Available online at www.sciencedirect.com
ScienceDirect
Genomics 89 (2007) 708–720
GENOMICS
www.elsevier.com/locate/ygeno

Flow-cytometric analysis of mouse embryonic stem cell lipofection using small and large DNA constructs

Samuel McLenachan, Joseph P. Sarsero *, Panos A. Ioannou

Cell and Gene Therapy Research Group, Murdoch Childrens Research Institute, Royal Children's Hospital, Flemington Road, Parkville, VIC 3052, Australia
Department of Paediatrics, University of Melbourne, Royal Children's Hospital, Flemington Road, Parkville, VIC 3052, Australia

Received 18 October 2006; accepted 6 February 2007
Available online 20 April 2007

Abstract

Using the lipofection reagent LipofectAMINE 2000 we have examined the delivery of plasmid DNA (5–200 kb) to mouse embryonic stem (mES) cells by flow cytometry. To follow the physical uptake of lipoplexes we labeled DNA molecules with the fluorescent dye TOTO-1. In parallel, expression of an EGFP reporter cassette in constructs of different sizes was used as a measure of nuclear delivery. The cellular uptake of DNA lipoplexes is dependent on the uptake competence of mES cells, but it is largely independent of DNA size. In contrast, nuclear delivery was reduced with increasing plasmid size. In addition, linear DNA is transfected with lower efficiency than circular DNA. Inefficient cytoplasmic trafficking appears to be the main limitation in the nonviral delivery of large DNA constructs to the nucleus of mES cells. Overcoming this limitation should greatly facilitate functional studies with large genomic fragments in embryonic stem cells.

Keywords: Flow cytometry; Embryonic stem cells; BAC; Transfection; Lipofection; TOTO-1

With the completion of the sequencing of the human genome, most genes are now available in fully sequenced bacterial artificial chromosome (BAC) vectors [1–3]. The combination of BAC library technology and the complete human genome sequence has enabled the isolation of BACs containing the loci for any gene of interest, providing convenient experimental access to large genomic sequences. Complementary technologies, such as bacterial homologous recombination, allow precise genetic alterations to be made, such as point mutations [4–8] or reporter gene insertion. BAC-based reporter constructs provide reliable recapitulation of endogenous gene expression patterns in cellular and transgenic models [7–11]. Moreover, due to the inclusion of large tracts of surrounding chromosomal sequences, BAC loci are isolated from positional effects on gene expression when integrated into a host genome. However, the large size of these DNA constructs, which renders them vulnerable to breakage and more difficult to purify, has provided additional challenges to their implementation as experimental or therapeutic materials. One of these challenges is the inefficient transfection of large DNA constructs.

A number of strategies have been developed for the introduction of DNA molecules to cells. Physical methods such as electroporation [12–14], ballistic delivery [15,16], and hydrodynamic pressure [17] enable direct access to the cytoplasm by transiently disrupting the integrity of the plasma membrane. On the other hand, viruses have evolved targeted entry mechanisms exploiting existing endocytic processes [18], which have been utilized in viral DNA delivery systems. In particular the lentiviral and adenoviral delivery systems have been shown to be highly efficient means of introducing exogenous DNA molecules into cells. However, there are significant safety issues involved in the use of viral technologies, and synthetic DNA delivery systems have been developed in an effort to address these issues.

Lipofection has emerged as a promising method for nonviral DNA delivery. Cationic lipids interact with DNA to form encapsulated lipoplexes, which are taken up at high efficiency by many cell types, both in vitro [19–22] and in vivo [23,24].

* Corresponding author. Department of Paediatrics, University of Melbourne, Royal Children's Hospital, Flemington Road, Parkville, VIC 3052, Australia. Fax: +61 3 8341 6212.
E-mail address: joe.sarsero@mcri.edu.au (J.P. Sarsero).

0888-7543/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygeno.2007.02.006

Lipoplexes gain access to the cell interior by binding to cell surface proteoglycans and cadherins [23,25,26], followed by adsorptive endocytosis. The endocytic pathway offers distinct advantages for the nonviral delivery of DNA to eukaryote cells. Endosome trafficking can facilitate the movement of DNA molecules toward the nucleus [27], overcoming diffusion difficulties and offering protection from cytoplasmic nucleases. However, the default pathway for endosomal trafficking is lysosomal fusion, which results in the degradation of transfected DNA by the exonuclease DNase II [28].

Endosomal maturation and lysosomal fusion are thought to require dissociation and degradation of the transfection reagent. Large and stable complexes or nonbiodegradable reagents inhibit these processes, while small complexes or biodegradable reagents are transported to the lysosomal compartment [28,29]. Electron and confocal microscopy studies have shown that endosomes containing DMRIE-C/DOPE lipoplexes do not fuse with lysosomes. Instead, these endosomes fuse with each other, forming large vesicles in the perinuclear region. However, this method did not result in detectable levels of DNA in the cytoplasm or nucleus and DNA was still complexed with the cationic lipid, suggesting that these lipoplexes remained trapped in perinuclear vesicles [27]. It has been proposed that cationic lipids interact with anionic lipids in the endosomal membrane, causing them to flip-flop from the cytoplasmic-facing side to form complexes with the transfection reagent. In turn, this causes the displacement of DNA from the lipoplex and facilitates its release into the cytosol [30]. However, an optimal reagent formulation is likely to be crucial in promoting endosome escape by this mechanism [27].

Following endosome escape, transfected DNA molecules are exposed to the hazardous, reducing environment of the cytoplasm. The cytoplasmic environment is densely crowded, with 10–15% of the available volume occupied by macromolecules. DNA fragments over 2 kb are essentially immobilized in the cytoplasm with respect to free diffusion [31], suggesting that even small DNA plasmids require active intracellular transport to reach the nucleus. In this context, it is interesting that protamine, histones, and other basic nuclear proteins have been reported to increase the efficiency of transfection [32–34]. It is possible that interactions between transfected DNA and nuclear-targeted DNA-binding proteins (such as histones and transcription factors) that are normally synthesized in the cytoplasm could result in the active translocation of both species into the nucleus.

Nuclear delivery of transfected DNA molecules is greatly enhanced in cells undergoing mitosis [35,36]. During cell division, transfected DNA may be sequestered into the reforming nuclear compartment through interactions with host chromatin. In the absence of mitosis, nuclear delivery is thought to involve the nuclear pore, which can accommodate particles up to 26 nm in diameter [37]. Herpesviruses are known to thread their large genomes through the nuclear pores by interacting with host import machinery [38], and the inclusion of peptides with nuclear localization signals in the DNA complex has been shown to provide enhanced nuclear delivery of DNA molecules [24,39], suggesting that nuclear import machinery may also be targeted by modular, synthetic transfection vehicles.

In an effort to identify limiting factors, we have examined the delivery of small and large DNA molecules to mouse embryonic stem (mES) cells using flow cytometry. Transfection of mES cells underpins all murine transgenic technologies, thus high-efficiency DNA delivery methods are of significant interest to researchers involved in the generation of transgenic and knockout mouse lines. Lipofection of mES cells has the potential to replace expensive microinjection techniques and has proven to be a reasonable efficacious method for delivering large DNA constructs [19,20]. However, lipofection of mES cells with large constructs remains significantly impaired compared with small plasmids, suggesting further improvements can be made.

To follow the physical uptake of DNA lipoplexes we have employed the dimeric cyanine dye TOTO-1. This compound forms highly fluorescent complexes with DNA [40] that dissociate with a half-life of about 11 h, providing a convenient approach to measure the physical uptake of DNA by flow cytometry. Using this approach we have optimized conditions for efficient lipoplex uptake and examined uptake across the plasma membrane independent of nuclear delivery. We find that under optimal conditions small and large DNA constructs are taken up with equivalent efficiencies. However, lipoplex uptake was limited by the proportion of uptake-competent mES cells, which increased with exposure time. Furthermore, we find that despite similar uptake efficiencies, nuclear delivery of BAC molecules is severely compromised compared with small plasmid molecules. This effect was observed using equivalent mass or molar concentrations of different-sized constructs, suggesting a cytoplasmic blockade to BAC transfection.

Results

Flow-cytometric analysis of mES transfection

Expression of a reporter gene, the standard method of measuring transfection efficiency, reflects the overall effect of a diverse number of cellular processes on transfected DNA, each of which may be differentially affected by transfection parameters. To follow the physical uptake of DNA molecules by mES cells readily, independent of any requirements for gene expression, we have employed the dimeric cyanine dye TOTO-1, which forms highly fluorescent, stable complexes with DNA. Figs. 1a–c illustrate the flow-cytometric profiles obtained from a typical DNA uptake experiment. After 5 h of exposure to lipoplexes containing labeled DNA, mES cells were trypsinized and analyzed by flow cytometry. Fig. 1a shows a typical forward scatter (FSC) versus side scatter (SSC) density plot obtained from an mES cell transfection. The live single-cell population was gated (R1) to exclude debris and cellular aggregates in the suspension from the analysis. The gated population was then analyzed on a FSC/FL1 density plot. Untransfected and vehicle-only controls exhibited little autofluorescence, forming a single FL1-negative population (Fig. 1b). Using these controls, thresholds were set to include all untransfected cells in the lower right quadrant. mES cells that had taken up TOTO-1-labeled DNA formed a clearly distinguishable population in the upper right quadrant (Fig. 1c), enabling rapid determination of uptake

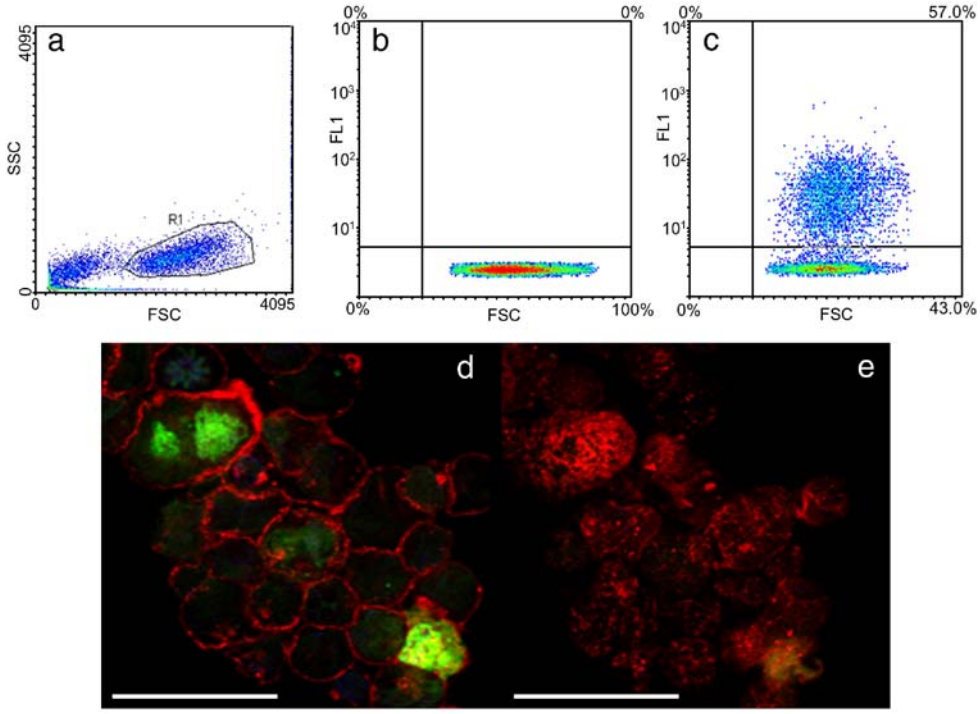

Fig. 1. Flow cytometry of mES cells. LipofectAMINE 2000 was used to transfect mES cells with TOTO-1-labeled DNA. 5 h after addition of lipoplexes, cells were trypsinized and analyzed by flow cytometry or confocal microscopy. (a) FSC vs SSC density plot of mES cells analyzed by flow cytometry. R1 indicates a typical gate used to analyze live cell data. (b) FSC vs FL1 density plot showing gated untransfected cells. (c) FSC vs FL1 density plot showing cells transfected with TOTO-labeled DNA (DNA:TOTO ratio of 50:1). (d and e) Confocal image stacks showing a cross section (d) as well as a focal plane corresponding to the cell surface (e). Scale bars indicate 50 μm.

efficiency. To quantitate DNA uptake, mean FL1 values were calculated for the upper right quadrant population.

We considered the possibility that TOTO-1-labeled DNA attached to the cell surface would not be distinguishable by flow cytometry from DNA that had gained access to the cell, potentially confounding the cellular uptake measurement. However, we reasoned that the stringent trypsinization used during the analysis would likely be sufficient to remove lipoplexes from the membrane surface. To explore this possibility further, we examined transfected cells by confocal microscopy. mES cells were transfected with TOTO-1-labeled DNA, harvested by trypsinization, and then stained with fluorescent probes for the plasma membrane (red) and the nucleus (blue). Image stacks were then collected on a confocal microscope. Fig. 1d shows a cluster of transfected cells, with the TOTO-1 signal (green) clearly localized inside the cells. Moreover, when we examined focal planes corresponding to the cell surface, we observed no TOTO-1 labeling of the cell exterior (Fig. 1e). These results confirm that surface-bound lipoplexes were removed during the harvesting of transfected cells and that the TOTO-1 signal observed by flow cytometry reflects internalized DNA.

Lipoplex condensation

The efficiency of lipoplex condensation is the first limiting step in lipofection, determining the proportion of input DNA that becomes available for uptake. To identify conditions promoting efficient lipoplex condensation the ratio of DNA to LipofectAMINE 2000 (LF2000) was varied during complex formation.

Lipoplexes were formed at three different DNA:LF2000 ratios (1:2, 1:4, and 1:8) using a constant, limiting amount (500 ng) of DNA and used to transfect mES cells. To follow lipoplex uptake independent of nuclear delivery and gene expression, DNA was prelabeled using the fluorescent dye TOTO-1. As condensation dynamics may be altered with increasing plasmid size, both small (6.6 kb) and large (200 kb) DNA constructs were compared. After 5 h of lipoplex exposure, cells were washed, trypsinized, and analyzed by flow cytometry.

For both small and large constructs, increasing LF2000 concentrations during lipoplex formation led to a greater proportion of cells taking up DNA (Fig. 2a), suggesting the production of greater numbers of lipoplexes. For the 6.6-kb plasmid, we observed a trend toward lower DNA uptake per cell with increasing LF2000 concentration (Fig. 2b). These changes in DNA availability are consistent with a mode of condensation in which excess LF2000 promotes the generation of greater numbers of lipoplexes with lowered DNA content.

In contrast to lipoplexes formed with small plasmids, increasing LF2000 concentration during the formation of BAC lipoplexes resulted in increases in both the number of cells taking up DNA and the DNA uptake per cell (Figs. 2a and 2b). As lipoplexes formed with BACs are thought to contain single DNA molecules [41], the large size of these constructs may preclude them from alterations in lipoplex copy number. In this case, the generation of greater numbers of competent BAC

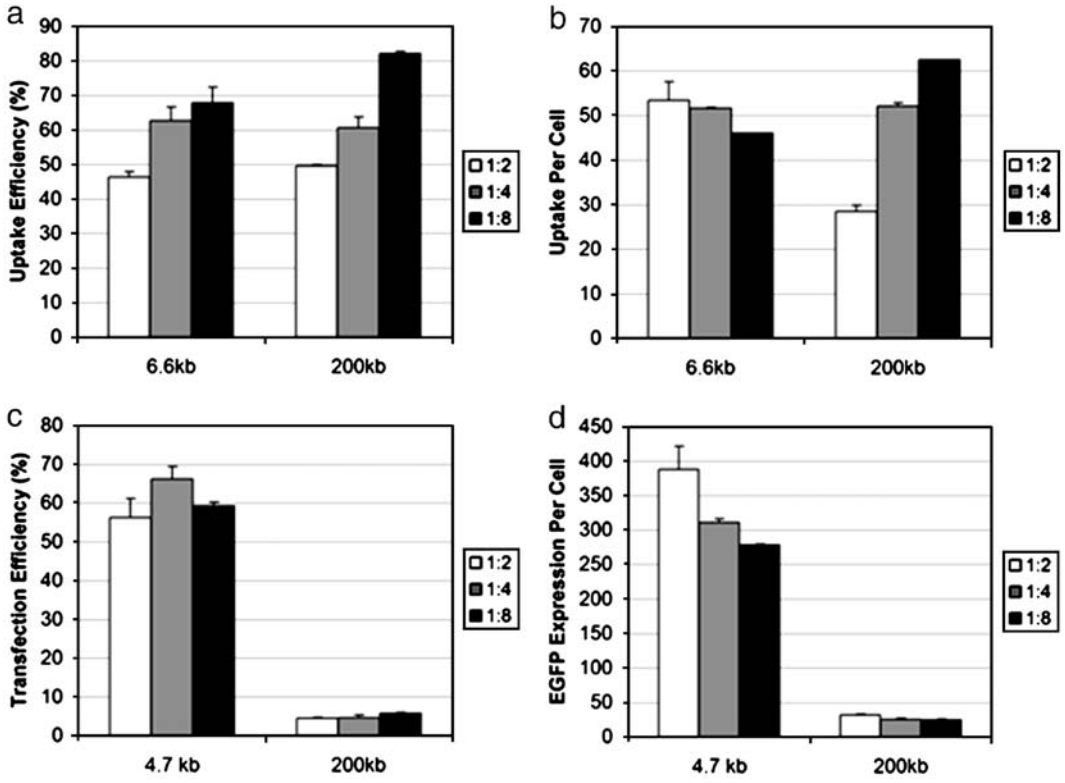

Fig. 2. Effect of DNA:LF2000 ratio on lipoplex condensation. mES cells were transfected with 500 ng of DNA using LF2000. Three different DNA:LF2000 ratios were compared: 1:2, 1:4, and 1:8. Mean values and standard error were calculated from three or four independent transfections. (a and b) Cellular uptake. mES cells were transfected with either TOTO-1/pGETrec plasmid (6.6 kb) or TOTO-1/BAC265 DNA (200 kb). 5 h after the addition of lipoplexes, cells were trypsinized and analyzed by flow cytometry. (a) The percentage of TOTO-1/DNA-positive cells was determined from FSC/FL1 density plots. (b) DNA uptake per cell was determined from the mean peak fluorescence of the $FL1^+$ cell population. (c and d) Nuclear delivery. mES cells were transfected with either the pEGFP-N22 plasmid (4.7 kb) or the pEBAC148G BAC (200 kb) DNA. 5 h after lipoplex addition, the medium was changed and cells were cultured overnight. EGFP expression was analyzed by flow cytometry at 24 h. (c) Transfection efficiency was determined using FSC/FL1 density plots. (d) EGFP expression per cell was determined from the mean peak fluorescence of the $FL1^+$ population.

lipoplexes at higher LF2000 concentrations appears to lead to more lipoplexes being taken up by individual cells.

In contrast with the cellular uptake data, the transfection efficiency of a small (4.7 kb) CMV–EGFP reporter plasmid was optimal at a DNA:LF2000 ratio of 1:4. In addition, the level of EGFP expression per cell decreased with increasing LF2000 concentration (Figs. 2c and 2d), suggesting the formation of lipoplexes with lower DNA content. Despite similar cellular uptake of small and large constructs by mES cells, transfection of a large (200 kb) construct containing a CMV–EGFP reporter cassette was severely reduced compared with the small plasmid. As a DNA:LF2000 ratio of 1:4 provided similar DNA uptake for both small and large plasmids (Figs. 2c and 2d), this ratio was used in further experiments.

Lipoplex concentration

The relationship between an optimal lipoplex concentration and the efficiency of cellular DNA uptake is a balance between having enough DNA and having too many lipoplexes in the culture. Insufficient concentrations of DNA will result in poor transfection efficiencies, while an excess of lipoplexes will compromise cell viability. To examine whether cellular uptake could be increased by elevating the lipoplex concentration, mES cells were transfected with increasing amounts of TOTO-1-labeled pGETrec (6.6 kb) using LF2000 and analyzed after 5 h by flow cytometry (Figs. 3a and 3b).

At DNA concentrations below 1000 ng per well, transfection efficiency was proportional to lipoplex concentration for both small and large plasmids. However, increasing DNA concentration from 1 to 4 μg per well resulted in no further increase in the proportion of cells taking up DNA (Fig. 3a and data not shown). In contrast, DNA uptake per cell was proportional to lipoplex concentration for both small and large constructs across all concentrations tested (Fig. 3b). These data are consistent with the saturation of the mES cell culture at 1 μg DNA lipoplexes per well and suggest that under the conditions used here a proportion of cells (30–50%) are refractory to lipoplex uptake during their exposure to lipoplexes.

Despite exhibiting similar saturation thresholds (1 μg DNA), addition of BAC lipoplexes to mES cells resulted in a lower proportion of cells (50%) taking up DNA than when small plasmids were used (65%) (Fig. 3a). Since uptake efficiency failed to increase to the levels achieved by small plasmids with increasing BAC lipoplex concentrations, this suggests that the uptake competence of mES cells may be

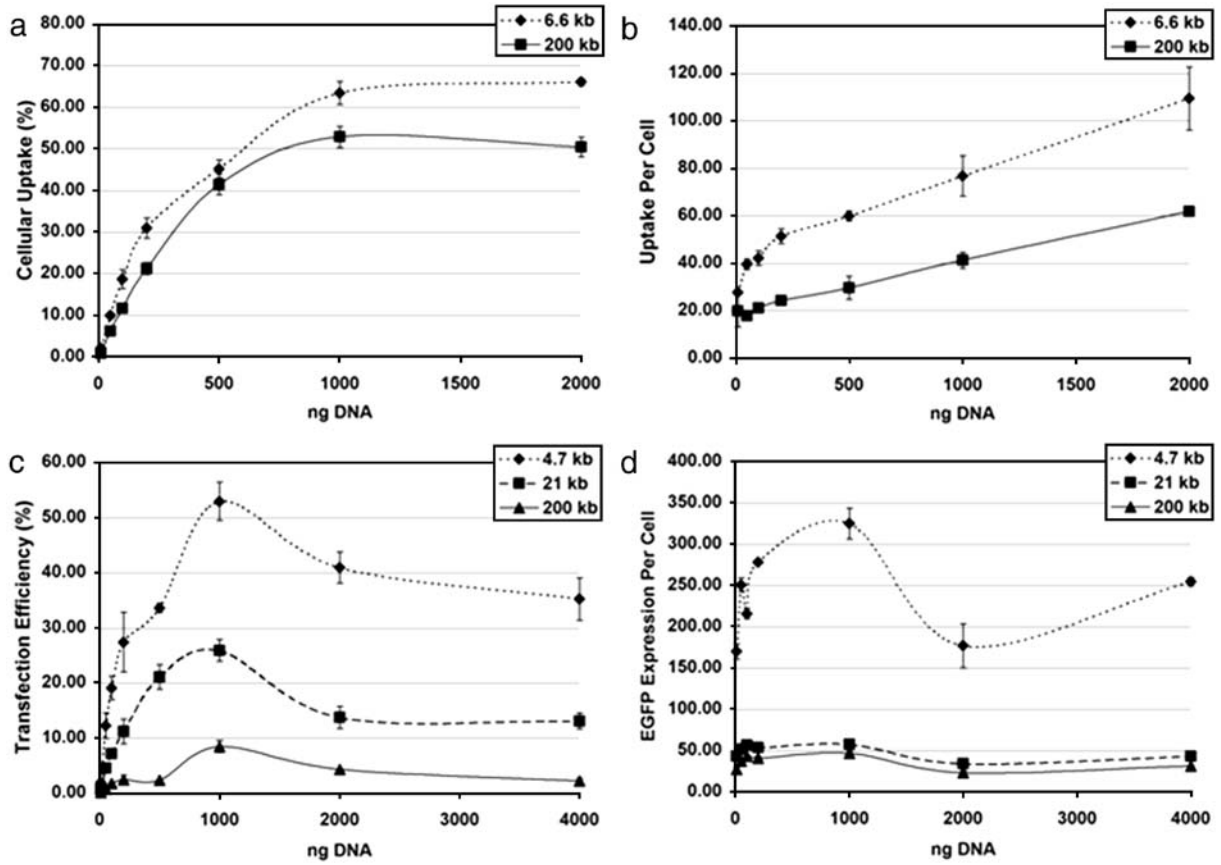

Fig. 3. Effects of lipoplex concentration on transfection efficiency. (a and b) mES cells were exposed to lipoplexes containing 5–2000 ng of TOTO-1/pGETrec (6.7 kb) or TOTO/BAC265 (200 kb) (DNA:LF2000 ratio of 1:4). DNA uptake was measured by flow cytometry after 5 h. (a) Cellular uptake efficiency and (b) MPFs were averaged across four independent transfections. Error bars represent the SEM. (c and d) mES cells were transfected with 5–2000 ng of pEGFP-N22 (4.7 kb), pEBAC160G (21 kb), or pEBAC148G (200 kb). EGFP expression was measured by flow cytometry 24 h after transfection. (c) Mean transfection efficiency and (d) MPFs were averaged across four independent transfections. Error bars indicate the SEM.

constrained by the amount of lipofection reagent used, rather than the number of competent lipoplexes produced. In this case, increasing LF2000 levels beyond 4 μg per well might be expected to reduce uptake competence through toxicity and/or the homeostatic mechanisms that protect mES cells from toxic shock.

Transfection efficiencies were also measured as a function of DNA concentration for three constructs of different sizes: the 4.7-kb pEGFP-N22 plasmid, the 19.5-kb pEBAC-160G BAC vector, and the 200-kb 148G BAC. All of these constructs contained a CMV–EGFP reporter cassette. For all constructs 1 μg of DNA was sufficient to attain maximum transfection efficiencies (Figs. 3c and 3d). Exposing mES cells to higher concentrations of lipoplexes resulted in decreased transfection efficiencies, which may reflect a decrease in mES cell viability at high lipoplex concentrations. In support of this interpretation, EGFP expression per cell was also decreased at concentrations above 1 μg of DNA per well, suggesting a selection bias for the survival of cells taking up fewer lipoplexes following transfection under these conditions.

As plasmid size increased, transfection efficiency dramatically decreased (Fig. 3c). This was correlated with a significantly diminished level of EGFP expression per cell (Fig. 3d), which may reflect a lower plasmid copy number in the nucleus. In fact, the level of EGFP expression per cell was the same for the two larger constructs (20 and 200 kb) and across all concentration ranges. The EGFP expression of cells transfected at very low DNA concentrations may reflect transcription from single transfected molecules. Thus, it is possible that even under saturating conditions, cells transfected with large constructs carry single transfected plasmids.

Effects of increasing DNA size

In the transfections described above, DNA mass was kept constant, allowing equal amounts of transfection reagent to be used in experiments comparing large and small plasmids. These experiments were not corrected for the differences in molar concentration between equal masses of small and large DNA constructs. Thus, although cells take up equal amounts of DNA (Fig. 2b), this equates to a 40-fold difference in the number of intracellular molecules between a construct of 5 kb and one of 200 kb. It has been suggested that lipofection is achieved through a “mass-action” mechanism in which intracellular defenses against invading DNA molecules are outcompeted by the large numbers of transfected plasmids [42] and that the molar insufficiency of large constructs fully accounts for their diminished transfection efficiencies [43].

To control for the differences in the number of molecules between large and small constructs, the mass of DNA used in each transfection was adjusted to contain 6 or 15.5 μmol of plasmid molecules. Because of the large difference in the size range of the constructs examined, a 40-fold difference between the mass of the smallest and the mass of the largest construct was required. To keep LF2000 concentration constant, this difference in DNA mass was compensated for by the addition of the pGETrec plasmid (6.6 kb), which is devoid of eukaryotic expression elements. Two micrograms (15.5 μmol) or 800 ng (6 μmol) total DNA was used in each transfection. Two micrograms of the 200-kb 148G BAC contains the same number of molecules (15.5 μmol) as 195 ng of the 19.5-kb pEBAC160G vector or 45 ng of the 4.5-kb pEGFP-N22. pGETrec stuffer DNA (1.8 and 1.95 μg) was used to bulk up the mass of the smaller constructs during lipoplex condensation. No stuffer DNA was used in the 148G BAC transfections. Expression of EGFP was measured by flow cytometry 24 h after transfection (Fig. 4).

Despite exposure to equivalent molar concentrations of each construct, lipofection of mES cells was highly size dependent (Fig. 4a). As construct size increased, transfection efficiency declined. As previously observed, the larger constructs achieved considerably lower levels of EGFP expression per cell (Fig. 4b). These data are consistent with the possibility that the transfection of large constructs is limited by their difficulties in moving through the cytoplasm.

Lipoplex exposure time course

To examine the dynamics of DNA uptake during lipoplex exposure, mES cells transfected with TOTO-1-labeled DNA (6.6 kb) were analyzed at four time points during the 6-h incubation period (Fig. 5).

Two hours after the addition of lipoplexes around 30% of cells had taken up DNA. By 5 h, the proportion of DNA-positive cells had doubled (Fig. 5a), after which mES cell viability appeared increasingly compromised when observed microscopically. In contrast, after 2 h of lipoplex exposure, the mean peak fluorescence of transfected cells was only slightly less than after 6 h (Fig. 5b), indicating that individual mES cells become saturated with DNA after a short lipoplex exposure time.

When mES cells were transfected with unlabeled pEGFP-N22 plasmid, transfection efficiency was similarly dependent on exposure time. In wells exposed to lipoplexes for 2 h, 30% of cells expressed EGFP 24 h after transfection (Fig. 5c). Transfection efficiency increased with increasing exposure time, reaching a maximum of around 50% after 6 h of lipoplex exposure. In contrast, EGFP expression per cell reached maximal levels after 2 h exposure time and remained stable with increasing exposure times (Fig. 5d). Exposing mES cells to lipoplexes for more than 6 h resulted in reductions both in transfection efficiency and in EGFP expression per cell (Figs. 5c and 5d), suggesting that 6 h of lipoplex exposure is optimal for mES cell lipofection, with increased exposure times adversely affecting mES cell viability.

To extend these observations further, we performed similar experiments using lipoplexes formed with EGFP mRNA prepared by in vitro transcription. Following lipofection, mRNA was translated and EGFP fluorescence could be detected by flow cytometry, providing an alternate method for the quantitation of lipoplex uptake (Figs. 5e and 5f). Results obtained with mRNA lipoplexes largely mirrored those obtained using DNA lipoplexes; however, intriguingly, extended mRNA lipoplex exposure (up to 8 h) did not appear to compromise lipofection efficiency, which may suggest that mRNA lipoplexes are better tolerated by mES cells than DNA lipoplexes.

Thus, the proportion of cells taking up lipoplexes was dependent on the exposure time, while DNA uptake per cell reached maximal levels after about 2 h. These data indicate that the cellular uptake of lipoplexes is limited by the proportion of cells that become competent for lipoplex uptake during the

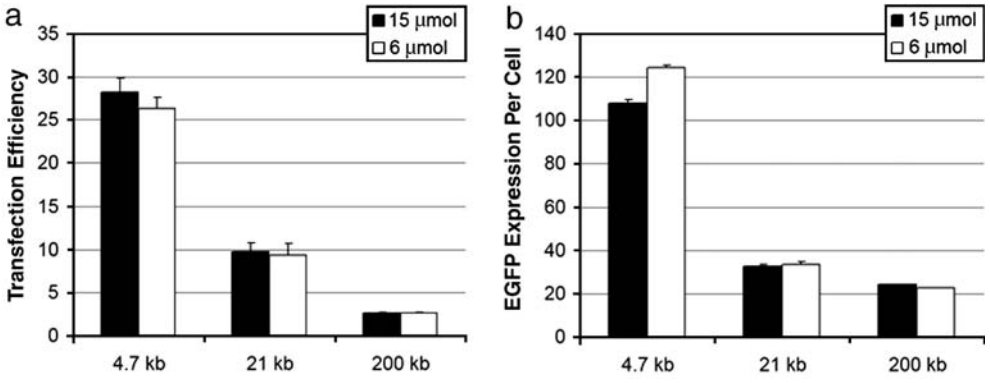

Fig. 4. Effects of DNA size on transfection. mES cells were transfected with equimolar amounts of one of three DNA constructs of different sizes, each containing a CMV–EGFP reporter cassette: pEGFP-N22 (4.7 kb), pEBAC160G (19.5 kb), and pEBAC148G (200 kb). Two DNA concentrations (15.5 and 6 μmol) were compared for each construct, amounting to 47/19 ng of pEGFP-N22 plasmid, 197/79 ng pEBAC160G vector, and 2000/800 ng pEBAC148G BAC. Each lipofection reaction was formulated to contain a total mass of 2 μg or 800 ng, respectively, using the pGETrec plasmid (6.6 kb) plasmid. (a) Transfection efficiencies and (b) EGFP expression per cell were analyzed 24 h after transfection.

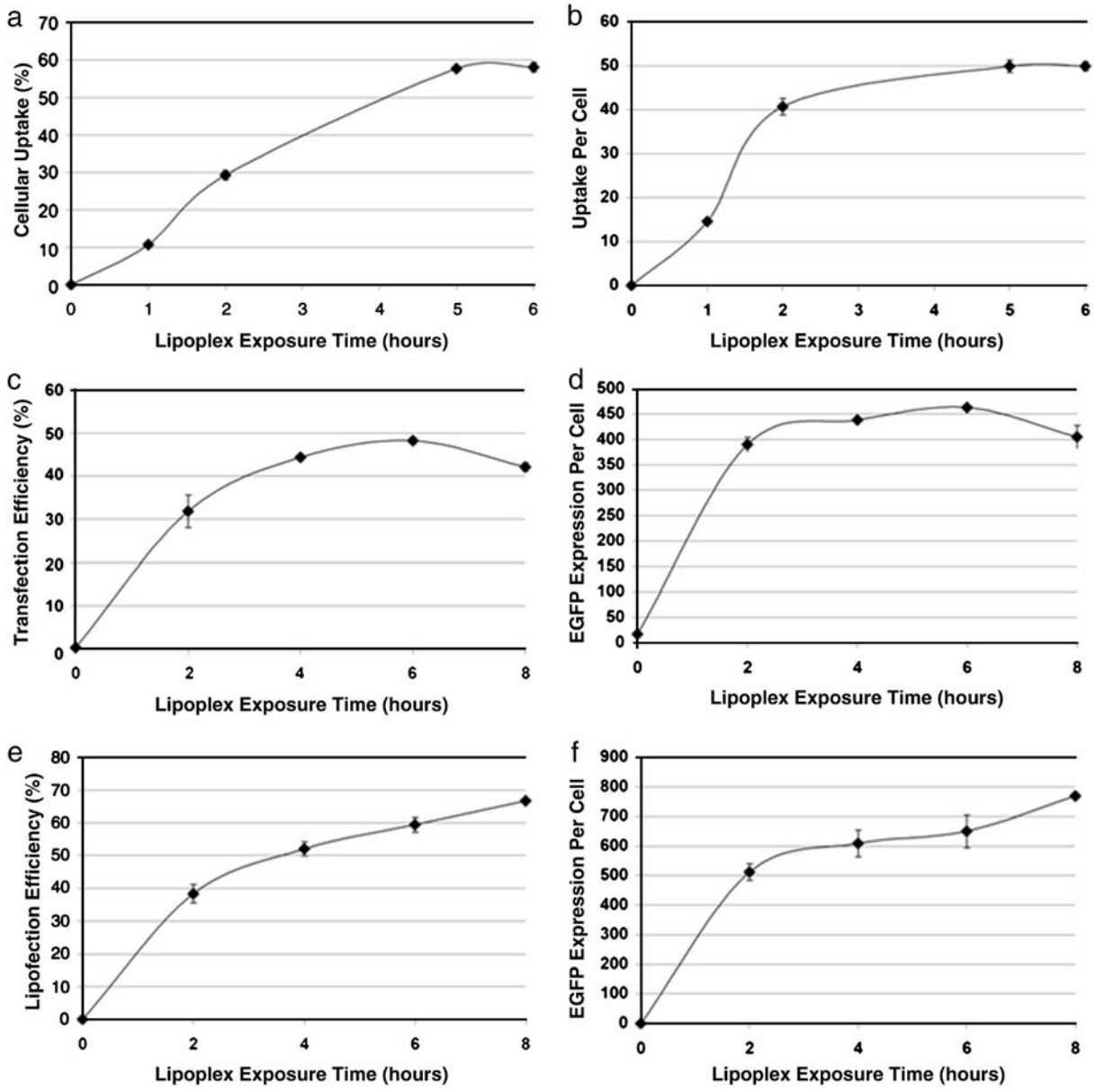

Fig. 5. Lipofection time course. (a and b) mES cells were transfected with 1 μg TOTO-1/pGETrec (6.6 kb) using LF2000. 1, 2, 5, or 6 h after addition of lipoplexes, cells were harvested and analyzed by flow cytometry. (a) Cellular uptake efficiencies and (b) uptake per cell were averaged over four independent transfections. Error bars indicate the SEM. (c and d) mES cells were transfected with 1 μg pEGFP-N22 DNA using LF2000. After 2, 4, 6, or 8 h exposure, lipoplexes were washed out and the cells cultured overnight. Each well was harvested and analyzed by flow cytometry 24 h after transfection. (a) Transfection efficiency and (b) EGFP expression per cell were averaged over four independent transfections. Error bars indicate the SEM. (e and f) mES cells were transfected with lipoplexes containing 1 μg of EGFP mRNA prepared by in vitro transcription (mRNA:LF2000 ratio of 1:2). After 2, 4, 6, and 8 h exposure, lipoplexes were washed out and cells cultured overnight. (e) Lipofection efficiencies and (f) EGFP expression per cell were analyzed 24 h after transfection.

exposure time and that individual mES cells become saturated with lipoplexes within 1–2 h.

Effects of lipofection on mES cell culture

To examine the effects of lipofection on the rate of cell growth in culture, cell counts were performed before, during, and after lipofection (Fig. 6). At the time of transfection (time 0), 1.25×10^5 cells were present in each well and some wells were exposed to DNA lipoplexes for 6 h. By 14 h, untransfected cells increased in number fourfold (Fig. 6), indicating that two population doublings had occurred during that time. In contrast, exposure to 1 μg of DNA lipoplexes prevented an increase in cell number at 14 h (Fig. 6).

At 14 h, cells were trypsinized and replated at 10^5 cells per well. Fourteen hours later (28 h postlipofection), the cell counts for both transfected and untransfected cells had increased by fourfold (Fig. 6), indicating that two cell divisions had taken place and suggesting a cell cycle length of around 7 h for exponentially growing mES cells. Thus, lipofection of

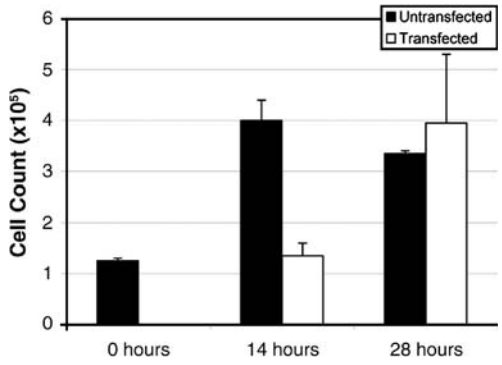

Fig. 6. Effects of lipofection on the rate of cell division. mES cells were plated into gelatin-coated 48-well plates and cultured overnight. The following day, cell counts were performed (0 hours) and some wells were transfected with 1 μg pEYFP-N1 DNA using LF2000 (1:4 DNA:LF2000 ratio). After 6 h of lipoplex exposure wells were washed and cultured for a further 8 h. Control wells were not transfected. Cells were then trypsinized and cell counts performed (14 hours) before the cells were replated into gelatin-coated 48-well plates (10^5 cells/well) and cultured for a further 14 h before final cell counts were performed (28 hours).

mES cells reduces the rate of cell growth for at least 14 h. Full recovery of growth rate was observed after a single cell passage.

Effect of plasmid linearization on transfection efficiency

To examine the effects of plasmid linearization on transfection efficiency, pEGFP-N22 was linearized by digestion with *Afl*III and purified by phenol–chloroform extraction. One microgram of linear or circular DNA was used to transfect 10^5 plated mES cells and transfection efficiency was measured by flow cytometry after 24 h. Transfection efficiencies were reduced by 60% when linear DNA was compared with circular plasmids (Fig. 7a). Furthermore, linear DNA transfection resulted in reduced EGFP expression per cell, yielding only 20% of the fluorescence per cell achieved using circular DNA (Fig. 7b).

To control for the influence of digestion and phenol–chloroform extraction on DNA quality, two control transfections were also performed. Mixed lipoplexes were formed using 1 μg of circular pEGFP-N22 and 1 μg of either circular pGETrec plasmid (Fig. 7, c+c) or linear pGETrec prepared by *Cla*I digestion and phenol–chloroform extraction (Fig. 7, c+l). The incorporation of linear, phenol–chloroform-purified DNA into lipoplexes had little effect on transfection efficiency (Fig. 7a), suggesting the formation of similar numbers of transfectable lipoplexes. EGFP expression per cell was slightly reduced (90%) compared with the value obtained using lipoplexes containing only circular DNA (Fig. 7b). This 10% reduction in the EGFP expression per cell may reflect the lower pEGFP-N22 plasmid copy number of mixed lipoplexes. Thus, reduced transfection of linear DNA was not due to the preparative methods. These results indicate that linear plasmids are less efficiently transfected into mES cells than circular plasmids.

Stable mES cell transfection using large DNA constructs

To examine the frequency of stable integration for small and large constructs, 10^6 mES cells were seeded into each well of a 12-well, gelatin-coated plate and cultured overnight. Each well was transfected with a total of 5 μg of DNA complexed with LF2000 at a ratio of 1:4. Circular pEGFP-N22 plasmid (4.7 kb) or *Peripherin-Green* BAC (187 kb) was compared with linear pEGFP-N22 (prepared by *Afl*III digestion followed by phenol–chloroform extraction) and linear *Peripherin-Green* (prepared by *Asc*I digestion followed by phenol–chloroform extraction).

Thirty-five hours after transfection, each well was harvested by trypsinization and cell counts were performed. All wells yielded $1.4–2.2 \times 10^6$ mES cells, indicating that the cells had recovered from the lipofection process and recommenced cell division. Twenty-five-microliter aliquots of each sample (containing $3.5–5.5 \times 10^4$ cells) were used to seed each well of a gelatin-coated 96-well plate and cultured under neomycin selection for 2 days (until colony formation). Surviving colonies were then counted.

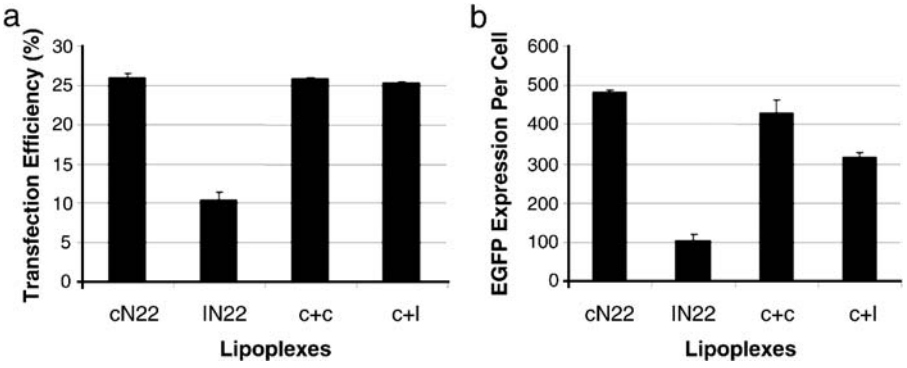

Fig. 7. Effects of plasmid linearization on transfection efficiency. mES cells were transfected with lipoplexes containing 1 μg of either circular pEGFP-N22 plasmid or linear pEGFP-N22, prepared by *Afl*III digestion followed by phenol–chloroform extraction. To control for the effect of phenol–chloroform extraction, mES cells were also transfected with lipoplexes formed using 1 μg circular pEGFP-N22 and 1 μg of either circular (c+c) or linear (c+l) pGETrec plasmid. Linear pGETrec was prepared by *Cla*I digestion followed by phenol–chloroform extraction. All lipoplexes were formed at a DNA:LF2000 ratio of 1:4. Each well was harvested and analyzed by flow cytometry 24 h after transfection. (a) Transfection efficiencies and (b) EGFP expression per cell were determined from FSC/FL1 live cell density plots. Error bars indicate the SEM.

Table 1
Frequency of stable transfection in mES cells

Construct	DNA (μg)	CFUs/cells plated	Stable transfection efficiency
Circular pEGFP-N22	5	$>100/5\times10^4$	–
Linear pEGFP-N22	5	$10/5\times10^4$	2×10^{-4}
Circular *Peripherin-Green*	10	$3/2\times10^6$	1.5×10^{-6}
Linear *Peripherin-Green*	10	$1/2\times10^6$	0.5×10^{-6}

No colonies were obtained in control wells exposed to LF2000 alone. However, circular pEGFP-N22 gave rise to >100 resistant colonies per well, while linear pEGFP-N22 gave rise to approximately 10 colonies per well. Given the density of colonies, the propensity of mES cells for aggregation, and the ability of circular plasmids to persist under selection due to replication from the SV40 origin, it was not possible to quantitate the frequency of stable transfection for the circular pEGFP-N22 plasmid transfections under these conditions. However, for linear pEGFP-N22, which cannot be replicated in transfected cells, colonies were surmised to have arisen from chromosomal integration events; thus stable transfection efficiencies could be calculated (Table 1).

mES cells transfected with the *Peripherin-Green* BAC gave rise to markedly fewer neomycin-resistant colonies. Both circular and linear *Peripherin-Green* transfections yielded stable clones. Under these conditions, we obtained a total of three colonies from 2×10^6 cells transfected with 10 μg of circular BAC DNA. Using linear BAC DNA a single resistant colony was obtained. All clones were surmised to have resulted from stable integrations as the *Peripherin-Green* BAC does not contain eukaryotic maintenance sequences that would allow it to persist in dividing cells as episomes. Stable transfection efficiencies for the BAC transfections are shown in Table 1.

Stable clones were maintained under selection for 22 days following transfection and were further cultured up to 33 days posttransfection. All clones maintained the characteristic morphology of mES cells, growing into tight colonies within 48 h.

To determine whether the *Peripherin-Green* BAC had integrated as an intact fragment, genomic DNA was prepared from each clone 28 days after transfection and analyzed by PCR. All clones were positive for the EGFP cDNA and amplification of exons 3–6 of the human *peripherin* locus yielded products of the expected size (data not shown), suggesting the locus had not undergone any major rearrangements. PCR was also performed on genomic DNA from each clone using primer pairs specific for the 5′ and 3′ regions of the P5 BAC, confirming the presence of distal sequences upstream and downstream of the *Peripherin-Green* locus following stable integration and suggesting the large BAC transgenes were incorporated into chromosomes as intact fragments (data not shown).

Discussion

The sequencing of the human and other genomes has made genes available for studies as intact functional units in sequenced PAC/BAC clones. However, functional studies with such clones are limited by their generally low efficiency of transfection. We have previously reported a large increase in the transfection efficiency of 20- to 200-kb BAC constructs that have the *oriP* sequence on the vector backbone in human erythroleukemia cells expressing the EBNA1 protein of Epstein–Barr virus, enabling the generation of stable cell lines for the identification of HbF inducers [7]. However, such an approach would not be possible or desirable for gene therapy with intact genomic loci in primary cells. We are therefore investigating the factors that limit the transfection of eukaryotic cells with large genomic DNA fragments, with a view to developing an efficient nonviral gene delivery method.

In this report, we have systematically examined the parameters influencing the transfection of mouse embryonic stem cells with small and large DNA constructs using the lipofection reagent LF2000. Using the fluorescent DNA binding dye TOTO-1 and expression from an EGFP reporter construct, we have dissected the lipofection process into two main components: cellular uptake, which measures the efficiency at which lipoplexes gain access to the cell, and nuclear delivery, which measures the efficacy of intracellular DNA trafficking.

The TOTO-1 DNA labeling method described here allows the physical uptake of DNA molecules to be followed by flow cytometry, independent of nuclear uptake and gene expression. The high quantum yield of the fluorescent TOTO/DNA complex allows the use of low labeling ratios (up to a bp:dye ratio of 200:1, data not shown), minimizing nonspecific labeling and any effects on DNA and lipoplex structure. Control experiments in which mES cells transfected with labeled DNA were analyzed by confocal microscopy demonstrated that the stringent trypsinization process used here effectively removed any lipoplexes that may have remained attached to the cell surface (Fig. 1e). Furthermore, cellular uptake data using TOTO-1/DNA lipoplexes was supported by additional experiments using EGFP mRNA lipoplexes (Figs. 5e and 5f and data not shown), which require internalization and translation (but not nuclear uptake) for detection. These observations confirm that the TOTO-1/DNA signal detected in the flow-cytometric analyses described here reflects intracellular DNA.

Factors affecting the cellular uptake of DNA lipoplexes by mES cells

The cellular uptake of DNA lipoplexes is affected by two main considerations: the properties of the lipoplex and the uptake competence of the cell. The formation of lipoplexes under optimal conditions is crucial to ensure the efficient generation of particles that are suitable for transfection. Transfectable lipoplexes are generally thought to comprise an irregular condensed DNA core coated in a layer of cationic lipids with particles ranging from 80 to 400 nm in size [22,41]. However, optimal conditions must be determined to promote the formation of discrete DNA–lipid particles over the formation of larger aggregates, which sequester DNA and reduce its availability for uptake.

Cationic lipids interact with DNA through the electrostatic interaction of their positively charged amine groups with the

negatively charged DNA backbone, leading to the condensation of DNA into lipoplexes. We have demonstrated that lipoplex condensation is more efficient at higher LF2000 concentrations, with increased numbers of cells taking up DNA at lower DNA:LF2000 ratios (Fig. 2a). During the condensation process, high LF2000 concentrations may increase the rate of plasmid encapsulation, generating greater numbers of lipoplexes with fewer plasmids being recruited to each lipoplex. Indeed, although lipoplexes formed with small plasmids were generated at higher frequency using lower DNA:LF2000 ratios, DNA uptake per cell declined, supporting this interpretation (Fig. 2b). Conversely, we have observed that increasing the concentration of DNA during lipoplex formation reduces the efficiency of condensation, leading to fewer transfected cells following transfection (data not shown). Together, these observations suggest that lipoplexes are formed most efficiently under conditions that serve to isolate plasmids from one another. Conditions that favor interactions between plasmids during their encapsulation (such as low reagent or high plasmid concentrations) are likely to cause aggregation, leading to the sequestering of DNA molecules in large, untransfectable particles.

Rapid condensation would be particularly important for the formation of lipoplexes using large DNA constructs such as BACs, which are thought to contain single plasmid molecules [41]. Inefficient condensation of BAC lipoplexes may leave loops of BAC DNA exposed, promoting interactions with other poorly formed lipoplexes and leading to aggregation. In contrast to small plasmids, BAC lipoplexes formed using higher LF2000 concentrations delivered increasing amounts of DNA per cell (Fig. 2b). Due to their large size, BACs are likely to be less amenable to alterations in the copy number of each lipoplex. Increased LF2000 concentration may promote the formation of competent BAC DNA lipoplexes in greater numbers, without influencing the DNA payload per lipoplex. Indeed, the amount of DNA delivered per cell was proportional to the efficiency of lipoplex formation for BACs (Fig. 2b). These observations support previous studies showing that, while the copy number is altered, the structure of lipoplexes formed using plasmids up to 52.5 kb is not affected by construct size [41] and further suggest that this may also be true for BACs up to 200 kb in size.

While efficient lipoplex condensation conditions may provide sufficient numbers of transfectable particles for lipofection, the proportion of cells that are competent for DNA uptake may further limit the access of lipoplexes to the cell interior. The data presented in Fig. 3 demonstrate that increasing lipoplex concentrations up to fourfold results in increased DNA uptake per cell, with only minimal increases in uptake efficiency. This suggests that only a fraction of the live cell population is competent for DNA uptake during exposure to lipoplexes at saturating concentrations. Furthermore, during the 6 h lipoplex exposure period, the proportion of cells taking up DNA continued to increase, while the amount of DNA uptake per cell reached 80–90% of the maximum value within 2 h (Fig. 5). Similarly, when mES cells were exposed to lipoplexes containing the 4.7-kb plasmid, transfection efficiency was limited by exposure time, while EGFP expression per cell was not. Thus, lipoplexes are not taken up by all cells simultaneously; otherwise, the data in Fig. 5 would have shown a stable proportion of transfected cells accumulating fluorescence over time. Nor are cells able to take up DNA for the entire duration of the exposure time, as DNA uptake per cell is stable despite increasing uptake efficiency (Fig. 5). Instead, the data support the existence of a transient window of DNA uptake competence, which cells move into and out of during lipoplex exposure.

Lipofection was shown to reduce the rate of cell growth in culture, although it was not determined if this was due to a reduced rate of proliferation or an increase in cell death (Fig. 7). mES cells exposed to lipoplexes for longer than 5–6 h exhibited gross signs of cell death. Moreover, when mES cells were oversaturated with lipoplexes EGFP expression per cell was reduced at 24 h, despite increased DNA uptake at 5 h (Fig. 3). These observations may reflect the preferential survival of cells taking up low levels of DNA, suggesting that lipoplex uptake is toxic to mES cells at high levels.

Together, the results indicate that the uptake of lipoplexes by mES cells may be limited both by the number of cells that enter an uptake-competent state during the exposure time and by a ceiling effect on DNA uptake created by the toxicity of lipoplexes at high concentrations. Using LF2000, optimal transfection efficiency of mES cells is achieved using 1 μg of DNA per 10^5 cells at a DNA:LF2000 ratio of 1:4. Preferential accumulation of lipoplexes at the cell surface late in the G1 phase of the cell cycle has been reported in HeLa cells [44], and we suggest that mES cells may become competent for lipoplex uptake as a function of the cell cycle. If this is the case, synchronization of mES cells prior to transfection and coordination of lipofection timing may enable transfection of the entire mES cell population with lipoplex exposure times of 2–3 h.

Factors affecting the intracellular trafficking of DNA

Despite similar, high-efficiency uptake of lipoplexes containing either BAC or small plasmid DNA, the transfection efficiencies obtained using large constructs were significantly reduced compared with small plasmids (Figs. 2 and 3). Some studies have implicated the intracellular concentration of DNA as the prime determinant of transfection efficiency for small plasmids [42] as well as for BAC DNA [43]. This hypothesis implies that competition between the molar concentration of transfected plasmids and the rate of intracellular degradation determines transfection efficiency. Under this model DNA lipofection is achieved by a “mass-action” mechanism [42] whereby intracellular defenses against invading nucleic acids are overcome by the large numbers of transfected DNA molecules. It follows that due to constraints on the total mass of DNA that can be taken up, the delivery of large constructs is limited by a reduced ability to saturate these defenses effectively.

We have shown that, while increasing lipoplex concentration resulted in higher levels of intracellular DNA, this had little effect on the transfection efficiencies of large constructs (Fig. 3). Furthermore, when mES cells were transfected with equimolar concentrations of small and large plasmids, transfection efficiency was more dependent on construct size than on

the amount of DNA used in each transfection. Transfection of larger plasmids was significantly reduced compared with smaller plasmids but was only slightly affected by lipoplex concentration (Fig. 4). Thus, factors other than the intracellular concentration of DNA molecules may limit the trafficking of large plasmids to the nucleus.

Given the immobility of DNA molecules over 2 kb in size in the cytoplasm [31], it is unlikely that DNA transit through the cytoplasm is achieved by passive diffusion. One possible scenario is that DNA binding proteins translated in the cytoplasm interact with transfected plasmids, resulting in cotransportation to the nucleus. Depending on the nature of the interaction between each macromolecule, the mobility of DNA–protein transit intermediates might range from loosely bound structures that transport DNA a short distance to stable complexes that transport DNA all the way to the nucleus. It seems likely that the mobility of transit intermediates through the cytoskeletal sieve would decrease with increasing plasmid size.

In support of this model, Dean et al. [45] demonstrated that a DNA sequence rich in transcription factor binding sites could enhance the transport of DNA from the cytoplasm to the nucleus of nondividing cells, suggesting cotransport of transcription factors and transfected DNA through the nuclear pore [45]. Furthermore, expression of the nuclear targeted EBNA-1 protein can enhance the transfection of DNA constructs containing the *oriP* origin of replication, to which the EBNA-1 protein binds [7]. However, Howden et al. [46] recently showed that this effect depends on the chromatin binding domain of EBNA-1, rather than the nuclear localization signal, which suggests that EBNA-1 may require cell division and the breakdown of the nuclear membrane to facilitate nuclear transport of transfected DNA.

This model supports the idea that the rate of cytoplasmic trafficking is a major determinant of transfection efficiency [42] and suggests that small plasmids evade degradation by rapid transit through the cytoplasm, rather than through the saturation of cellular defenses. In support of this conclusion, we observed EGFP expression as early as 5 h after lipoplex exposure in mES cells transfected with small plasmids, while cells transfected with BACs did not express EGFP at this time (data not shown). Thus, the amount of time large constructs spend navigating the cytoplasm may be a critical determinant of transfection probability.

In addition to the effects of plasmid size on DNA delivery, we have found that transfection of linear DNA is reduced in comparison to circular DNA (Fig. 7). This may reflect an increased sensitivity of linear fragments to degradation. While circular DNA is invulnerable to exonucleases, linear DNA would be quickly degraded by these enzymes. Alternatively, linear DNA contains free ends that are likely to be detected as double-strand breaks by nuclear repair machinery. Cells respond to double-strand breaks in one of two ways: repair or apoptosis. When double-strand breaks are detected in large numbers, apoptotic signaling overtakes repair signaling, leading to the fragmentation of the genome and the orderly demise of the cell. The activation of these cellular defenses by transfected linear DNA molecules may constrain transfection efficiencies by causing apoptosis at high concentrations. However, the incorporation of linear DNA fragments into lipoplexes did not affect the cotransfection of a circular plasmid, suggesting that apoptotic signaling does little to inhibit transfection under our conditions. Thus, intracellular degradation of linear DNA fragments is likely to explain their reduced transfection efficiencies.

Using the optimized lipofection methods described above, we have demonstrated the stable transfection of mES cells with a 187-kb BAC at a frequency of around 1 in 500,000 cells. Stable transfection efficiencies were increased at least 100-fold using small plasmids. PCR analysis of 5′ and 3′ sequences of this BAC indicated the integration of intact human genomic sequences into the mouse genome.

Concluding remarks

Lipofection is a high-efficiency method for the delivery of large DNA constructs to mES [19,20] and other cell lines [21,26,47,48]. In this paper, we have investigated the parameters influencing mES cell lipofection. Our results suggest that the delivery of small plasmids is primarily limited by lipoplex uptake and that asynchronously dividing mES cells are a heterogeneous population with regard to lipoplex uptake competence. In addition, we have demonstrated that although lipoplexes formed with large plasmids and those formed with small plasmids are taken up with similar efficiencies, nuclear delivery may be limited by the rate of intracellular transit. In addition to DNA delivery, we have demonstrated the delivery and translation of in vitro-transcribed mRNA using LF2000. mRNA delivery represents a novel strategy for protein expression in mES cells and may enable more precise control over intracellular protein levels as well the ability to deliver transient waves of protein expression for temporary roles, such as intracellular trafficking. We have recently demonstrated the facility of this approach using EBNA mRNA cotransfection to enhance transfection efficiency [46].

Methods

DNA constructs

pEGFP-N22 is a 4693-bp CMV immediate-early promoter-driven EGFP reporter plasmid (CMV–EGFP) [49] derived from the pEGFP-N1 construct (Clontech, Palo Alto, CA, USA) and containing a kanamycin/neomycin resistance gene. pEYFP-N1 is a 4733-bp CMV–EYFP reporter plasmid (Clontech). pEBAC160G is a 21.3-kb enhanced BAC vector developed in our lab [50]. It contains a number of eukaryotic expression cassettes including the hygromycin resistance gene for eukaryotic selection, the EBV *EBNA1* gene and *oriP* element for episomal maintenance, and a CMV–EGFP reporter cassette. pEBAC/148βG is a 200-kb BAC comprising the RPCI 1-148O22 PAC clone insert retrofitted into the pEBAC160G vector [7]. The 183-kb-long insert contains the intact human β-globin locus. pGETrec is a 6578-bp plasmid encoding the components of the *GET Recombination* system [49]. The genes on this plasmid cannot be expressed in eukaryotic cells. BAC265 (*RPCI 11-265B8*) is a 197-kb BAC containing the entire human *FXN* locus [8]. This locus encodes the frataxin protein, which is involved in mitochondrial iron–sulfur cluster biosynthesis. *Peripherin-Green* is a 187-kb genomic reporter construct in which the EGFP cDNA has been placed in frame with the coding sequences of the *peripherin*

locus in the P5 BAC (*RPCI 11-234P5*) (unpublished data). Due to their vulnerability to shearing, BAC constructs were handled using wide-bore pipette tips.

TOTO-1 labeling of DNA

Labeling of DNA molecules with the dimeric cyanine dye TOTO-1 (Molecular Probes, Eugene, OR, USA) was performed in 0.5× TE buffer to produce stable TOTO/DNA complexes with defined base pair:dye molecular ratios [40]. TOTO-1 was diluted to the appropriate concentration (3 μM for a DNA bp:dye molecular ratio of 50:1, 1.5 μM for a ratio of 100:1) and DNA was added to a final concentration of 100 ng/μl. Labeling reactions were incubated for at least 60 min at 4 °C and were used immediately. In the experiments described herein a bp:dye ratio of 50:1 to 100:1 was used, although we have found that labeling ratios of up to 200:1 are suitable for the analysis of transfection by flow cytometry (data not shown).

In vitro transcription

The Ambion SP6 mMESSAGE mMACHINE Kit (Ambion) was used for the production of EGFP mRNA. In vitro transcription template DNA was amplified by PCR from the pEGFP-N22 plasmid using the EGFPivt5 (5′-ATTTAGGTGACACTATAGAAGCGCCACCATGGATGTGAGCAAGGGCGAGGAGC-3′) and EGFPivt3 (5′-GGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAGATTATAGAGTCGCGGCCGCTTTAC-3′) primers. Eight 50-μl reactions were pooled and purified using the QIAquick PCR purification kit (Qiagen). In vitro transcription was performed according to the manufacturer's recommendations. RNA was quantitated by UV spectroscopy and gel electrophoresis.

mES cell culture and transfection

W9.5 mES cells were maintained under feeder-free culture conditions in gelatin-coated flasks, using standard ES medium (DME+15% fetal calf serum +0.1 mM amino acids +0.1 mM β-mercaptoethanol+1000 U/ml LIF). Cells were passaged every 2 to 3 days. For transfections, mES cultures were trypsinized, counted, and used to seed 24- or 48-well gelatin-coated plates (10^5 cells/well), for transfection the following day. We did not observe any overt differences in the growth or transfection of mES cell colonies between 24- and 48-well plates. For stable transfections, mES cells were grown under neomycin (Invitrogen) selection (300 μg/ml) as indicated.

LipofectAMINE 2000 (Invitrogen, San Diego, CA, USA) was used for all transfections. DNA (0.5–4 μg) or in vitro-transcribed EGFP mRNA was diluted in OptiMEM (Invitrogen) to a final volume of 50 μl. LipofectAMINE 2000 (1–16 μg) was diluted in OptiMEM to a final volume of 50 μl. The two solutions were mixed by pipetting and incubated at room temperature for 20 min. A DNA:LipofectAMINE ratio of 1:4 was routinely used, unless otherwise specified.

Flow cytometry

To harvest mES cells for flow cytometry, medium was removed and cells were washed one to three times with phosphate-buffered saline (PBS). After the final wash, PBS was replaced with 100 μl 0.25% trypsin/EDTA. The cells were then incubated for 5–10 min at 37 °C, dispersed by pipetting, and transferred into 400 μl PBS supplemented with 1% fetal calf serum.

Single-cell suspensions were kept on ice until data collection. Flow cytometry was performed on FACStar or FACSCalibur systems (Becton–Dickinson, Franklin Lakes, NJ, USA). Cells ($1–2\times10^4$) were analyzed for each sample. Data analysis was performed using WinMDI 2.8 (Joseph Trotter) and Excel (Microsoft) software. Untransfected cells and vehicle-only controls were included in every experiment and typically exhibited minimal autofluorescence. Evaluation was gated to include only living cells and cutoff thresholds for quantitation of the fluorescent cell population were determined from these experimental controls. Transfection and cellular uptake efficiencies refer to the percentage of cells included in the fluorescent fraction of the live cell population. In this report, "cellular uptake" refers to the internalization of DNA, while "transfection efficiency" is used to indicate nuclear delivery and expression of transfected DNA containing an EGFP expression cassette. As a measure of the TOTO/DNA uptake per cell or the level of EGFP expression per cell, the mean peak fluorescence of the fluorescent fraction was calculated using the WinMDI 2.8 software and expressed in arbitrary units.

Confocal microscopy

Single-cell suspensions were prepared in the same manner as for flow cytometry. Wheat germ agglutinin–AlexaFluor594 conjugate (Molecular Probes) and/or TOPRO-3 (Molecular Probes) was added to cell suspensions (2.5 μg/ml and 2 μM, respectively) and incubated on ice for 30 min. Cells were pelleted, resuspended in PBS+4% paraformaldehyde, and left on ice for a further 20 min before being transferred onto slides for confocal analysis. Microscopy was performed on a Zeiss Axioplan confocal microscope. Images and three-dimensional stacks were analyzed using the Confocal Assistant software.

Acknowledgments

Panos A. Ioannou passed away in April 2005. This work was inspired and led by him, and his colleagues dedicate this research to his memory. We thank members of the Cell and Gene Therapy Research Group for informative discussions and reagents. This work was supported by grants from the Brockhoff Foundation and the Ronald Geoffrey Arnott Foundation and a block grant from the National Health and Medical Research Council of Australia (No. 973051). Samuel McLenachan was the recipient of a Melbourne Research Scholarship.

References

[1] H. Shizuya, et al., Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in Escherichia coli using an F-factor-based vector, Proc. Natl. Acad. Sci. USA 89 (1992) 8794–8797.
[2] P.A. Ioannou, et al., A new bacteriophage P1-derived vector for the propagation of large human DNA fragments, Nat. Genet. 6 (1994) 84–89.
[3] K. Osoegawa, et al., An improved approach for construction of bacterial artificial chromosome libraries, Genomics 52 (1998) 1–8.
[4] M. Orford, et al., Engineering EGFP reporter constructs into a 200 kb human beta-globin BAC clone using GET Recombination, Nucleic Acids Res. 28 (2000) E84.
[5] M. Nefedov, R. Williamson, P.A. Ioannou, Insertion of disease-causing mutations in BACs by homologous recombination in Escherichia coli, Nucleic Acids Res. 28 (2000) E79.
[6] D. Jamsai, et al., Insertion of common mutations into the human beta-globin locus using GET Recombination and an EcoRI endonuclease counterselection cassette, J. Biotechnol. 101 (2003) 1–9.
[7] J. Vadolas, et al., Development of sensitive fluorescent assays for embryonic and fetal hemoglobin inducers using the human beta-globin locus in erythropoietic cells, Blood 100 (2002) 4209–4216.
[8] J.P. Sarsero, et al., Upregulation of expression from the FRDA genomic locus for the therapy of Friedreich ataxia, J. Gene Med. 5 (2003) 72–81.
[9] S.M. Magdaleno, T. Curran, Gene dosage in mice—BAC to the future, Nat. Genet. 22 (1999) 319–320.
[10] N. Heintz, BAC to the future: the use of BAC transgenic mice for neuroscience research, Nat. Rev. Neurosci. 2 (2001) 861–870.
[11] J.P. Sarsero, et al., Human BAC-mediated rescue of the Friedreich ataxia knockout mutation in transgenic mice, Mamm. Genome 15 (2004) 370–382.
[12] K. Shigekawa, W.J. Dower, Electroporation of eukaryotes and prokaryotes: a general approach to the introduction of macromolecules into cells, BioTechniques 6 (1988) 742–751.
[13] H. Kimoto, A. Taketo, Initial stage of DNA-electrotransfer into E. coli cells, J. Biochem. (Tokyo) 122 (1997) 237–242.
[14] N. Eynard, F. Rodriguez, J. Trotard, J. Teissie, Electrooptics studies of

Escherichia coli electropulsation: orientation, permeabilization, and gene transfer, Biophys. J. 75 (1998) 2587–2596.
[15] C.Y. Lu, et al., Gene-gun particle with pro-opiomelanocortin cDNA produces analgesia against formalin-induced pain in rats, Gene Ther. 9 (2002) 1008–1014.
[16] Y.C. Chuang, et al., Gene therapy for bladder pain with gene gun particle encoding pro-opiomelanocortin cDNA, J. Urol. 170 (2003) 2044–2048.
[17] G. Zhang, et al., Hydroporation as the mechanism of hydrodynamic delivery, Gene Ther. 11 (2004) 675–682.
[18] H. Nagel, et al., The alphavbeta5 integrin of hematopoietic and nonhematopoietic cells is a transduction receptor of RGD-4C fiber-modified adenoviruses, Gene Ther. 10 (2003) 1643–1653.
[19] J.T. Lee, R. Jaenisch, A method for high efficiency YAC lipofection into murine embryonic stem cells, Nucleic Acids Res. 24 (1996) 5054–5055.
[20] W.M. Strauss, et al., Germ line transmission of a yeast artificial chromosome spanning the murine alpha 1(I) collagen locus, Science 259 (1993) 1904–1907.
[21] N. Joshee, D.R. Bastola, P.W. Cheng, Transferrin-facilitated lipofection gene delivery strategy: characterization of the transfection complexes and intracellular trafficking, Hum. Gene Ther. 13 (2002) 1991–2004.
[22] W.J. Montigny, S.F. Phelps, S. Illenye, N.H. Heintz, Parameters influencing high-efficiency transfection of bacterial artificial chromosomes into cultured mammalian cells, BioTechniques 35 (2003) 796–807.
[23] L.C. Mounkes, W. Zhong, G. Cipres-Palacin, T.D. Heath, R.J. Debs, Proteoglycans mediate cationic liposome–DNA complex-based gene delivery in vitro and in vivo, J. Biol. Chem. 273 (1998) 26164–26170.
[24] T. Tagawa, et al., Characterisation of LMD virus-like nanoparticles self-assembled from cationic liposomes, adenovirus core peptide mu and plasmid DNA, Gene Ther. 9 (2002) 564–576.
[25] K.A. Mislick, J.D. Baldeschwieler, Evidence for the role of proteoglycans in cation-mediated gene transfer, Proc. Natl. Acad. Sci. USA 93 (1996) 12349–12354.
[26] H. Keller, et al., Transgene expression, but not gene delivery, is improved by adhesion-assisted lipofection of hematopoietic cells, Gene Ther. 6 (1999) 931–938.
[27] J. Zabner, A.J. Fasbender, T. Moninger, K.A. Poellinger, M.J. Welsh, Cellular and molecular barriers to gene transfer by a cationic lipid, J. Biol. Chem. 270 (1995) 18997–19007.
[28] R. Wattiaux, N. Laurent, S. Wattiaux-De Coninck, M. Jadot, Endosomes, lysosomes: their implication in gene transfer, Adv. Drug Delivery Rev. 41 (2000) 201–208.
[29] N. Laurent, et al., Uptake by rat liver and intracellular fate of plasmid DNA complexed with poly-L-lysine or poly-D-lysine, FEBS Lett. 443 (1999) 61–65.
[30] O. Zelphati, F.C. Szoka Jr., Mechanism of oligonucleotide release from cationic liposomes, Proc. Natl. Acad. Sci. USA 93 (1996) 11493–11498.
[31] G.L. Lukacs, et al., Size-dependent DNA mobility in cytoplasm and nucleus, J. Biol. Chem. 275 (2000) 1625–1629.
[32] F.L. Sorgi, S. Bhattacharya, L. Huang, Protamine sulfate enhances lipid-mediated gene transfer, Gene Ther. 4 (1997) 961–968.
[33] B. Schwartz, M.A. Ivanov, B. Pitard, V. Escriou, R. Rangara, G. Byk, P. Wils, J. Crouzet, D. Scherman, Synthetic DNA-compacting peptides derived from human sequence enhance cationic lipid-mediated gene transfer in vitro and in vivo, Gene Ther. 6 (1999) 282–292.
[34] D. Balicki, C.D. Putman, P.V. Scaria, E. Beutler, Structure and function correlation in histone H2A peptide-mediated gene transfer, Proc. Natl. Acad. Sci. USA 99 (2002) 7467–7471.
[35] I. Mortimer, et al., Cationic lipid-mediated transfection of cells in culture requires mitotic activity, Gene Ther. 6 (1999) 403–411.
[36] M. Wilke, E. Fortunati, M. van den Broek, A.T. Hoogeveen, B.J. Scholte, Efficacy of a peptide-based gene delivery system depends on mitotic activity, Gene Ther. 3 (1996) 1133–1142.
[37] B. Alberts, et al., Molecular Biology of the Cell, 3rd ed. Garland, New York, 1994.
[38] P.M. Ojala, B. Sodeik, M.W. Ebersold, U. Kutay, A. Helenius, Herpes simplex virus type 1 entry into host cells: reconstitution of capsid binding and uncoating at the nuclear pore complex in vitro, Mol. Cell. Biol. 20 (2000) 4922–4931.
[39] K. Braun, et al., A biological transporter for the delivery of peptide nucleic acids (PNAs) to the nuclear compartment of living cells, J. Mol. Biol. 318 (2002) 237–243.
[40] H.S. Rye, et al., Stable fluorescent complexes of double-stranded DNA with bis-intercalating asymmetric cyanine dyes: properties and applications, Nucleic Acids Res. 20 (1992) 2803–2812.
[41] P. Kreiss, et al., Plasmid DNA size does not affect the physicochemical properties of lipoplexes but modulates gene transfer efficiency, Nucleic Acids Res. 27 (1999) 3792–3798.
[42] W.C. Tseng, F.R. Haselton, T.D. Giorgio, Transfection by cationic liposomes using simultaneous single cell measurements of plasmid delivery and transgene expression, J. Biol. Chem. 272 (1997) 25641–25647.
[43] R.E. White, et al., Functional delivery of large genomic DNA to human cells with a peptide-lipid vector, J. Gene Med. 5 (2003) 883–892.
[44] W.C. Tseng, F.R. Haselton, T.D. Giorgio, Mitosis enhances transgene expression of plasmid delivered by cationic liposomes, Biochim. Biophys. Acta 1445 (1999) 53–64.
[45] D.A. Dean, J.N. Byrd Jr., B.S. Dean, Nuclear targeting of plasmid DNA in human corneal cells, Curr. Eye Res. 19 (1999) 66–75.
[46] S.E. Howden, et al., Chromatin-binding regions of EBNA1 protein facilitate the enhanced transfection of Epstein–Barr virus-based vectors, Hum. Gene Ther. 17 (2006) 833–844.
[47] M. Colin, et al., Cell delivery, intracellular trafficking and expression of an integrin-mediated gene transfer vector in tracheal epithelial cells, Gene Ther. 7 (2000) 139–152.
[48] F. Pampinella, et al., Analysis of differential lipofection efficiency in primary and established myoblasts, Mol. Ther. 5 (2002) 161–169.
[49] K. Narayanan, R. Williamson, Y. Zhang, A.F. Stewart, P.A. Ioannou, Efficient and precise engineering of a 200 kb beta-globin human/bacterial artificial chromosome in E. coli DH10B using an inducible homologous recombination system, Gene Ther. 6 (1999) 442–447.
[50] K. Al-Hasani, et al., Development of a novel bacterial artificial chromosome cloning system for functional studies, Plasmid 49 (2003) 184–187.