cells both LMD and LND deliver pDNA(Cy3) to nuclei within 30–45 min incubation with cells. By contrast, pDNA is detected only in the cytoplasm in growth-arrested cells over the period of time investigated, and not in the nuclei. LD systems prepared from DC-Chol/DOPE cationic liposomes and pDNA(Cy3) behave similarly to LMD systems, which suggests that mu peptide is unable to influence trafficking events in this current LMD formulation, in spite of its strong NLS capacity. We further describe the effect of polyethyleneglycol (PEG) on cellular uptake. "Stealth" systems obtained by post-coating LMD particles with fluorescent-labelled PEG molecules (0.5, 5 and 10 mol% fluorescein-PEG^{5000}-N-hydroxysuccinimide) were prepared and shown to be internalised rapidly (mins) by cells, without detectable transgene expression. This result indicates that PEG blocks intracellular trafficking of pDNA.

KEYWORDS:

gene technology · gene therapy · lipids · nuclear localisation sequence · peptides

Introduction

Gene therapy strategies depend on efficient devices for the delivery of nucleic acids into target cells.[1] Peptide- and cationic-lipid-based gene transfer vectors have shown promise for gene therapy but are far less efficient than viral gene transfer vectors, which are currently the gene delivery vehicle of choice in the majority of gene therapy clinical trials undertaken to date.[2] However, nonviral vectors such as cationic liposomes are still the subject of intensive research and their efficacy continues to improve. Cationic liposomes are usually formulated from a cationic amphiphile and a neutral phospholipid. The choice of lipids is crucial for transfection efficiency and with respect to cell toxicity.[3] As a result, major efforts are being made to synthesise novel lipids with improved properties.[4, 5] Some lipid formulations are commercially available, such as lipofectin (Gibco BRL), Transfectam (Promega) and lipofectAMINE (Gibco BRL), or more recently, Trojene (Avanti Polar Lipids).[6] The role of the cationic lipids is to interact with or destabilise cell membranes. When

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[**] *LMD = liposome:mu peptide:DNA; LD = liposome:plasmid DNA*

  1439-4227/03/04/04 $ 20.00+.50/0 ChemBioChem **2003**, 4, 286 – 298

formulated with plasmid DNA (pDNA), cationic liposomes form cationic liposome:plasmid DNA (LD, lipoplex) particles that enter cells by endocytosis and cause endosome localisation.[7–11] However, LD particles are topologically still outside the cytoplasmic compartment of the cell, with a membrane separating them from the cytoplasm.[12] Certain lipids such as phosphatidylserine and dioleoyl L-α-phosphatidyl ethanolamine (DOPE) are thought to promote endosome escape of pDNA into the cytoplasm, probably by virtue of their tendency to adopt a hexagonal (H_{II}) phase when exposed to acidic environments and thus promote membrane fusion.[13, 14] This pivotal tendency allows pDNA escape into the cytosol and prevents the transport of lipoplexes to lysosomes, where enzymatic degradation may destroy LD particles.[15] The pDNA used in nonviral delivery systems is mainly of bacterial origin. Such pDNA can cause inflammation and toxicity at the doses required for in vivo application.[16] Components of the mammalian immune system are activated by bacterial, but not by vertebrate DNA, which suggests species-dependent structural differences.[17] This drawback is ascribed to the increased number and unmethylated status of bacterial CpG motifs compared to mammalian genomic sequences.[18] Delivery of high doses of LD particles to the mouse airway epithelium is associated with unacceptable levels of inflammation/toxicity.[16–19]

Increasingly, ternary nonviral systems are being developed that use a nucleic acid condensing species, liposomes and pDNA. Nucleic acid condensing compounds neutralise the negative nucleotide charges of pDNA, and consequently condense the pDNA volume to a fraction of its natural state.[20, 21] Often, the nucleic acid condensing compounds are cationic peptides, such as peptides containing poly- and oligo-L-lysine, protamine, polyimidazole, and oligo-L-lysine.[22–26] Bifunctional cationic peptides containing a receptor-targeting moiety have also been described.[27] Alternative nonpeptide species that have been used include histones, polyethyleneimine, poly[(2-dimethylamino)ethylmethacrylate], cationic peptoids and starburst dendrimers.[28–34] We have recently described a ternary nonviral gene delivery system referred to as liposome:mu:DNA (LMD).[35, 36] This vector is based on the cationic adenoviral core peptide μ (mu or M, MRRAHHRRRRASHRRMRGG) that condenses pDNA into MD (mu:DNA) nanoparticles (100 ± 20 nm in diameter) at an optimal ratio of 0.6 equivalents (w/w) peptide per pDNA molecule.[37]

The work presented herein aims to shed light on mechanistic and functional aspects of the intracellular trafficking of LMD systems compared to one other ternary system, LND, and a binary LD system, all prepared by using 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl]cholesterol (DC-Chol)/DOPE (60:40, m/m) cationic liposomes. LND is similar to LMD except that the mu peptide is substituted by an SV40 derived peptide (N; RRRPKKKRKVSRRR) comprised of the SV40 nuclear localisation sequence (NLS) flanked by arginine residues. We describe the preparation and properties of a fully orthogonal threefold-labelled LMD system and an orthogonal two-fold labelled LND system and make a comparison between LMD, LND and LD systems prepared from pDNA labelled with cyanine dye Cy3. Our data provide clear indications about the problems that remain to be solved before nonviral vector systems suitable for routine in vivo applications may be developed.

Results

Fluorescent and colorimetric properties of the particles

LMD

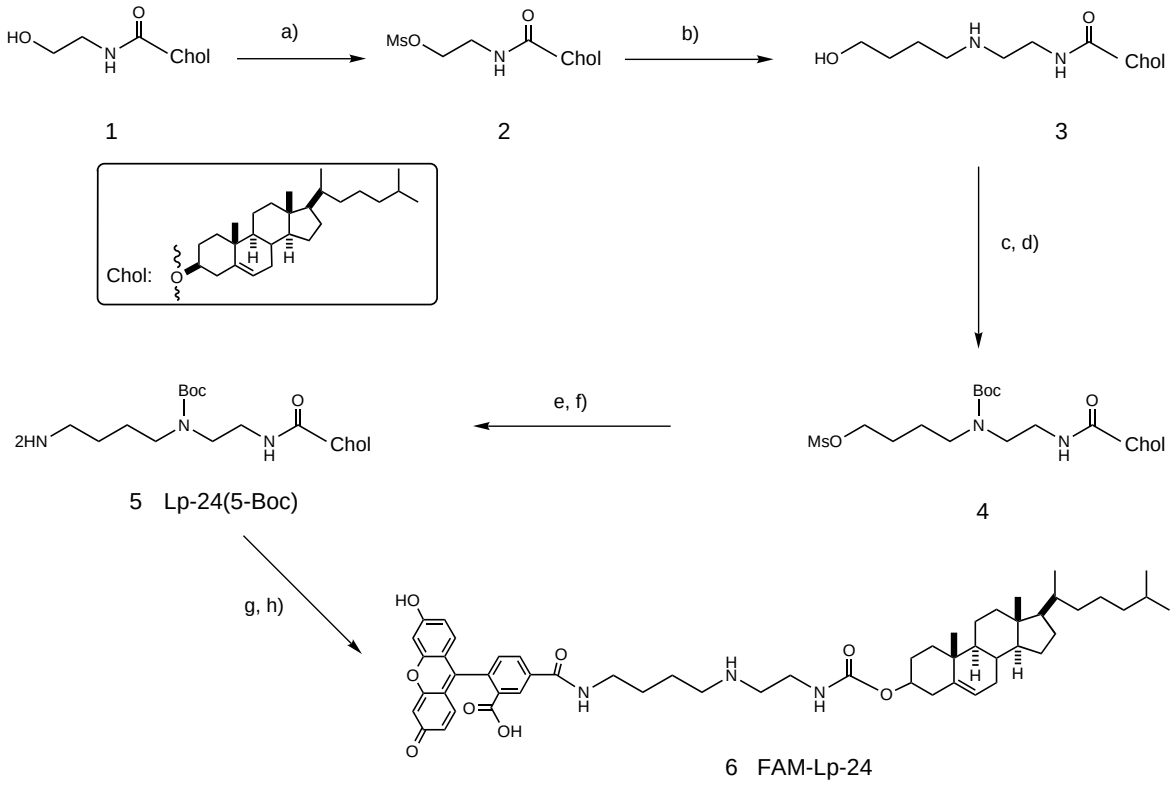
Prior to the formation of a fully orthogonal threefold-labelled LMD system, fluorescent- and dye-labelled components were prepared. First, mu peptide was synthesised and quantitatively labelled at its *N* terminus with the fluorophore tetramethylrhodamine (TAMRA, red emission, intensity maximum, $I_{max} = 570$ nm). Second, novel cationic lipid-24 (Lp-24) was synthesised and derivatised with the fluorophore 5-carboxyfluorescein (FAM, green emission, $I_{max} = 518$ nm) to give fluorescent lipid FAM-Lp-24 (Scheme 1). FAM-Lp-24 was formulated with DC-Chol and DOPE lipids to give DC-Chol/DOPE/FAM-Lp-24 cationic liposomes (57:40:3, m/m/m). Finally, the β-galactosidase reporter gene plasmid pUMVC1 (7528 bp) was labelled enzymatically with either cyanine dye Cy5 (blue colour, $\lambda_{max} = 649$ nm) or Cy3 (red, $\lambda_{max} = 568$ nm) by using a modified nick-translation procedure to give pDNA(Cy5) and pDNA(Cy3), respectively.[38] The fluorescent profile of the threefold-labelled LMD was dominated by the rhodamine-labelled mu peptide. We have previously shown that at equilibrium, 1200 – 1400 copies of mu peptide are involved in the stabilisation of the MD complex prepared from pUMVC1, a fact that underlines this observation.[37] By contrast, the fluorescence intensity of FAM-Lp-24 was quenched by approximately 30% when formulated into cationic liposomes, a result consistent with insertion into the lipid bilayer (results not shown). Colour emissions from the pDNA(Cy5) was found to be considerably weaker than for the mu peptide and FAM-Lp-24 lipid, probably as a result of the low levels of dye labelling. In general, the low sensitivity of Cy5 is an intrinsic problem for detection by confocal microscopy. Therefore, single-dye-labelled LMD was also formulated by using the more readily detectable pDNA(Cy3) for confocal microscopy studies involving a comparison between LMD, LND and LD systems, all prepared from DC-Chol/DOPE (60:40, m/m) cationic liposomes.

LND

SV40-derived peptide (N) that contains the nuclear localisation sequence (NLS) of the SV40 T large antigen,[39] was N_α-labelled with FAM on the solid phase after completion of the peptide synthesis. Two-fold-labelled LND was formulated with this FAM-SV40 derived peptide and pDNA(Cy5). A single-dye-labelled LND was prepared with pDNA(Cy3).

Particle sizes

DNA condensation studies were performed with quantitatively N_α-labelled peptides and pDNA prior to determining the number of equivalents of peptide required to condense pDNA(Cy3). Different peptide:pDNA ratios can afford different particle sizes and stabilities, especially when fluorescent-labelled peptides such as TAMRA-mu and FAM-SV40-derived peptides are employed. A profile of particle sizes as a function of peptide:pDNA ratios between 0.4 and 2.0 was obtained by photon correlation



Scheme 1. *Chemical synthesis of lipid FAM-Lp-24. Reagents used for the individual steps: a) MsCl (2.5 equiv), NEt_3 (3 equiv), DCM (0.2 M); b) 4-aminobutan-1-ol (10 equiv), NEt_3 (1.1 equiv), THF (0.2 M); c) $(BocO)_2$ (1.1 equiv), NEt_3 (1.1 equiv), DCM (0.2 M); d) MsCl (2.5 equiv), NEt_3 (3 equiv), DCM (0.2 M); e) NaN_3 (5 equiv), NaI (1 equiv), DMF (0.2 M), 80 °C; f) PMe_3 (1.15 equiv), H_20/NH_3, THF (0.1 M); g) FAM (0.83 equiv), HBTU (1.7 equiv), DIEA (2.5 equiv); h) TFA/H_20 (95:5), 2 h. Boc = butoxycarbonyl.*

spectroscopy (PCS) and is shown in Figure 1. Over the range of peptide equivalents tested, a particle diameter of 130 ± 20 nm was measured both for mu peptide:pDNA and for SV40-derived peptide:pDNA. For consistency with unlabelled LMD systems, a standard peptide:pDNA ratio of 0.6 equivalents peptide with respect to pDNA (w/w) was used for condensation throughout. Typically, a slight increase in size of about 20 – 35 nm occurred when MD or ND particles were complexed with cationic liposomes (results not shown). The average size of the three-fold-labelled LMD particles was 163 ± 20 nm, and that of the two-fold labelled LND particles was 150 ± 20 nm, as determined by dynamic light scattering.

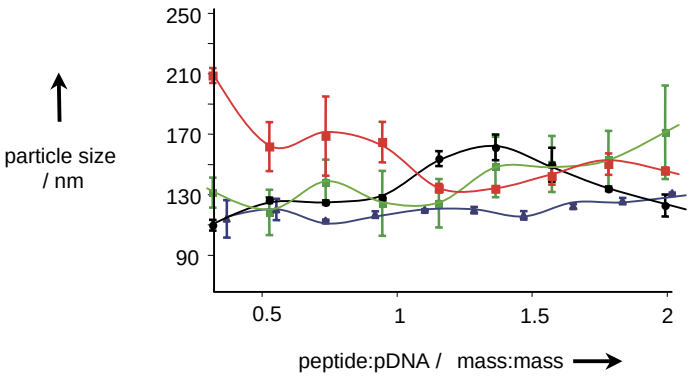


Figure 1. *Dynamic light scattering analyses with mu and SV40-derived peptides at different peptide:pDNA ratios. Blue triangles: mu:pDNA; black circles: SV40-derived peptide:pDNA; red squares, TAMRA-mu:pDNA; green squares: FAM-SV40:pDNA. Upon complexation of these peptide/pDNA nanoparticles with liposomes, a general size increase of 20 – 35 nm was observed (data not shown).*

Electrophoretic gel migration

Charge neutralisation and pDNA condensation were examined by observation of the electrophoretic migration of the pDNA in agarose gels. Retardation was observed at all labelled peptide:pDNA ratios (Figure 2). However, even in an excess of either TAMRA-mu or FAM-SV40-derived peptide (4 equivalents), complete retardation was not achieved. By contrast, total retardation of the pDNA was observed in an experiment with TAMRA-$[K]_{16}$ at 0.7 equivalents (w/w) of peptide:pDNA (results not shown). This indicates that TAMRA-$[K]_{16}$ binds more strongly with pDNA than either TAMRA-mu or FAM-SV40 peptide. In a separate lane, TAMRA-mu peptide in the absence of pDNA was found to migrate towards the negative pole of the gel (Figure 2 B, lane 13).

Unlabelled mu peptide at peptide:pDNA ratios (w/w) exceeding 0.7 also completely prevents migration of pDNA.[37, 40] We used equilibrium dialysis to determine the dissociation constant, K_d, for the interaction of TAMRA-mu with pDNA. A value of 23 mM was determined, which shows that the interaction with pDNA is weakened by at least an order of magnitude in comparison with unlabelled mu peptide when the N_α terminus of the peptide is capped with TAMRA.[37] Indeed, LMD particles prepared with

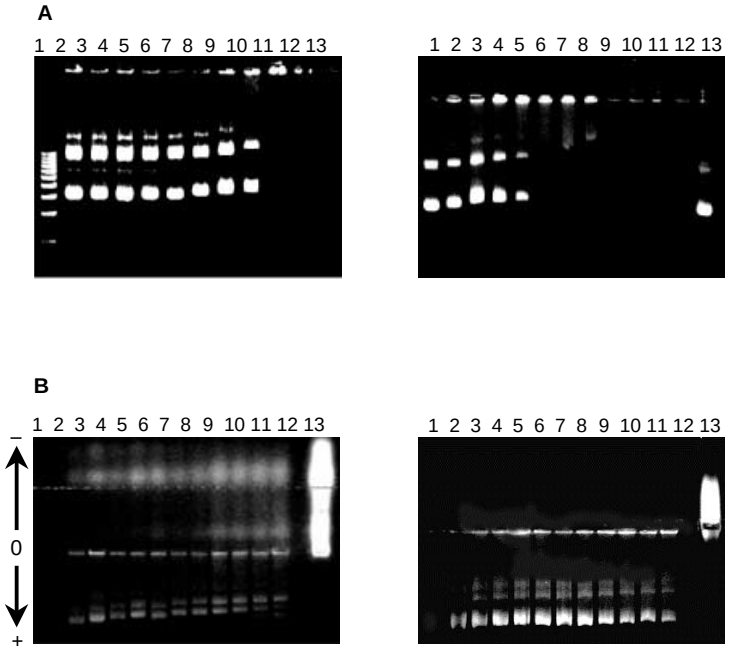


Figure 2. *Electrophoretic migration shift assays of peptide/pDNA complexes for peptide:pDNA (w/w). A) Left: mu peptide; lane 1, 1-kb ladder; lane 2, naked pDNA (1 μg); lanes 3 – 13 peptide:pDNA complexes with the amount of peptide:pDNA increasing by 0.1 mg per lane. Right: SV40-derived peptide; lane 1, 1 μg pDNA; lanes 2 – 13, peptide:pDNA complexes with the amount of peptide:pDNA increasing by 0.1 μg per lane. Note that both peptides retain pDNA in the wells for ratios exceeding 0.7. B) Left: detection of TAMRA-labelled mu; right: FAM-labelled SV40-derived peptide. Both gels were visualised above a UV source (254 nm) without addition of ethidium bromide. Lanes 13 contain the labelled peptides only and show that the peptides migrate to the negative pole. Even with an excess of peptide over pDNA, complete pDNA retardation is not achieved.*

TAMRA-mu peptide were generally found to be about 20 nm larger than those prepared with unlabelled mu peptide (Figure 1), a result that confirms differential interactions between the two peptide species with pDNA as a result of N_α-terminal labelling.

Confocal microscopy analysis of threefold-labelled LMD and LND

The cellular trafficking of the ternary LMD and LND systems was investigated by confocal microscopy. 56 FHTE8o$^-$ cells were grown on cover slips, transfected with threefold-labelled LMD or twofold-labelled LND and fixed after 15 minutes. Intriguingly, TAMRA-mu peptide (red label) and the FAM-SV40-derived peptide (green label) were localised substantially in cell nuclei after 15 minutes incubation of cells with either vector system. Both TAMRA-mu and FAM-SV40-derived peptides were seen predominantly localised in the nucleoli and nuclei, as demonstrated by the yellow colour in Figure 3 B (bottom panel, left), which is due to the overlay of the green and red colours. By contrast, both lipid FAM-Lp-24 (Figure 3 A, green label) and pDNA(Cy5) (Figure 3 A and B, blue label) were found predominantly in the cytosol after 15 minutes.

After 30 minutes incubation of the cells with LMD or LND, the majority of both the lipid and pDNA were visible as condensed spots outside the nucleus. However, detection of the pDNA was difficult and these results are therefore not conclusive. Some lipid also appeared to be in the nuclei of several cells, visible as yellow spots due to the overlay of red and green (data not shown).

Confocal microscopy analysis of single-labelled LMD(Cy3), LND(Cy3) and LD(Cy3)

The decreased particle integrity of threefold-labelled LMD led us to investigate the intracellular trafficking of pDNA on its own. The results described above suggest that separation of peptide and pDNA occurs within 15 minutes of cell entry, which might be due to decreased binding affinity between the peptide and pDNA owing to the presence of fluorescent and dye reporter groups. Consequently, we were concerned as to whether or not these threefold-labelled systems were appropriately describing the situation of intracellular trafficking involving unlabelled vector systems. Therefore, we elected to switch to single-dye-labelled systems in which pDNA was labelled with cyanine dye Cy3, which emits at red wavelengths and is consequently more readily detected than the other dyes considered.

A comparative study was then carried out between LMD(Cy3), LND(Cy3) and LD(Cy3) vector systems to investigate the relative influence of mu and SV40-derived peptides on the kinetics of pDNA nuclear trafficking. Cells were treated for just two minutes with each vector system in turn and then washed extensively to remove excess vector system and avoid the possibility of a continuous gradient from the extracellular medium into the cells during the course of the experiments. Cells were then incubated and fixed at 7, 15, 30 or 45 minutes of incubation to give a time course record. The intensive intracellular fluorescence of the Cy3 dye was a clear sign that substantial amounts of all vector systems were internalised by cells even within two minutes of incubation. The cellular trafficking of all three systems was characterised by rapid localisation of pDNA(Cy3) in the cytosol followed by entry into nuclei within 30 – 45 minutes of incubation. When cell growth was arrested by treatment with aphidicolin, pDNA(Cy3) was found to be rapidly internalised into cells ($<$ 2 minutes), as observed with dividing cells. However, after 45 minutes, pDNA(Cy3) was still found associated along nuclear membranes irrespective of whether LMD(Cy3), LND(Cy3) or LD(Cy3) systems were under observation (Figure 4).

Transfection efficiency

The steric demands of fluorescent labels led us to perform control transfection experiments to demonstrate that threefold-labelled LMD and two-fold labelled LND were still able to transfect the 56 FHTE8o$^-$ human tracheal epithelial cell line used in confocal experiments in spite of the presence of fluorescent labels. Fully labelled LMD and LND were shown to be able to

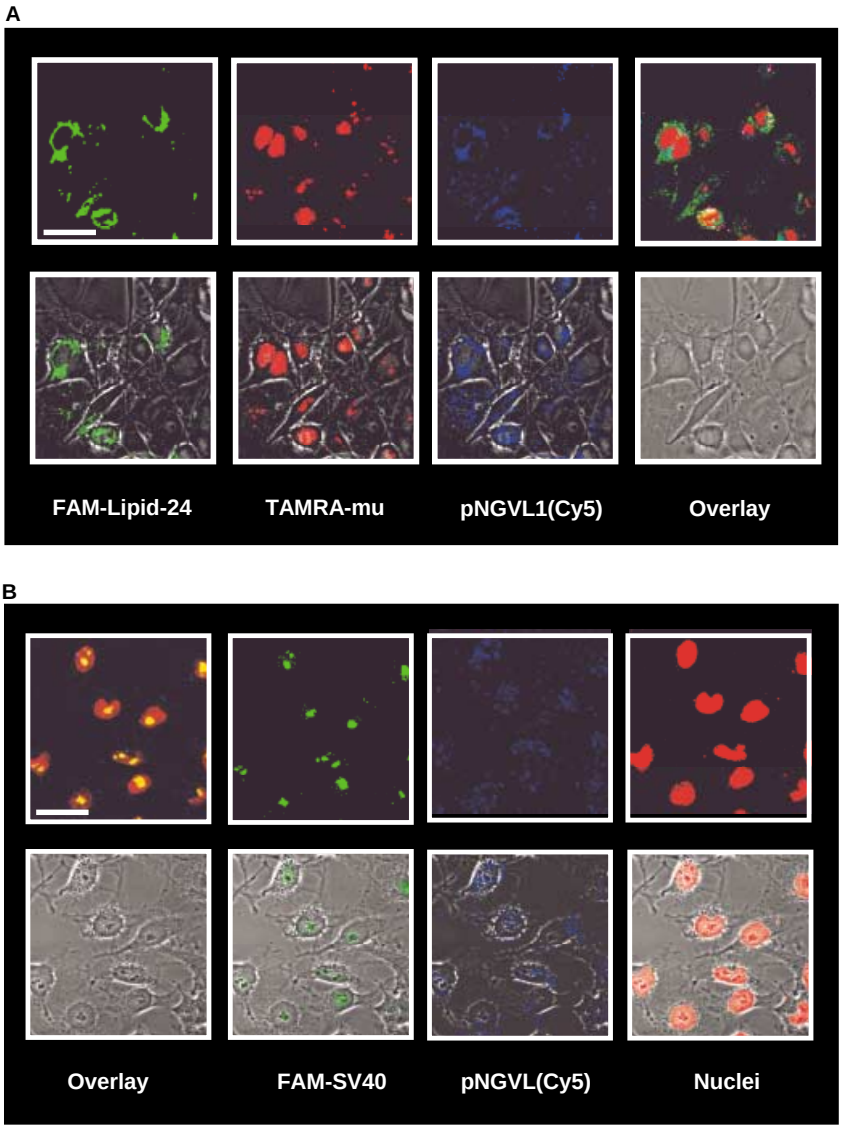


Figure 3. *Confocal microscopy analyses of threefold-labelled LMD (A) and twofold-labelled LND (B) after 15 minutes incubation of the vector system with 56 FHTE8o$^-$ cells. In the case of threefold-labelled LMD (A), TAMRA-mu is in red, the FAM-Lp-24 lipid in green and the pDNA(Cy5) in blue. For the twofold-labelled LND vector system (B), FAM-SV40-derived peptide is in green and the pDNA(Cy5) in blue. The red colour in panel B is caused by the staining of the nuclei with propidium iodide. Most of the peptide is localised in the nucleoli. The bar represents 10 μm.*

transfect 56 FHTE8o$^-$ cells 50 – 60% as efficiently as unlabelled LMD and LND systems (results not shown). Unlabelled LMD and LND systems were found to exhibit very similar transfection efficiencies, whereas LD systems showed less time and dose efficiency on this particular cell line (Figure 5), in agreement with previous observations.[36]

Effect of surface PEGylation on cellular uptake

Polyethylene glycol (PEG) lipids are commonly employed in liposome and other lipid-based drug delivery systems to provide a steric barrier at the particle surface, which inhibits protein binding and, therefore, opsonisation in vivo.[41–43] We applied a post-coating method to stabilise LMD vector systems with 0.5, 5 or 10 mol% fluorescein-PEG^{5000}-NHS (NHS = *N*-hydroxysuccinimide; green, I_{max} = 520 nm).[44] Excess (hydrolysed) PEG was removed by extensive dialysis. 56 FHTE8o$^-$ cells were incubated with fluorescein-PEGylated LMD vector systems, followed by extensive washing after two minutes to remove excess vector system. Confocal analysis revealed that rapid internalisation had taken place, as demonstrated by the intracellular localisation of the green colour derived from the fluorescein label of the PEG (Figure 6). However, these same fluorescein-PEGylated LMD vector systems were unable to mediate transgene expression of cells to levels significantly above that achieved by naked pDNA transfection (data not shown), in spite of the clear cellular internalisation.

Nuclear localisation and transcriptional activation properties of peptides

The potential nuclear localising properties of mu peptide were determined by using a series of recombinant expression plasmids (based on pUMVC1) that express β-galactosidase as an N-terminal fusion with peptide sequences that include the mu peptide sequence. The plasmid pUMVC1 described above harbours the β-galactosidase gene (*lacZ*) downstream of a nucleotide sequence coding for the NLS of SV40 large T antigen. When this fusion gene is expressed in mammalian cells, the corresponding fusion protein is detected microscopically in cell nuclei after X-gal staining. Fusion protein is expressed initially in the cytosol but becomes localised in the nucleus under the influence of the NLS. By means of site-directed mutagenesis, the nucleotide sequence coding for the NLS was removed and replaced by an *NaeI* restriction enzyme site to give a new plasmid pUMVC1-cyt. Double-stranded oligonucleotide sequences were then separately engineered into the *NaeI* restriction site coding for the NLS of SV40 large T antigen again (PPKKKRKVEDPK) as a positive control, into mu peptide (MRRAHHRRRRASHRRMRGG), reordered mu peptide (MHRHRGRAASRRRRHRRMG) and reverse mu peptide (RPSAGAAAAGRRPGPGAGA). Reordered mu peptide contains the same amino acid composition as mu peptide but with a substantially altered sequence, whilst reverse mu is a "nonsense" peptide that results from a reverse reading of the original mu peptide oligonucleotide coding sequence. The sequences of all these *NaeI* inserts were confirmed by DNA sequencing. Separate lipofectAMINE-mediated transfections of HeLa cells were then performed with each of the *NaeI* insert plasmids in turn. Cells were then incubated for a further 24 h

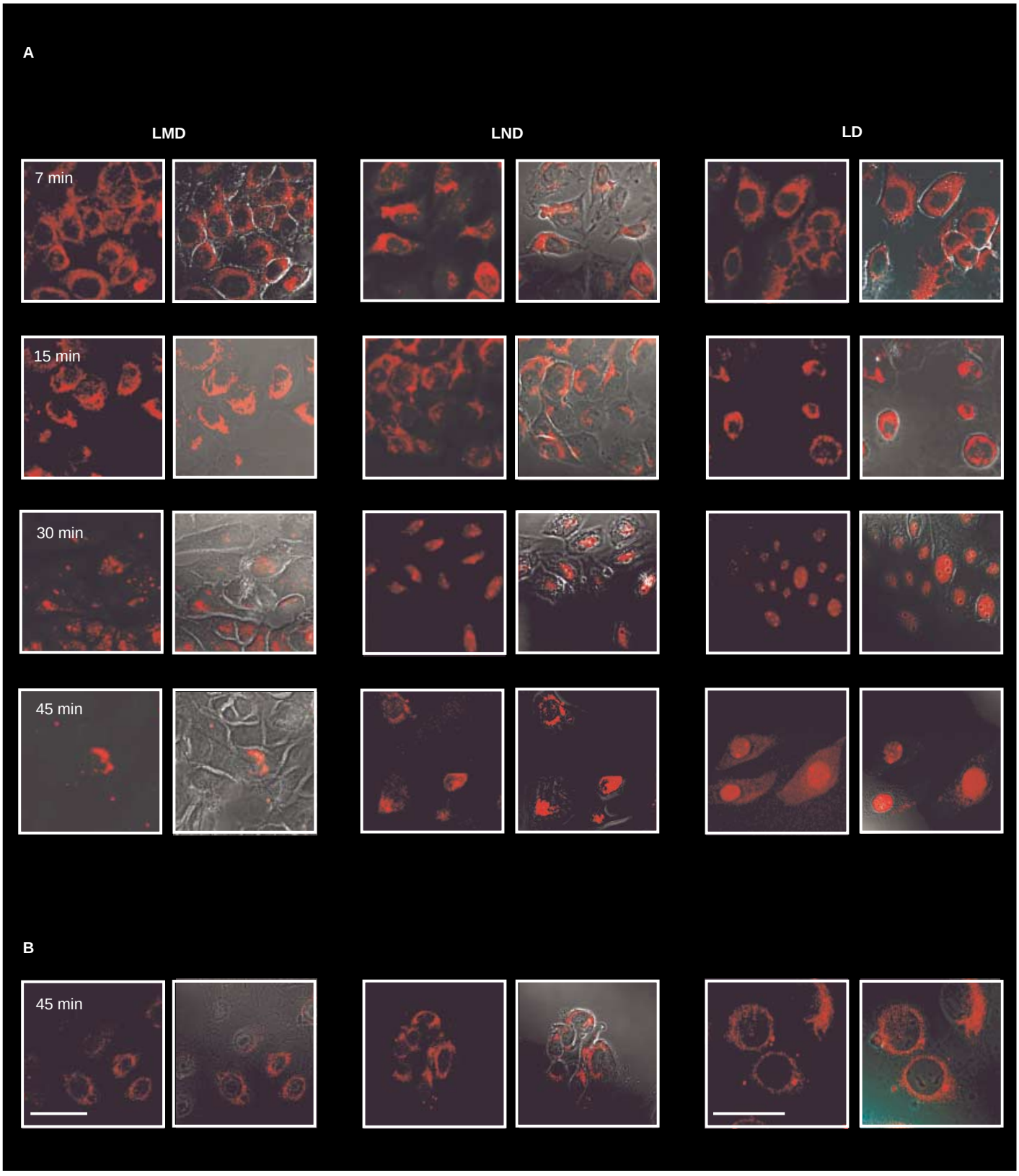


Figure 4. *Confocal microscopy images of 56 FHTE8o$^-$ cells transfected with Cy3-labelled LMD, LND and LD systems. The cells were washed thoroughly with PBS after two minutes incubation with the vectors and fixed with paraformaldehyde at the indicated times. A) Dividing 56 FHTE8o$^-$ cells. B) Synchronised (growth-arrested with aphidicolin) 56 FHTE8o$^-$ cells. No pDNA was detected in the nuclei of growth-arrested cells. The bar represents 10 μm.*

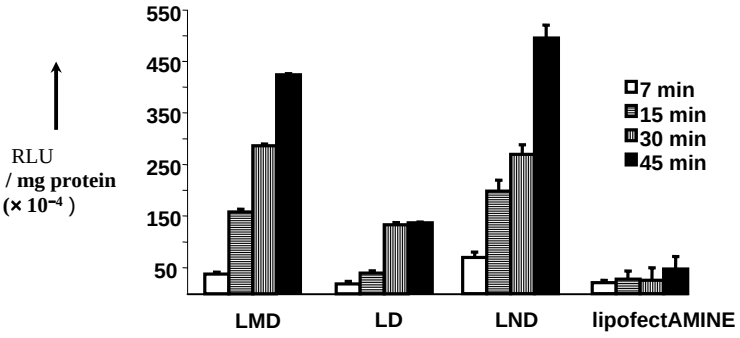


Figure 5. Transfection experiments on 56 FHTE8o⁻ cells. LMD, LND, LD and lipofectAMINE were transfected in normal growth medium (10% serum) for the indicated time periods, washed with PBS and incubated for 48 h at 37 °C. Identical experiments with synchronised cells (aphidicolin) gave transfection levels no different from naked plasmid DNA transfection (graphs not shown). RLU = relative light units.

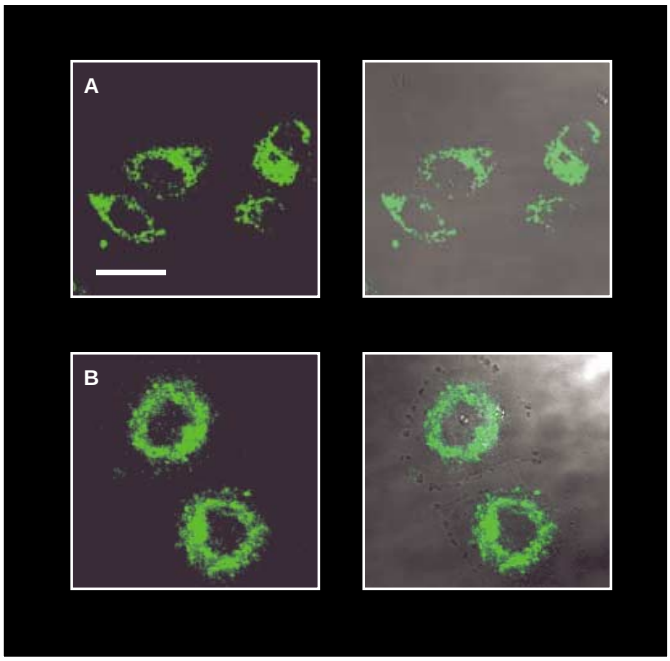


Figure 6. Confocal microscopy studies of LMD surface modified with fluorescent-labelled polyethylene glycol (fluorescein-PEG^{5000}-NHS); A) 0.5 mol%, B) 5 mol% fluorescein-PEG^{5000}-NHS. LMD was prepared as described and NHS-activated polyethyleneglycol-fluorescein was conjugated according to a post-coating protocol described by Wagner et. al.[44] After intensive dialysis, human tracheal cells (≈ 10^5) were incubated with the vectors (0.5 μg) for two minutes, then the wells were washed with PBS (3 ×) and incubated for 45 minutes. The cells were fixed with paraformaldehyde. Bright cytosolic fluorescence was detected by confocal microscopy analyses for both formulations A and B. The bar represents 10 μm.

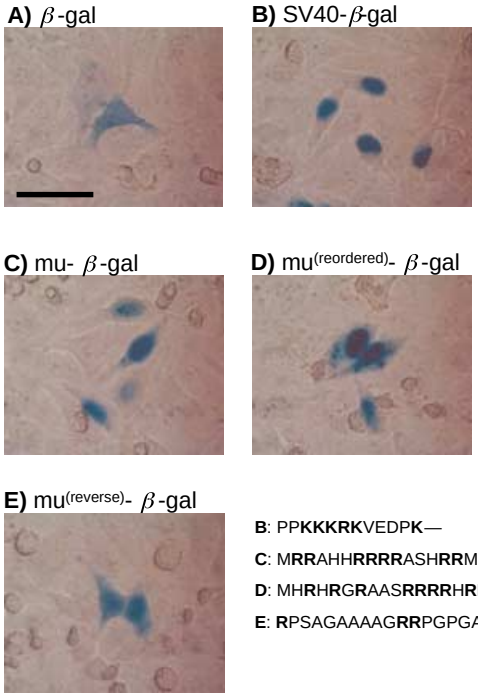


Figure 7. Nuclear localisation of β-galactosidase by NLS peptides. HeLa cells were transfected with expression plasmids encoding either β-galactosidase (A), fusion proteins consisting of β-galactosidase fused with the NLS peptide region from SV40 (large T antigen) (B), mu peptide (C), reordered mu peptide (D) or reverse mu peptide (E). The cells were incubated for 24 h to allow the recombinant protein to be expressed, then fixed and stained. The expression and distribution of the recombinant protein was visualised microscopically. The recombinant proteins containing the SV40 NLS, mu and reordered mu are clearly translocated from the cytoplasm to the nucleus, whilst the proteins comprising β-galactosidase alone or reverse mu are not relocated and remain distributed throughout the cytoplasm. The bottom right panel shows the sequences of the peptides fused to β-galactosidase. The bar represents 10 μm.

post transfection, then fixed and stained with X-gal. The subcellular distribution of expressed β-galactosidase fusion proteins was then assessed in each case by means of light microscopy. Representative cells post-transfection are shown in Figure 7 for each of the *NaeI* insert plasmids in turn and the control pUMVC1-cyt plasmid. Cells transfected with pUMVC1-cyt or the reverse mu insert plasmid clearly showed a pattern of cytosolic β-galactosidase expression consistent with a lack of expressed NLS. In contrast, all cells transfected with the remaining *NaeI* insert plasmids exhibited a clear pattern of β-galactosidase expression in the nucleus consistent with the expression of an active NLS.

The observed differences in transfection between binary and ternary vectors, at least in certain cell lines such as human tracheal cells (Figure 5), led us to investigate the possibility of an active impact of the mu and SV40-derived peptides on the cellular transcription process. For this purpose, we used pEGFP-C1, which encodes a red-shifted variant of wild-type green fluorescent protein (GFP), to transfect 56 FHTE8o⁻ cells with LD, LMD and LND vectors. The cells were then fixed after 15, 30, 45, 60, 120, 180, 240 minutes, respectively. Visualisation of the GFP fluorescence by confocal microscopy revealed no difference in the onset of green fluorescence caused by cellular production of green fluorescent protein (Figure 8). Cells transfected with binary and ternary vectors generated GFP as early as 15 minutes post transfection, and a substantial amount of protein was obtained only 4 h post transfection (Figure 8).

Discussion

We have recently devised an important new type of nonviral vector system known as liposome:mu:DNA (LMD). LMD systems

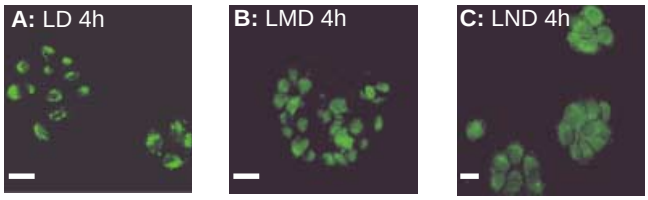


Figure 8. *Visual representation of the green fluorescence generated from GFP expression 4 h after transfection of 56 FHTE8o$^-$ cells with LD (A) LMD (B) and LND (C). Note that over all time courses investigated (15 minutes to 8 h), no difference in the onset of green fluorescence for the three formulations was observed by confocal microscopy, which indicates that the NLS peptides mu and SV40 do not play an active role in the transcription process. The bar represents 10 μm.*

can be formulated in a reproducible and scalable manner, they are stable to aggregation in low ionic strength medium, amenable to long-term storage and able to deliver reproducible transfection results in vitro, ex vivo and to a certain extent in vivo.[36] However, as we have already made clear, LMD systems are not yet matured to a state that allows them to compete with viral vector systems for clinical applications. To achieve this goal a clear understanding about the real limitations of these LMD systems will be required. The biophysical and mechanistic studies described here represent an important first stage in the process of revealing the limitations of LMD systems and lead to clear suggestions about future directions that should be taken. The behaviour of LMD systems was compared and contrasted with the behaviour of a comparable LND system and an equivalent LD system. In all cases, DC-Chol/DOPE (60:40, m/m) cationic liposomes were used as the basis of the system. The SV40-derived peptide (N) was prepared to provide a positive comparison with the mu peptide (μ or M) in order to determine if mu peptide possesses any unique characteristics over and above the characteristics of a cationic, arginine-residue-rich peptide with pDNA condensing and packaging properties.

The basic tenet of mechanistic confocal studies of the type described above is that the introduction of fluorescent labels does not substantially impair either the structural integrity or the transfection characteristics of any one nonviral vector system so that conclusions about the intracellular trafficking behaviour of fluorescent- and dye-labelled components can be extrapolated to the behaviour of unlabelled components. Our data suggest that even with threefold-labelled LMD systems, this basic tenet is broadly upheld, although the inclusion of fluorescent labels does perturb biophysical properties. Both TAMRA-mu and FAM-SV40-derived peptides produced larger MD and ND particles upon interaction with pDNA, respectively, than the corresponding unlabelled peptides (Figure 1). This observation suggests that the interaction of both fluorescent-labelled peptides with pDNA is adversely perturbed compared with unlabelled peptides by the presence of N-terminal fluorescent groups. Data from agarose gel retardation assays (Figure 2), and the evaluation of an elevated dissociation constant, K_d, of 23 mM for the interaction between TAMRA-mu peptide and pDNA are certainly consistent with this suggestion. Fortunately, these biophysical differences between labelled and unlabelled peptides did not appear to seriously impair either the formulation of threefold-labelled LMD and twofold-labelled LND systems or the functional transfection of human tracheal epithelial cells (56 FHTE8o$^-$ cells) in vitro, although the transfection occurred at lower levels than with completely unlabelled systems (Figure 5). Therefore, confocal data obtained with both threefold- and twofold-labelled systems should be qualitatively accurate with respect to the behaviour of unlabelled systems. The observation that peptides and pDNA rapidly dissociate ($<$15 minutes) after entry of either nonviral vector system into 56 FHTE8o$^-$ cells is thus likely to be representative of the behaviour of unlabelled peptides and pDNA (Figure 3), with the caveat that TAMRA-mu and FAM-SV40-derived peptides should separate from pDNA(-Cy5) more readily than corresponding unlabelled components owing to their generally weaker mutual association properties. The fact that the FAM-SV40-derived peptide was able to enter the nucleus or nucleolus of each transfected cell so rapidly ($<$15minutes) whilst the pDNA(Cy5) is left in the cytosol is not surprising since this peptide possesses a well-known NLS sequence anyway,[45] whose efficacy has been further demonstrated by the results of *NaeI* insert plasmid transfections (Figure 7). Significantly, TAMRA-mu peptide behaviour was sufficiently similar to that of FAM-SV40-derived peptide to suggest that mu peptide also possesses an NLS (Figure 3), a suggestion in line with the results of *NaeI* insert plasmid transfections (Figure 7C). Therefore, adenoviral mu peptide has clear NLS properties in addition to being an efficient and reliable template for viral DNA condensation in the adenovirus core, and pDNA condensation in LMD and MD preparation.[37]

Other peptide-based nonviral vector systems that have been studied, such as the lipid:integrin-targeting peptide:DNA (LID) system formulated as lipofectAMINE:$[K]_{16}$RGD: pDNA,[46] the peptide:DNA system formulated as AKRARLSTSFNPVYPYEDES-$[K]_{20}$:pDNA and other similar complexes,[25] have all been found to behave very similarly to threefold- and twofold-labelled LMD and LND systems, respectively. Therefore, in spite of the caveat mentioned above, there appears little reason to doubt the qualitative conclusions of the data produced with threefold- and twofold-labelled LMD and LND systems, respectively. In the cases of the LID and other peptide:DNA systems mentioned above, the majority of peptide was associated with cell nuclei after 30 minutes incubation of the vector systems with cells, whereas pDNA appeared to be associated with cell nuclei only after 2 h. On the basis of this information, these peptides have been described as "vectors of nuclear uptake of pDNA".[25] In our view, this is an exaggeration since such a claim can only really be justified if peptide and pDNA *co*-localise in the nucleus simultaneously. On the other hand, there has been some evidence provided to suggest that $[K]_{16}$ peptides might be actively involved in pDNA transport into the nucleus by mediating a direct interaction between pDNA and nuclear pore complexes.[47] However, this evidence was generated by using substantial amounts of $[K]_{16}$ peptides that could have made the nuclear membrane permeable or otherwise modified the nucleus, thereby favouring nuclear penetration artificially.[47] Moreover, in the absence of data on growth-arrested cells that demonstrate a beneficial effect of the peptides, for instance in active transport of pDNA within the time frame of peptide import into nuclei, the

putative role of peptides as mediators of direct interactions between pDNA and nuclear pore complexes (NPCs) should be regarded as provisional at best.

NLS regions such as that belonging to the SV40 T large antigen sequence are generally rich in lysine and arginine residues, and are therefore both highly cationic and basic in character.[39] Classical NLSs of proteins are recognised by the cytosolic protein importin-β, which interacts with importin-α in order to mediate import into the nucleus through NPCs.[48] Examination of the mu peptide sequence with the program PSORT resulted in an NLS score of 3.37, considerably higher than the NLS score of 1.95 obtained for the NLS sequence of the SV40 T large antigen (proteins devoid of an NLS sequence such as serum albumin are not transported into the nucleus and have an average NLS score over the whole protein of -0.47). The theoretical NLS score of mu peptide further reinforces our experimental conclusions that mu peptide possesses NLS region(s) (see Figure 3 and 7). Variations of the mu peptide sequence tested in the *NaeI* insert plasmid transfections (Figure 7) are a clear demonstration of the critical importance of sequential arginine residues as a determinant of NLS capacity. Particularly noteworthy are the results for reordered mu peptide, whose sequence (MHRHRGRAASRRRRHRRMG, Figure 7D) has six contiguous arginine residues broken only by a single histidine residue. This sequence fused to β-galactosidase appeared to be just as potent as, if not more than the mu peptide sequence for concentrating the enzyme in cell nuclei (Figure 7C). These data contrast well with the data obtained with the reverse mu peptide sequence (RPSAGAAAAGRRPGPGAGA) fused to β-galactosidase, where the fusion protein remained in the cell cytosol (Figure 7E) presumably because the reverse mu peptide has insignificant NLS characteristics given the almost complete absence of sequential arginine residues in its sequence.

Even if mu peptide possesses clear NLS region(s), our confocal microscopy data suggest clearly that current LMD formulations are unable to take advantage of this capacity to facilitate pDNA transport into nuclei. The direct comparison between LMD, LND and LD transfections of 56 FHTE8o$^-$ cells carried out by using pDNA(Cy3) to monitor intracellular pDNA trafficking by confocal microscopy illustrated very clearly that pDNA trafficking rates into the nuclei of dividing cells are the essentially same (30–45 minutes) irrespective of the nonviral vector system under investigation (Figure 4A). Furthermore, where aphidicolin-treated nondividing cells are concerned, pDNA was unable to enter cell nuclei to any significant degree after 45 minutes of incubation in all cases (Figure 4B). Such kinetic data not only highlight a key limitation of the current binary and ternary formulations but underscore two key facts: neither the extracellular membrane barrier nor the intracellular endosomal membrane barrier are major barriers for the LMD or LD transfection process; the nuclear barrier will likely remain a major barrier, as reported previously, until such time as the NLS region(s) of the mu peptide can be properly harnessed in a subsequent step of evolution of the LMD system.

Given the apparent equality of intracellular pDNA trafficking rates, the differences between LMD and LND transfection efficiency on the one hand, and LD transfection efficiency on the other, need to be explained (Figure 5). These differences could be accounted for if mu and even the SV40-derived peptide were able to operate as transcriptional enhancers. However, since a direct comparison between LMD, LND and LD transfections of 56 FHTE8o$^-$ cells with GFP plasmid showed essentially no differences in the rates at which GFP fluorescence emerged in the cytosol (Figure 8), the possibility that mu and even the SV40-derived peptides are transcriptional enhancers does not appear likely. Instead, we are now of the opinion that LMD and LND are more efficient in transfection than the LD system largely because the former two systems are significantly more stable in the cell culture medium used during in vitro transfection than the LD system, as we have noted previously.[36] Therefore, we shall primarily be seeking to develop future nonviral vector systems by building upon the LMD or LND platform systems and paying particular attention to harnessing the demonstrated capacity of the NLS region(s) of mu peptide to promote pDNA access to the nuclei of cells across the nuclear membrane through the narrow inner diameter of NPCs (10–20 nm).[49–51]

One final significant point needs to be addressed. Previously, we observed that the development of clinically useful LMD systems competitive with viral vector systems could only be achieved with the development of triggerable LMD (trigLMD) systems; that is, systems stable and nonreactive in extracellular fluids but unstable once recognised and internalised by target cells in a target organ of choice. TrigLMD systems will almost certainly require surface protection of some description. Such protection has been shown to effectively protect liposomes and enhance in vivo circulation times.[52, 53] Therefore, we employed a post-coating strategy to add fluorescent-labelled polyethylene glycol (fluorescein-PEG^{5000}) molecules to the surface of LMD particles in order to monitor the transfection competence of these surface-modified particles and their cellular trafficking properties.[44] As expected, surface-modified LMD particles were not transfection competent in standard transfection assays of the type described herein (results not shown). However, we were extremely surprised to see that rather than being excluded from cellular uptake as a result of camouflage of the positive surface charge, particles were apparently taken up freely into cells only to be stored in perinuclear vesicles, consistent with endosome entrapment (Figure 6). These results have serious implications. PEG molecules have been used widely in liposomal drug delivery systems to protect liposomes from the deleterious consequences of uncontrolled interactions with biological fluid and tissue components such as varieties of proteins, lipids and carbohydrates found in serum. Accordingly, PEG molecules have also been introduced to some nonviral vector systems, such as the stabilised plasmid lipid particle (SPLP) system.[54] However, our data clearly suggest that PEG molecules actively inhibit the transgene expression process even at a low amount of surface protection (0.5 mol%), by a mechanism that appears to involve endosome entrapment. In other words, whilst our LMD and LD transfections appear to be little troubled by the endosome compartment (Figure 4), transfections involving surface-pegylated LMD particles certainly are affected (Figure 6).

Our observations are in complete agreement with Song et al., who studied the delivery of oligodeoxynucleotides (ODNs) to

cells mediated by a variety of unilamellar DODAC/DOPE/PEG-lipid cationic liposome systems. Song et al. demonstrated that different amounts and chemical structures of PEG-lipids have little effect on the binding and subsequent endocytosis of cationic liposome/ODN complexes but severely inhibit endosomal release of ODNs into the cytoplasm and thereby prevent ODNs from having any biological effect inside the cell at all.[55] On the basis of these data, credible alternatives to PEG as a surface-stabilising molecule for nonviral vector systems that give protection in biological fluids and tissues without impairing transfection efficiency must be found. Alternatively, new mechanisms and new chemistry must be established to allow inhibitory PEG molecules to be completely released once vector particles are inside endosome compartments after endocytosis, to yield "naked" nonviral vector particles that should be able to disrupt endosome membranes and facilitate the escape of pDNA into the cytosol.[13] Either or both strategies may have to be adopted to develop the first generation of trigLMD systems. However, given the potent NLS properties of the mu peptide demonstrated in this paper, the focus for attempts to achieve competent in vivo nonviral vectors is clear. New chemical innovations will be needed to provide temporary extracellular protection of particle surfaces, and a way to fully exploit the NLS function of mu peptide must be found so as to direct the transgene into the nuclei and achieve maximum transfection efficacy in vivo.

Experimental Section

General: OptiMEM was purchased from Gibco BRL (Lexington, KY, UK); ammonium chloride from Merck (Harlow, Essex, UK), paraformaldehyde, bovine serum albumin (BSA; fraction V), propidium iodide and 1,4-diazobicyclo[2.2.2]octane from Sigma (Pampisford, Camridgshire, UK); RNAse A (free of DNAse) from Boehringer (Ingelheim, G); glycerol from Dako (Ely, UK); phosphate-buffered saline (PBS) from Life Technology (Lexington, KY, UK). All PBS used for confocal microscopy contained 2% BSA. The Cy5 and Cy3 nick-translation labelling kit was purchased from Amersham – Pharmacia (Little Chalfont, Buckinghamshire, UK). All other fluorescent probes were purchased from Molecular Probes (Eugene/OR, USA). ^{1}H NMR spectra were recorded on either Brucker DRX_{300} or Jeol GX-270Q, with residual isotopic solvent ($CHCl_3$, $\sigma_H = 7.26$ ppm) as an internal reference. Mass spectra were recorded on VG-7070B or Jeol SX-102 instruments. Silica chromatography refers to flash column chromatography on Merck-Kieselgel 60 (230 – 400 mesh).

Lipids: DOPE was purchased from Avanti Polar Lipids (Alabaster, AL, USA). DC-Chol was synthesised in our laboratories according to published procedures.[4] The chemical synthesis of the novel lipid Lp-24 (Scheme 1) was carried out by using standard organic synthesis procedures, as described previously for similar compounds.[4] Lp-24 was labelled with the fluorophore FAM and fully characterised by analytical HPLC, mass spectrometry, fluorescence spectroscopy, UV analysis and ^{1}H NMR spectroscopy.

Synthetic procedure: A solution of methanesulfonyl chloride (0.19 mL, 2.40 mmol) was added dropwise to a solution of 2-(cholesteryloxycarbonyl)aminoethanol **1**[4, 5] (0.45 g, 0.96 mmol) and triethylamine (0.40 mL, 2.88 mmol) in dichloromethane (DCM; 10 mL) at 0 °C. After one hour, ice was added to the reaction mixture followed by addition of saturated ammonium chloride (15 mL) and extraction with diethyl ether. Purification was carried out by flash chromatography over silica (diethyl ether) to yield 2-(cholesteryloxycarbonyl)aminoethane-1-sulphonate **2** (89%). 4-amino-butan-1-ol (2 mL, 7.50 mmol) was added to **2** (0.43 g, 0.75 mmol) dissolved in tetrahydrofuran (THF; 2.5 mL). After completion of the reaction and purification over silica (diethyl ether), 7-cholesteryloxycarbonyl-(5,7-aza)-heptan-1-ol **3** (75%) was isolated. ^{1}H NMR (300 MHz, σ_H): $\delta =$ 5.27 – 5.33 (1 H, m, H-6′), 4.40 – 4.44 (1 H, m, H-3′), 3.52 – 3.56 (2 H, m, H-6), 3.27 – 3.30 (2 H, m, H-1), 2.75 – 2.80 (2 H, m, H-2), 2.65 – 2.71 (2 H, m, H-3), 2.21 – 2.36 (2 H, m, H-4′), 1.77 – 2.09 (5 H, m, H-2′, H-7′ and H-8′), 0.99 – 1.50 (28 H, m, H-4, H-5, H-1′, H-9′, H-11′, H-12′, H-14′ to H-17′, H-20′, H-23′ to H-25′), 0.96 (3 H, s, H-19′), 0.60 – 0.88 (3 H, d, $J =$ 7.0 Hz, H-21′), 0.81 – 0.86 (6 H, dd, H-26′, H-27′), 0.64 (3 H, s, H-18′) ppm. MS (FAB, *m/z*): 567 $[M+Na]^+$, 545 $[M+H]^+$, 369 $[Chol]^+$.

"Bocylation" of N^5 was achieved by addition of di-*t*butyl-dicarbonate (0.04 g, 0.18 mmol) to a solution of **3** (0.10 g, 0.18 mmol) and triethylamine (0.03 mL, 0.23 mmol) in DCM (0.9 mL) and the product was purified over silica (diethylether) to give 7-cholesteryloxycarbonyl-(5,7-aza)-(5-Boc)-heptan-1-ol (95%). Mesylation of this compound was carried out as described above on a 1.15 mmol scale to give **4** in 88% yield after purification over silica (diethyl ether). Conversion to the azide derivative was achieved by reacting **4** (1.40 g, 1.97 mmol) with sodium azide (0.63 g, 9.74 mmol) and sodium iodide (0.30 g, 1.95 mmol) under nitrogen in anhydrous DMF at 80 °C, followed by purification over silica (petroleum/diethyl ether, 1:1) to yield 1.24 g pure product (95%). Reduction of this azide compound with the free amine was achieved by reacting the azide (0.8 g, 1.2 mmol) with trimethylphosphine (106 mg, 1.4 mmol) in THF (9.4 mL) followed by treatment with aqueous ammonia (1 mL) for one hour and purification over silica (chloroform/methanol, 3:1) to afford 7-cholesteryloxycarbonyl-(1,7-diamino)-(5-aza-*tert*.-butyloxycarbonyl)-heptane **5** (Lp-24[5-Boc]; 75%) as a white solid. Coupling of Lp-24(5-Boc) **5** (20 mg, 32 μmol) with FAM (10 mg, 26 μmol) was carried out in the presence of *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU; 20 mg, 52 μmol) and *N*,*N*,-diisopropylethylamine (DIEA; 4.6 μL, 80 μmol) in DMF (1 mL). All solvent was evaporated and replaced by trifluoroacetic acid (TFA)/water (9:1, 2 mL) and the product was purified by semipreparative HPLC (C_{18}). The fraction containing the product was lyophilised to give 2 mg (8%) pure product, FAM-Lp-24 **6**. HPLC (0 – 100% acetonitrile, 20 mins): $t_R = 15.08$ min, single peak ($\lambda = 214$ nm). MS (MALDI, *m/z*): 900.85; ^{1}H NMR (270 MHz, σ_H): $\delta = 10.1$ (1 H, s, −OH), 8.5 – 6.5, 9 H, aromatics of FAM), 5.36 – 5.39 (1 H, m, H-6′), 4.45 – 4.55 (1 H, m, H-3′), 3.21 – 3.32 (6 H, m, H-1, H-2, H-3), 2.74 (2 H, t, $J =$ 6.5 Hz, H-6), 2.22 – 2.35 (2 H, m, H-4′), 1.84 – 2.03 (5 H, m, H-2′, H-7′, H-8′), 1.47 (9 H, *s*, Me of *t*Bu), 1.06 – 1.57 (25 H, m, H-4, H-5, H-1′, H-9′, H-11′, H-12′, H-14′ to H-17′, H-20′, H-22′ to H-25′), 0.96 (3 H, s, H-19′), 0.92 (3 H, d, $J =$ 6.5 Hz, H-26′), 0.90 (3 H, d, $J =$ 6.5 Hz, H-27′), 0.68 (3 H, s, H-18′) ppm.

Liposomes: A lipid film consisting of DC-Chol:DOPE (60:40; m/m) was formed in a round-bottomed flask by removal of the DCM under reduced pressure. After one hour of drying under vacuum, the lipids were suspended in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; 4 mM, pH 7) under sonication (20 minutes) at a final concentration of 4.5 mg mL^{-1}. Unilamellar vesicles were prepared by extruding the lipids through two 100 nm membranes (10 ×, Millipore, Ireland). The content of organophosphorus was determined as described by Stewart.[56] Determination of the size and estimation of the homogeneity of the resulting liposomes was carried out by photon correlation spectroscopy. Fluorescent FAM-Lp-24 was incorporated into these liposomes by adding a solution of FAM-Lp-24 (3 mL, 1 mg mL^{-1}, 4 mM HEPES, pH 7) to the liposomes

described above (15 μL, 4.5 mg mL^{-1}) while thoroughly vortexing the mixture. Fluorescence quenching of 32% compared to quenching by free FAM-Lp-24 proved incorporation into liposomes had occurred.

Peptides: The adenoviral core peptide mu (MRRAHHRRR-RASHRRMRGG) and the SV40 NLS-containing peptide (H-RRRPKKKRKVSRRR-NH_2)[39] were synthesised according to standard 9-fluorenylmethoxycarbonyl (Fmoc) based Merrifield solid-phase peptide chemistry on Wang and Rink amide resin, respectively.[57] In order to avoid failure sequences being produced by incomplete coupling steps, pseudoproline dipeptides were incorporated where appropriate.[58–61] TAMRA and FAM were coupled on resin to N_α-Met and N_α-Arg, respectively, by using standard peptide protocols. Peptide purification was carried out on a Hitachi semipreparative HPLC system at a flow rate of 20 mL min^{-1}, with a LiChrospher C_{18} (300 Å, 5 μm) column. All peptides were desalted on Sephadex G25 M (PD 10) columns (Amersham – Pharmacia) prior to use. Analytical HPLC was run on a Hitachi system with a Purospher RP-18 endcapped column (5 μm) at a flow rate of 1 mL min^{-1}, gradient 0 – 100% acetonitrile (20 mins). All peptides were fully characterised by mass spectrometry (FAB or MALDI) and analytical HPLC (Hitachi) before use.

DNA: The plasmid pUMVC1 was obtained from the University of Michigan Vector Core (http://www.med.umich.edu/vcore/Plasmids/) and amplified/purified by Bayou Biolabs, Qharahan, LA, USA. Labelling with cyanine dyes Cy5 and Cy3 was carried out according to a modified published procedure by a nick-translation technique. We found it unnecessary to relax the pDNA with $MgCl_2$ prior to performing the nick-translation procedure.[38] UV absorption of the Cy5 or Cy3 labelled pDNA proved insufficient to visualise the pDNA under a UV source without post-run staining with ethidium bromide. A UV/Vis wavelength scan between 200 and 700 nm revealed two maxima: $\lambda_1 = 260$ nm (adenin bases) and $\lambda_2 = 649$ nm (Cy5) or 568 nm (Cy3). Plasmid pEGFP-C1 (4.7 kb) was purchased from Clontech (Palo Alto, CA, USA) and used without further purification.

Pegylation of LMD: Fluorescein-PEG^{5000}-NHS was purchased from Shearwater (Huntsville, AL, USA). Post-coating onto LMD was carried out as described by Wagner and co-workers for a similar system.[44]

Preparation of LMD, LND and LD: All LMD and LND particles were prepared at the optimised lipid:peptide:pDNA ratio of 12:0.6:1 (w/w/w) and LD vector systems at 12:1 (lipid:pDNA, w/w).[35] To this end, pDNA (1 mg mL^{-1}) was rapidly added to a solution of mu peptide (0.1 mg mL^{-1}) in HEPES (4 mM, pH 7) under vortexing to form the MD complex. This complex was added dropwise to the liposomes (4.5 mg mL^{-1}) under heavy vortexing to form LMD particles at a concentration of 50 μg mL^{-1} (pDNA), a slightly modified version of the procedure previously reported. LND particles were prepared in an equivalent manner.[4, 35, 36] The fluorescent and/or dye-labelled particles were prepared in the same way as the nonfluorescent systems described above, except that the peptide, DNA and liposomes were replaced with equimolar amounts of fluorescent- or dye-labelled entity.

Fluorescence spectroscopy: Fluorescence determinations were carried out at 20 °C on a Shimazu RF-5301 spectrofluorimeter with a water-cooled cryostate (Grant LTD 6). An excitation wavelength near the UV absorbance maximum for the fluorophore was chosen and results were recorded at medium scanning speed at a sampling interval of 0.2 nm and with a slit width of 5 nm (FAM and TAMRA) or 15 nm (Cy5) for all excitation and emission spectra.

Photon correlation spectroscopy: The particle sizes of the LMD, LND and LD systems were measured by using dynamic light scattering on a Coulter N4 plus. All measurements were performed at 20 °C at an angle of 90° with an equilibration time of 1 min and running times of 300 sec at concentrations of 5 μg mL^{-1} pDNA in HEPES (4 mM, pH 7). The refractive index of the buffer was set to 1.333. Unimodal analysis was used to calculate the mean particle size.

Cell culture: Foetal human tracheal epithelial 56 FHTE8o$^-$ cells provided by Dr Dieter Gruenert[62] were cultured in 50% Dulbecco's modified Eagle's medium (DMEM) 50% Ham F12, supplemented with 10% foetal calf serum (FCS; normal supplemented medium). HeLa cells were maintained in DMEM supplemented with 10% FCS. Cells were seeded on 12-well culture plates for transfection experiments and on Labteck slides for confocal microscopy analysis, and grown until semi-confluent.[46] Cells were growth arrested by incubation with aphidicolin (5 μg mL^{-1}) for 16 h.[63]

Transfection:

Dividing cells: LMD, LND and LD vector systems were prepared as described above at a final concentration of 50 μg mL^{-1} pDNA. For LMD vectors, labelled liposomes of composition DOPE:DC-Chol:-FAM-Lp-24 = 40:57:3 (m/m/m) were used, whereas LND vectors were prepared with nonlabelled liposomes. The lipid:peptide:DNA ratio was 12:0.6:1 (w/w/w) for each vector system. For transfections, each vector (30 μL, 50 μg mL^{-1}) was diluted with OptiMEM (870 μL; Gibco BRL) and added to three wells (300 μL each) of a 12-well plate containing semiconfluent tracheal cells. The plate was incubated for times indicated in the text. The plates were washed with DMEM and incubated for 48 h at 37 °C in normal growth medium. The pDNA dose per well was 0.5 μg. The activity was detected with a chemiluminescent reporter assay kit (Galacto-Light, Tropix Inc., Paris, F). The protein concentration of cell lysates was determined by using the Bradford method and activities were expressed as relative light units per milligram protein.[64] Cells transfected by using lipofectAMINE (Gibco BRL) were transfected as indicated by the manufacturer.

The expression and distribution of β-galactosidase in situ was visualised as follows: transfected cells were fixed in 1% formaldehyde/PBS and stained (0.4 mg mL^{-1} X-gal, 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 0.1 mM $MgCl_2$) overnight at room temperature, protected from light. After staining the cells were rinsed in PBS and stored in 1% formaldehyde/PBS.

Growth-arrested cells: Cells were grown until semiconfluent. The medium was replaced by normal growth medium (10% FCS) containing aphidicolin (Sigma, UK; 5 μg mL^{-1}) before incubating the cells for 16 h. Transfections were carried out as described above.

Electrophoretic migration assay: Electrophoretic mobility shift assays on 1% agarose gels were performed at peptide:pDNA ratios between 0.1 and 2.5. Both labelled and native peptides were investigated. The peptide/pDNA complexes were prepared by rapid addition of pDNA to a solution of peptide (0.1 mg mL^{-1}) in HEPES (pH 7, 4 mM). The sizes of the resulting complexes were determined by PCS as described above.

Confocal laser scanning microscopy: Tracheal cells were grown in glass wells for 48 h in normal supplemented medium and then thoroughly washed (3 × 300 μL) with the same medium. LMD (10 μL, 50 μg mL^{-1} pDNA) was diluted with OptiMEM (90 μL) then added to the cells. TAMRA-mu (3.5 μg mL^{-1}, 100 μL) was used as a negative control and pUMVC1(Cy5) (5 μg mL^{-1}, 100 μL) as the positive control. After each well's designated incubation time at 37 °C, the respective wells were rinsed three times with PBS (3 × 300 μL) then treated with a solution of polyformaldehyde (4%) and left for 20 minutes. The supernatant was removed and the wells washed with PBS and treated with NH_4Cl (50 mM, 4 × 5 min). Wells were incubated for ten minutes with RNAse A (1 mg mL^{-1} in PBS) then the nuclei were labelled by treatment with propidium iodide (3 μg mL^{-1} in PBS) for three minutes. Next, the wells were incubated in 1,4-diazabicy-

clo[2.2.2]octane (10 mg mL^{-1} in PBS) for ten minutes at room temperature before addition of glycergel. Glass cover slips were fixed between the wells on narrow strips of scotch tape and the slides were incubated at 4 °C in order to allow the glycergel to solidify. Confocal microscopy was performed with a TCS SP Leica (Lasertechnichk GmbH) microscope, equipped with a 40 × objective (plan apo corrections; numerical aperture = 1.25). An argon – krypton ion laser adjusted to 568 and 647 nm was used for propidium iodide and Cy5 excitation, as previously described.[46, 65]

In order to improve pDNA detection, which is hampered by the low sensitivity of the Cy5 dye, the pDNA was labelled with Cy3 (emitting in red). LMD(Cy3) vectors were prepared with labelled pDNA(Cy3) as the sole marker. LD(Cy5) particles were separately formed at the same lipid:pDNA ratio (12:1, w/w) as LMD(Cy5). Transfection and preparation of cover slides were carried out as described above with the exception that propidium iodide was replaced by 4′,6-diamidino-2-phenylindole, which stains the nuclei blue. In order to ensure that cellular uptake of vectors does not interfere with intracellular trafficking, the cells were washed extensively with PBS after two minutes incubation to clear off remaining extracellular vector before addition of natural growth medium (DMEM). The cells were then fixed as described above after the indicated times. Analyses were carried out at $\lambda = 488$ nm (95%) on an LSM 510 Zeiss instrument with an 8-bit scan mode plane, stack sizes of 1024 × 1024 (130.3 × 130.3 mm), a pixel time of 1.12 ms and a plan apochromate objective (100 ×, 1.4 oil Ph3). The filter used was a Ch1 (LP 560), pinhole 224 μm.

Production of recombinant expression plasmid vectors: Construction of a cloning vector based upon pUMVC1 was achieved by using site-specific mutagenesis to exclude the NLS sequence of SV40 large T antigen and replace it with an Nae1 restriction enzyme site with the primer sequence 5′-TTCCGGAATTCCGCAGCCGGCAGGACTTTCCTTCAG-3′ (Sigma-Genosys, UK). High fidelity Pfu DNA polymerase was used in the PCR reaction in order to prevent errors due to the length of template to be amplified. Template DNA and the PCR product were separated by restriction digestion followed by agarose gel electrophoresis, extraction and purification (Qiagen). The following oligonucleotides (with complementary strands) were then blunt-end cloned into the NaeI site:

SV40 NLS: 5′-AGAGGAAGAGGCCGGCCACCACCTAAAAAGAAGAGAAAGGTAGAAGACCCCAAGGGGCCCGGCAGAATAG-3′

Mu: 5′-AGAGGAAGAGGCCGGCCAATGCGGCGGGCCCACCACCGGCGGCGGCGGGCCTCCCACCGGCGGATGCGGGGCGGCGGGCCCGGCAGAATAG-3′

Mu reordered: 5′-AGAGGAAGAGGCCGGCCAATGCACCGGCACCGGGGCCGGGCCGCCTCCCGGCGGCGGCGGCACCGGCGGATGGGCGGGCCCGGCAGAATAG-3′

Mu Reverse: 5′-CTATTCTGCCGGGCCCGCCGCCCCGCATCCGCCGGTGGGAGGCCCGCCGCCGCCGGTGGTGGGCCCGCCGCATTGGCCGGCCTCTTCCTCT-3′

The plasmids were then transferred into *Escherischia coli* DH5α competent cells (Invitrogen Life Technologies) and screened by restriction digest analysis by using the enzyme EarI (New England Biolabs) to identify recombinant plasmids and the orientation of the inserted fragment. Positive clones were then grown up in the presence of kanamycin and plasmid constructs were extracted (Qiagen). Each construct (2 μg) was then used to transfect semi-confluent HeLa cells by using lipofectAMINE (Gibco BRL) in a 1:12 (w/w) DNA:liposome ratio. Transfection was allowed to take place for a period of 4 h and cells were left to express for 16 h. Visualisation of expression and localisation was achieved by fixation and staining as described earlier.[27]

The authors thank Dr. Toshiaki Tagawa for helpful discussions and Mr. Andreas Zumbühl for preliminary efforts to synthesise FAM-Lp-24. Michael Keller thanks the Swiss National Science Foundation for supporting this work (Grant nos. 83EU-056143 and SSMBS1070). The Imperial College Genetic Therapies Centre thanks Mitsubishi Chemical Corporation for financial support. The contribution of C. C. and R. B. H. was supported by the March of Dimes Birth Defects Foundation. M. C., L. V., S. M., C. C. and C. B.-H. acknowledge the support of Vaincre la Mucovicidose.

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Received: September 18, 2002
Revised version: January 28, 2002 [F490]