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ARTICLE

Analysis of Differential Lipofection Efficiency in Primary and Established Myoblasts

Francesca Pampinella,[1] Delphine Lechardeur,[2] Elena Zanetti,[1] Ian MacLachlan,[3] Mohammed Benharouga,[2] Gergely L. Lukacs,[2] and Libero Vitiello[1,*]

[1]*Department of Biology, University of Padova, Padova, Italy 35121*
[2]*Department of Laboratory Medicine and Pathobiology, University of Toronto and Hospital for Sick Children, Research Institute, Program in Lung and Cell Biology, Toronto, Ontario, Canada M5G 1X8*
[3]*Protiva Biotherapeutics Inc., Burnaby, B.C., Canada V5J 5J8*

**To whom correspondence and reprint requests should be addressed. Fax: +39 049 827 6209. E-mail: vitiello@bio.unipd.it.*

In this study we have compared the process of lipid-mediated transfection in primary and established myoblasts, in an attempt to elucidate the mechanisms responsible for the scarce transfectability of the former. We determined the metabolic stability of cytoplasmically injected and lipofected DNA in primary and established myoblasts and carried out a comparative time course analysis of luciferase reporter-gene expression and DNA stability. The efficiency of the transcription-translation machinery of the two cell types was compared by intranuclear injection of naked plasmid DNA encoding luciferase. Subcellular colocalization of fluorescein-labeled lipopolyplexes with specific endosomal and lysosomal markers was performed by confocal microscopy to monitor the intracellular trafficking of plasmid DNA during transfection. The metabolic stability of plasmid DNA was similar in primary and established myoblasts after both lipofection and cytoplasmic injection. In both cell types, lipofection had no detectable effect on the rate of cell proliferation. Confocal analysis showed that nuclear translocation of transfected DNA coincided with localization in a compartment devoid of endosome- or lysosome-specific marker proteins. The residency time of plasmid DNA in this compartment differed for primary and established myoblasts. Our findings suggest that the lower transfectability of primary myoblasts is mostly due to a difference in the intracellular delivery pathway that correlates with more rapid delivery of internalized complex to the lysosomal compartment.

Key Words: gene therapy, transfection, lipopolyplex, endosome, primary myoblast, plasmid stability

INTRODUCTION

Several features make cationic liposomes a promising tool for gene therapy: there is no size limitation to the DNA they can transfer, they are suitable for large-scale production, and their low immunogenicity and cytotoxicity make them good candidates for repeated *in vivo* administration. Despite their widespread use, however, their mechanisms of action are still scarcely known.

It is generally accepted that DNA/cationic liposome complexes enter the cell through an endocytic process [1]. This conclusion has been drawn from indirect observations of the cellular metabolism of lipofection and from morphological observations. Substances that interfere with the endolysosomal pathway (such as wortmannin, cytochalasin B, and nigericin) inhibit lipofection [2,3]. Agents with known endosome-destabilizing activity, such as adenoviral particles [4] and fusogenic peptides [5,6] enhance transfection efficiency. Direct observation using fluorescence and transmission-electron microscopy has shown that lipoplexes are internalized through a process that is kinetically very similar to that of endocytosis, and that gold-labeled lipoplexes are found inside vesicles that resemble endosomes [7,8]. Although extensively studied in established cell lines, the mechanism of cationic lipid-mediated transfection of primary myoblasts has yet to be described.

We have previously reported that lipofection is up to 50-fold more efficient in established compared with primary myoblasts [9]. However, the reasons for the high transfectability of the former are still not understood. Possible explanations may involve a more efficient internalization of the complexes and/or an increased nuclear uptake of plasmid DNA in established myoblasts. Also, the rate of DNA degradation in the cytoplasm, one of the

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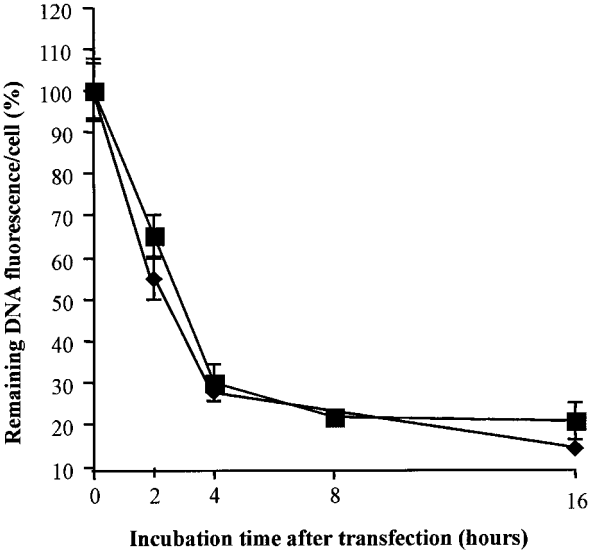

FIG. 1. Turnover rate of plasmid DNA after transfection. Metabolic stability of plasmid DNA in transfected primary (square) and C2C12 (diamond) myoblasts was measured by quantitative single-cell fluorescence video-image analysis. The remaining fluorescence signal derived from FITC-plasmid DNA was plotted as a function of incubation time and expressed as percentage of the initial value.

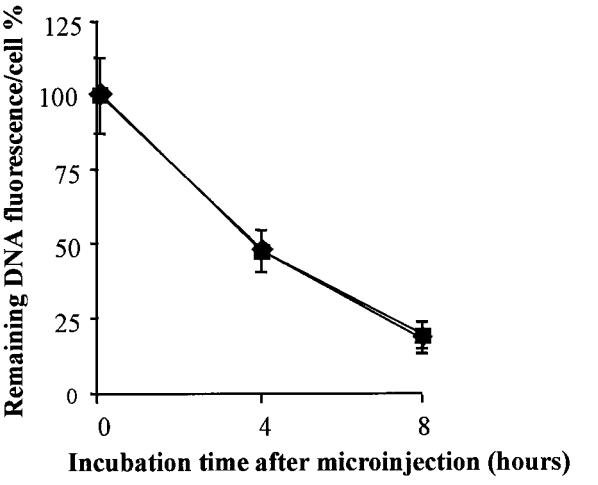

FIG. 2. Turnover rate of naked plasmid DNA in the cytoplasm of myoblasts. The disappearance of cytoplasmically microinjected FITC-labeled DNA was determined in primary (square) and C2C12 (diamond) myoblasts using quantitative single-cell fluorescence video imaging as for Fig. 1.

barriers to nuclear delivery of plasmid DNA [10], could be substantially reduced in C2C12 compared with primary cells. To determine the reasons behind the difference in the transfection efficiency, we compared the intracellular stability of plasmid DNA, administered both by lipofection and cytoplasmic injection, as well as the amount of lipopolyplexes internalized after transfection. The effect of lipofection on cell proliferation and the time course of reporter gene expression in the two cell types were also analyzed. Lastly, the intracellular trafficking pathway of lipofected plasmid DNA was determined by laser fluorescence confocal microscopy, in relation to specific markers of the endolysosomal compartments. To the best of our knowledge, this is the first study that has analyzed the intracellular trafficking of lipopolyplexes in primary cells using such an approach. Our findings show that the higher transfectability of established myoblasts could not be explained by differential uptake of lipopolyplexes or by differential stability of the plasmid DNA in the endolysosomal or cytoplasmic compartments. Instead, the results indicate that a slower delivery of lipopolyplexes to the lysosomal compartment is one of the factors responsible for the pronounced transfectability of established myoblasts.

RESULTS

Half-life of Lipofected DNA

To monitor the intracellular stability of lipofected DNA, supercoiled double-stranded pL018 plasmid was FITC-labeled and used to form DODAC-PEG lipopolyplexes. Cultured human primary myoblasts and C2C12 cells were exposed to such complexes for 2 hours and were then re-plated, left undisturbed for 6 hours, and fixed at different time points. We carried out this procedure to remove all of the noninternalized complexes from the cell surface. The internalized DNA, visualized by fluorescence microscopy, appeared as punctate clusters, with aggregates of different size showing a clear perinuclear distribution (data not shown). Quantitative single-cell fluorescence video-image analysis revealed that the half-life of plasmid DNA was approximately 2.5 hours in C2C12 cells and 3 hours in primary myoblasts (Fig. 1). DNA disappearance was quite rapid in the initial phase (between 0 and 8 hours in Fig. 1) and slowed down considerably in the following period. No fluorescence was ever observed inside the nuclei at any of the time points. Confirming previous observations [9], the amount of plasmid DNA found inside primary cells was higher than that found in C2C12. In particular, at the zero time point (that is, 6 hours after the end of transfection) the average fluorescence intensity within C2C12 and primary myoblasts was approximately 2.6×10^6 and 5.3×10^6 arbitrary units per cell, respectively.

Turnover Rate of the Plasmid DNA in the Cytosol

It has recently been shown that the cytosol contains nucleases that actively degrade microinjected DNA [10]. We therefore speculated that a difference in such activity could have a role in the enhanced transfectability of established versus primary myoblasts. To assess this possibility, we analyzed the metabolic turnover of naked plasmid DNA in the cytoplasm of microinjected primary and established

TABLE 1: BrdU incorporation analysis in primary and established myoblasts

	% of BrdU-positive human myoblasts		% of BrdU-positive C2C12 myoblasts	
Hours after transfection	transfected	non-transfected	transfected	non-transfected
1	26.3 ± 13.1	25.1 ± 0.8	53.2 ± 6.8	49.2 ± 7.3
5	19.5 ± 3.9	32.4 ± 6.4	63.4 ± 11.1	50.8 ± 4.7
7	28.2 ± 5.1	30.8 ± 8.0	43.9 ± 1.6	62.3 ± 8.9
22	32.9 ± 14.3	27.7 ± 3.6	53.6 ± 11.3	52.8 ± 4

Cells were transfected with standard procedure for 2 hours. After removing the complexes, BrdU was added to the wells to a final [10 μM] and left in place for 1 hour before fixing the cells. BrdU-positive cells were stained with an anti-BrdU antibody by standard immunofluorescence techniques.

myoblasts. Following cytosolic co-injection of FITC-labeled DNA and TRITC-dextran, the time course of plasmid DNA disappearance was monitored by quantitative single-cell fluorescence video-image analysis. The cytosolic half-life of the DNA was found to be around 4 hours both in primary and C2C12 cells (Fig. 2).

Time Dependence of Luciferase Expression after Lipofection

In parallel with the DNA stability measurements, we also analyzed the levels of transgene expression by carrying out time-course experiments with unlabeled plasmid. As a preliminary step, transfections were performed both with and without trypsinization and re-plating, to determine whether such a procedure had an effect on the expression profile of the transgene. We then analyzed luciferase levels 6, 8, 10, 12,14, 18, and 22 hours after removing the lipopolyplexes and no significant differences were found between re-plated and undisturbed cells (data not shown). The re-plating step was therefore omitted, so that we could measure the levels of luciferase activity also at earlier time points (starting at 1 hour after the end of transfection; Fig. 3). In both cell types, the kinetics of reporter gene expression was not linear and the two curves were quite similar for the first 8–10 hours. Luciferase activity was detected as early as 1 hour after removal of the complexes, and increased rapidly over the following 4 hours and more slowly from 6 to 10 hours after the end of transfection. After this time, the expression reached a plateau in primary myoblasts, whereas in C2C12 cells luciferase levels continued to increase until reaching a maximum 18 hours after complex removal. In primary myoblasts luciferase expression was virtually identical at 10 and 14 hours after the end of transfection (1636 ± 360 and 1600 ± 160), whereas in C2C12 cells luciferase expression almost tripled in the same time interval (5800 ± 1532 and 15666 ± 2868). Such a difference was highly significant in a *t*-test analysis ($P = 0.002$). The maximal level of luciferase expression found in primary cells was much lower than that of C2C12 (~ 1.5 ng luciferase/mg total protein extract, versus 21 ng/mg, respectively).

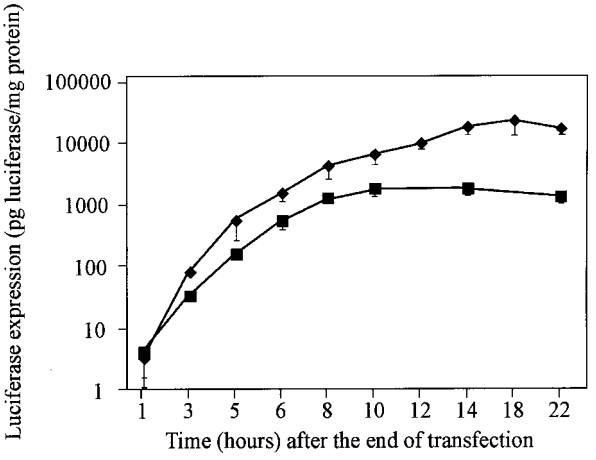

FIG. 3. Time-course of luciferase expression upon transfection of myoblasts. Following standard (2 hours) lipofection of primary (square) and C2C12 (diamond) myoblasts, luciferase activity was measured at the indicated times. The results are normalized for cellular protein content and expressed as amount of luciferase.

Effect of Transfection on Cell Proliferation

As lipid-mediated transfection is more efficient in the presence of active cell proliferation [11], we examined whether increased cell division rate was responsible for the augmented transfection potential of established myoblasts or, by the same token, whether an impaired proliferation of primary cells was responsible for their poor luciferase expression. We measured the proliferative activity in primary and C2C12 myoblasts, both transfected and non-transfected, by treating the cells with bromodeoxyuridine (BrdU) to label the actively duplicating DNA in nuclei. BrdU was added to the culture medium 0, 3, and 6 hours after the end of transfection. At all time points measured, approximately 30% of the primary and 50% of the established myoblasts demonstrated BrdU incorporation. Such findings were in good agreement with the shorter doubling time found for C2C12 compared with primary myoblasts during routine *in vitro* expansion. It is important to note that in both cases no significant difference was found in the number of BrdU-positive nuclei between transfected and non-tranfected myoblasts, indicating that the transfection process *per se* had not affected the rate of cell proliferation (Table 1).

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TABLE 2: Luciferase expression after intranuclear injection of naked pL018 plasmid

	fg luciferase/cell 2 hours postinjection	fg luciferase/cell 4 hours postinjection
C2C12	783 ± 38	5105 ± 199
Primary myoblasts	532 ± 42	3251 ± 187

After 2 and 4 hours of incubation postinjection, cells were lysed and luciferase assay was performed on the lysate. The expression data were normalized for cell survival rate, 80% and 60% in C2C12 and primary myoblasts, respectively.

Intracellular Trafficking of Lipopolyplexes in Primary Myoblasts

To study the intracellular trafficking pathway of lipofected plasmid DNA, we established the localization of FITC-conjugated plasmid DNA relative to specific endosomal and lysosomal markers at various time points post-transfection. Initially, cells were exposed to the complexes for 30 minutes and fixed after a 5-minute chase to identify the intracellular compartment encountered by plasmid DNA in the initial phase of transfection. After such a short exposure to lipopolyplexes, the amount of FITC-conjugated plasmid DNA found inside the cells was very small. Internalized plasmid DNA was co-localized with the Rab5B marker of the early endosomal compartment as well as with the late-endosomes marker TRITC-dextran (Fig. 4A).

The subsequent fate of internalized plasmid DNA was studied in cells exposed to lipopolyplexes for 2 hours (that is, the standard procedure) before trypsinization and replating. At 6 hours after the removal of the complexes, cells were immunostained using lysosome-specific antibodies (LAMP-2 and CD-63) or an antibody specific for early endosomes (Rab5B). Again, TRITC-dextran was used to label late endosomes. Our observations revealed that at this time point only a portion of the plasmid-associated FITC signal colocalized with lysosomes and that none of the plasmid signal was associated with early or late endosomes (Fig. 4B). With time, the fraction of plasmid DNA that colocalized with lysosomes increased, paralleling the disappearance of the FITC-DNA signal. At 10 hours after the end of transfection nearly all of the plasmid DNA colocalized with lysosomes (Fig. 4C). These experiments suggest that the intracellular trafficking of the plasmid DNA follows the route of a fluid phase marker, albeit with significantly slower kinetics. In fact, whereas fluid phase markers require approximately 60 minutes to be chased from the endosomal compartment into lysosomes, it took 16 hours (from the end of transfection) before most of the lipopolyplex-associated plasmid DNA could be found inside the lysosomes.

Intracellular Trafficking of Lipopolyplexes in C2C12 Myoblasts

The trafficking of lipofected plasmid DNA was also followed in C2C12 cells, after both 30-minute and 2-hour exposures to lipopolyplexes. In the first case, the pattern of colocalization with labeled dextran and Rab-5 antibody was equivalent to that found in primary cells, albeit with a smaller amount of internalized DNA (data not shown). Colocalization of plasmid DNA with the lysosomal compartment was followed with a LAMP-1 antibody. As opposed to what was seen with primary myoblasts, only a fraction of the labeled plasmid DNA was found inside the lysosomes 14 hours after the end of a 2-hour transfection; an almost complete colocalization was found after an additional 8 hours (Fig. 5).

Evaluation of Transcription-Translation Efficiency in Primary and Established Myoblasts

To compare the efficiency of the transcription-translation machinery between primary and established myoblasts, we carried out nuclear injections of the pL018 plasmid and the reporter gene expression was quantitated 2 and 4 hours post-injection. The amount of protein detected was approximately 50% higher in C2C12 than in primary myoblasts at both time points considered (Table 2). The variation in transgene expression between 2 and 4 hours after injection (sixfold) was very similar in both cell types.

DISCUSSION

The lower transfectability of primary versus established cells has been reported for several different cell types [12,13], including myoblasts [9,14]. Determining the mechanisms responsible for such phenomena would be of great help in designing an efficient nonviral gene-delivery system for *in vivo* or *ex vivo* gene therapy. In this paper we set out to elucidate the reasons for the poor transfectability of primary versus established myoblasts. It is now generally accepted that internalization of complexes occurs mainly, if not exclusively, by endocytosis and the internalized plasmid DNA is targeted for degradation into the lysosomal compartment [7,8,15,16].

There are several mechanisms that could explain the enhanced transfectability of the established myoblasts. Among these are enhanced uptake of plasmid DNA, attenuated degradation rate in the endolysosomal compartment, augmented escape from endolysosomes, mitigated hydrolysis in the cytoplasm by nucleases, and more efficient transcription/translation of plasmid DNA. The experiments performed here were designed to investigate these possibilities.

We had shown previously that, 24 hours after lipofection, primary myoblasts contain more plasmid DNA than C2C12 cells but the percentage of intact versus degraded DNA is higher in established myoblasts [9]. Using single-cell-fluorescence video imaging, here we have therefore analyzed the intracellular plasmid DNA content in both lipofected and microinjected cells. It is important to note that in previous experiments with HeLa and COS cells we have performed a comparative analysis between the

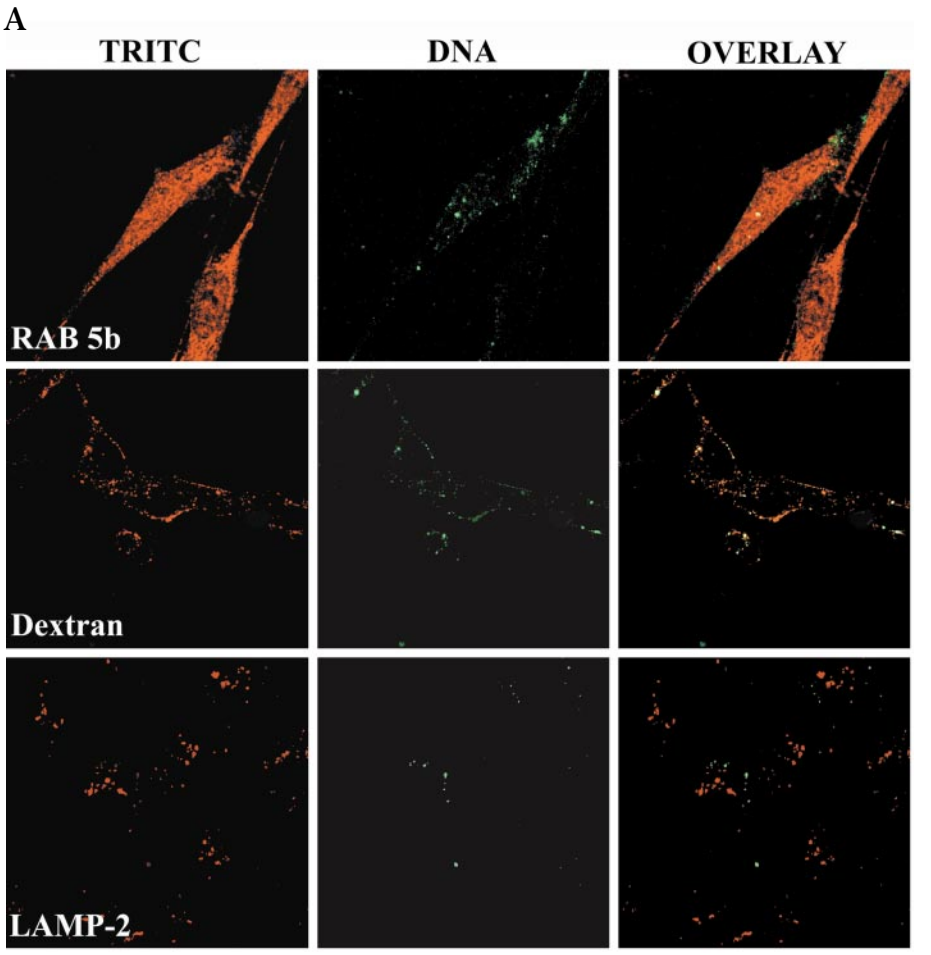

FIG. 4. Intracellular trafficking of the complexes in primary myoblasts transfected with lipopolyplexes containing FITC-labeled plasmid. Images refer to a focal plane selected in the central portion of the cells; in the overlays a yellow signal denotes the colocalization of two fluorochromes. Original magnification, ×100. (A) Cells were exposed to the lipopolyplexes for 30 minutes and fixed 5 minutes after removal of the complexes. Lysosomes and early endosomes were stained with LAMP-2 and Rab5B specific primary antibodies, respectively. Late endosomes were labeled by TRITC-dextran internalization. Note that in this experiment the cells had not undergone the re-plating procedure and therefore a considerable part of the signal comes from lipopolyplexes that were not actually inside the cells. (B) (page 166) Cells were fixed at the zero time point (that is, 6 hours after removal of the complexes) and stained with antibodies against lysosomal markers, (LAMP-1 and CD 63), and early endosomal marker (Rab5B). Late endosomes were stained by TRITC-dextran loading. (C) (page 167) Cells were fixed at different times after the end of transfection and immunostained for LAMP-2.

turnover of microinjected intact plasmid DNA using FISH and that of biotinylated (by UV-crosslinking of photoactivable biotin) and FITC-labeled plasmid DNA. No significant difference could be discerned in the turnover rate of microinjected DNA by these detection techniques, suggesting that fluorescein-labeled plasmid is indeed representative of intact plasmid DNA when monitoring the degradation rate (D.L. and G.L.L., unpublished data). The results reported here demonstrate that the rates of degradation of internalized DNA in the hours immediately following the end of transfection were almost identical in the two cell types ($T_{1/2}$ = 2.5 and 3 hours in primary and established myoblasts, respectively). Also, the amount of DNA internalized by primary myoblasts was indeed higher than that internalized by C2C12 cells. The rate of DNA degradation when plasmid DNA was microinjected into the cytoplasm of single cells was the same as when plasmid DNA entered via lipofection. Altogether, these results indicate that neither increased internalization nor delayed degradation of the plasmid DNA could explain the differential transfectability of primary and established myoblasts. It is interesting to note that plasmid DNA stability in muscle cells appeared to be quite different from that observed in other cell types. The half-life of lipofected DNA was found to be 12–14 hours in HeLa and COS cells (D.L. and G.L.L., unpublished data), whereas that of the cytoplasmically microinjected DNA was only 90 minutes [10].

The intracellular processing of complexes was analyzed by monitoring the time course of transgene expression. The two cell types demonstrated similar trends for the first 8–10 hours after removal of complexes. Luciferase could be detected as soon as 1 hour after removal of the complexes, and increased rapidly during the following 5 hours. This time period is considerably shorter than the 8 hours reported in experiments with cytoplasmic microinjections [17]. This difference could stem from the different cell types they used or from the less-sensitive β-gal assay used in their experiments. Luciferase expression began to decline in the primary myoblasts after 10 hours, whereas in the established cells it kept increasing for 8 more hours. In absolute terms, luciferase expression was higher in C2C12 cells at all time points considered. These data suggest that there is a difference in the efficiency of lipofected DNA release from the vesicular compartment into the cytoplasm and that this process is less efficient in primary myoblasts. This hypothesis is also supported by the data from confocal microscopy. Another possibility was that the two cell types had significant differences at the level of transcription/translation processes. To test such a

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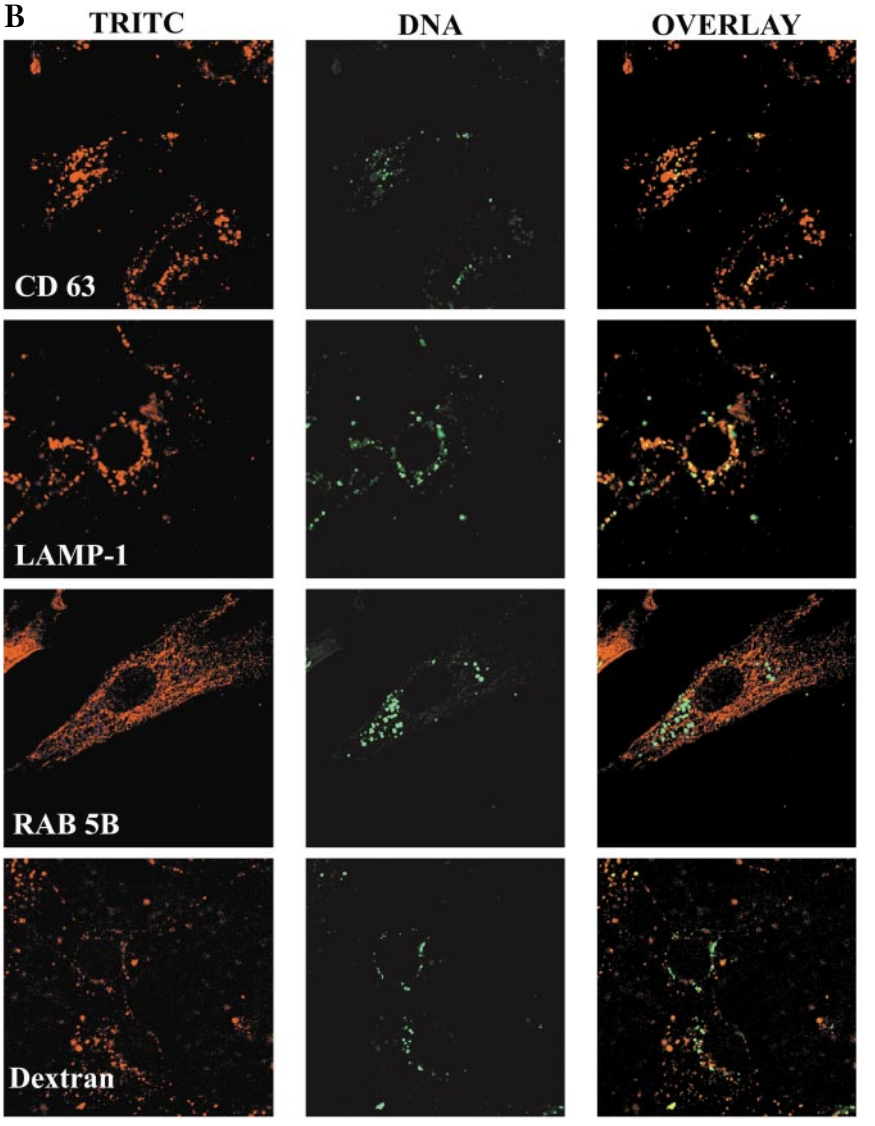
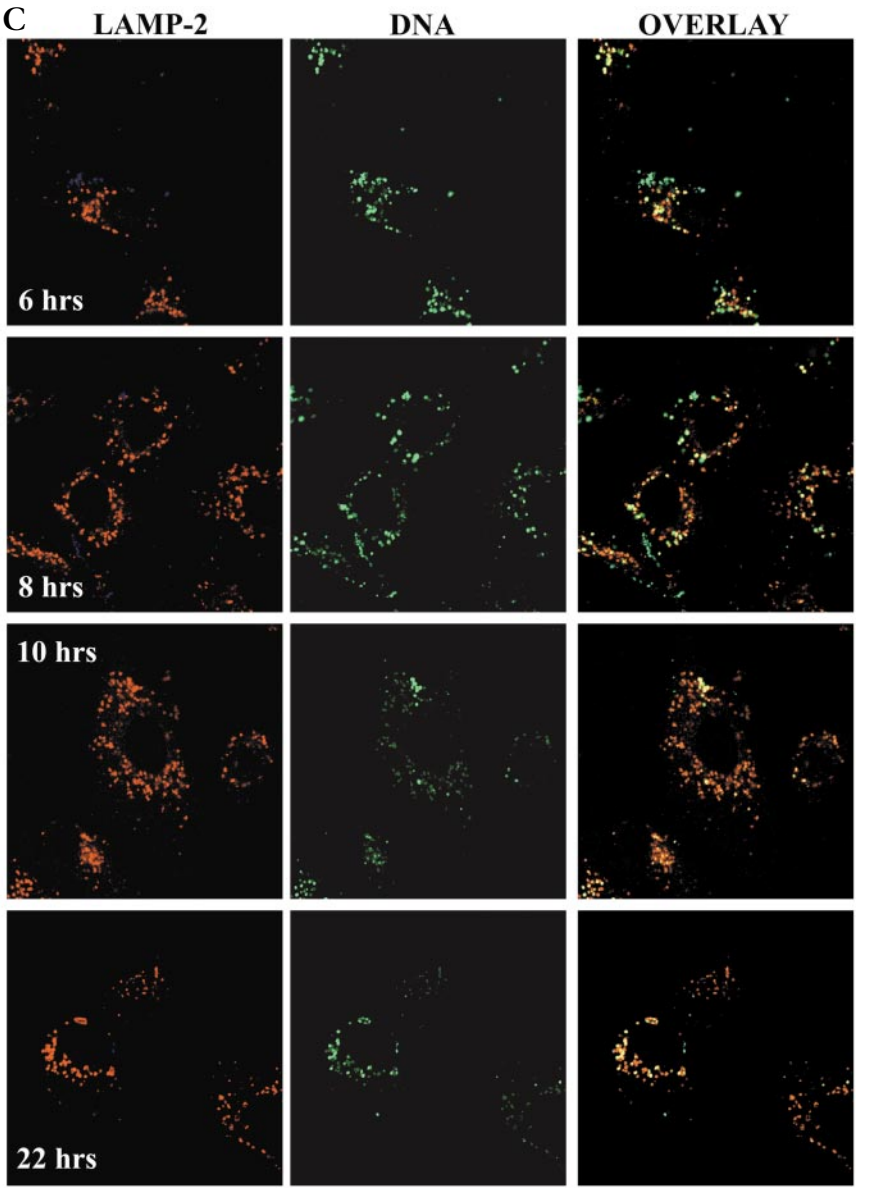


hypothesis, we carried out direct microinjections of plasmid DNA in the nuclei of the two cell types. Our findings showed that, in these experiments, C2C12 myoblasts did express higher levels of reporter gene than primary cells, but the difference was at most 50%. This finding is in good agreement with data that show how cell lines with different transfectability do yield similar expression levels following intranuclear injection of plasmid DNA [17].

Another factor that is known to influence the efficiency of lipid-mediated transfection is cell proliferation [11,18]. We used BrdU incorporation assays to determine if lipofection had a negative effect on the rate of myoblast division. These experiments did not reveal any significant difference between the rate of mitosis in transfected and nontransfected myoblasts (both primary and established, Table 1). As expected, the rate of C2C12 cell proliferation was higher than that of primary cells, but the barely twofold difference could not likely account for the drastically lower transfectability of primary myoblasts.

Finally, we have directly analyzed the intracellular trafficking of lipopolyplexes in primary and established myoblasts. To the best of our knowledge, this is the first report that used confocal microscopy to establish the localization of FITC-labeled DNA/lipid complexes relative to endosomal- and lysosomal-specific markers in primary cells. The results confirmed that lipopolyplexes enter myoblasts via endocytosis, as indicated by their colocalization with the endosomal marker Rab5B, and at later time points are delivered into the lysosomes. The timing of lysosomal colocalization was in good agreement with the findings of the expression time course. In fact, 10 hours after the end of transfection, most of DNA appeared to be inside the lysosomes in primary cells, whereas in C2C12 most of labeled plasmid was still outside the lysosomal compartment at 14 hours. On the other hand, the colocalization experiments brought up a different question, that is, the nature of the compartment in which plasmid DNA is localized during the time in which translocation to the nucleus takes place. Endosome half-life is approximately 30 minutes [reviewed in 19], but it took several hours before most transfected DNA could be found inside the lysosomes. Therefore, during this period of time the DNA could be associated with endolysosomal transport vesicles lacking the characteristic lysosomal membrane proteins or, in part, free in the cytosol. This latter possibility would be consistent with the observation that the distribution pattern of microinjected DNA in the cytoplasm is similar to that seen after lipofection. On the other hand, the measured DNA half-life in the cytoplasm was about 4 hours, whereas measurements carried out in transfected primary myoblasts between 6 and 14 hours after transfection showed a half-life of just 2.5 hours. This observation would then indicate that during this time only a fraction of the plasmid DNA could be free in the cytosol.

The data presented here indicate that the differences between the cell proliferation rate and the efficiency of the transcription/translation machinery contribute only marginally to the higher transfectability of established versus primary myoblasts. On the other hand, the good



correlation of the luciferase expression time course with the differential trafficking of the complexes between the two cell types suggests that the latter process is the main limiting factor for primary myoblast transfectability.

MATERIALS AND METHODS

Cell cultures. Primary human myoblasts, obtained from fetal skeletal muscle as described [20], were grown in F12 medium containing 20% fetal bovine serum (FBS) plus epidermal growth factor (EGF; 10 ng/ml), fibroblast growth factor (FGF; 1 ng/ml), insulin (10 μg/ml), and gentamicin (10 μg/ml). C2C12 murine myoblasts were grown in Dulbecco's minimal essential medium (DMEM) containing 10% FBS and gentamicin (10 μg/ml). Cultures were maintained at 37°C and 5% CO_2. Transfections were performed in 24-well plates (each well 2 cm^2). For the microinjection and colocalization experiments, cells were seeded on laminin-coated coverslips in 24-well plates. Culture medium, growth factors, serum, antibiotic, and plastics were from Life Technologies Inc. Insulin was from Sigma-Aldrich.

DNA. Transfections and microinjections were performed with pL018 plasmid DNA, which carries a modified luciferase gene (derived from the pGL3 plasmid by Promega) under the control of a CMV intermediate-early promoter and SV40 enhancer. DNA was prepared by standard double CsCl purification, followed by ethanol precipitation and dialysis. For the measurements of DNA-associated fluorescence, supercoiled plasmid DNA was FITC-labeled using the LabelIT kit (Panvera, WI) as recommended by the manufacturer.

Liposomes and preparation of lipopolyplexes. DODAC liposomes were prepared with the monocationic lipid dioleyldimethilammonium chloride (DODAC, designed and synthesized at INEX Pharmaceuticals) plus 1,2,-sn-dioleylphosphatidyl ethanolamine (DOPE) and 1-OH-(2′-(w-methoxy-polyethylen-glicol) succinoyl-2-*N*-octylsphyngosine) (PEG ceramide C8) in a 45:50:5 molar ratio, respectively. The PEG chain had a molecular weight of 3000 Da. The appropriate amounts of lipids were dissolved in chloroform, dried under nitrogen stream, and lyophilized overnight. The lipid film was then re-suspended in distilled water and extruded through two stacked 100-nm filters to obtain DOD-PEG Large Unilamellar Vesicles (LUV) with an average diameter of 110 ± 30 nm. In the final vesicles, PEG chains were inserted in the external side of the bilayer through their lipidic anchor. The presence of PEG prevented the aggregation and precipitation of lipopolyplexes, allowing the consistent preparation of complexes with a final diameter of approximately 220 nm.

Lipopolyplexes were prepared essentially as described [9]. Briefly, plasmid DNA was precondensed with poly-L-lysine hydrobromide (approximate MW 17,000; Sigma) at a lysine/nucleotide ratio of 0.3 on ice for 15 minutes. After adding mannitol to a final concentration of 5% (w/v), the DNA/poly-lysine solution was added dropwise to the desired amount of DOD-PEG liposomes, dissolved in 5% mannitol on ice. The total lipid/DNA ratio was 7.5 nmoles/μg. Such ratio translated in a calculated charge ratio (+/–) of approximately 1.3, or 1.8 if considering also the poly-L-lysine. Such a ratio was chosen from lipid titration experiments in which primary myoblasts were transfected with a fixed amount of plasmid DNA/poly-L-lysine complexed with liposomes at a lipid/DNA ratio ranging from 5 to 20 nmoles of lipid/μg of DNA. Lipopolyplexes were incubated on ice for 15 minutes and then diluted in FBS (200 μl per well) before being layered onto the cells.

Transfections and luciferase assays. Each 2-cm^2 well was transfected with 20 μl of lipopolyplexes containing 4.5 μg of DNA diluted in 200 μl of FBS. Complexes were left in contact with the cells for 2 hours (or for 30 minutes for the "short-time" colocalization experiments) and were then removed by rinsing the wells twice with the appropriate culture medium. Where indicated, cells were rinsed thoroughly in PBS, trypsinized, and re-plated to remove all the noninternalized complexes from the cell surface.

For luciferase analysis, transfected cells were rinsed twice in phosphate buffered saline (PBS) and then lysed with 100 μl of cell lysis buffer (Promega). Cell lysate (5 μl) was mixed with 100 μl luciferase assay substrate (Promega) and the light emission (integrated over 10 seconds) was

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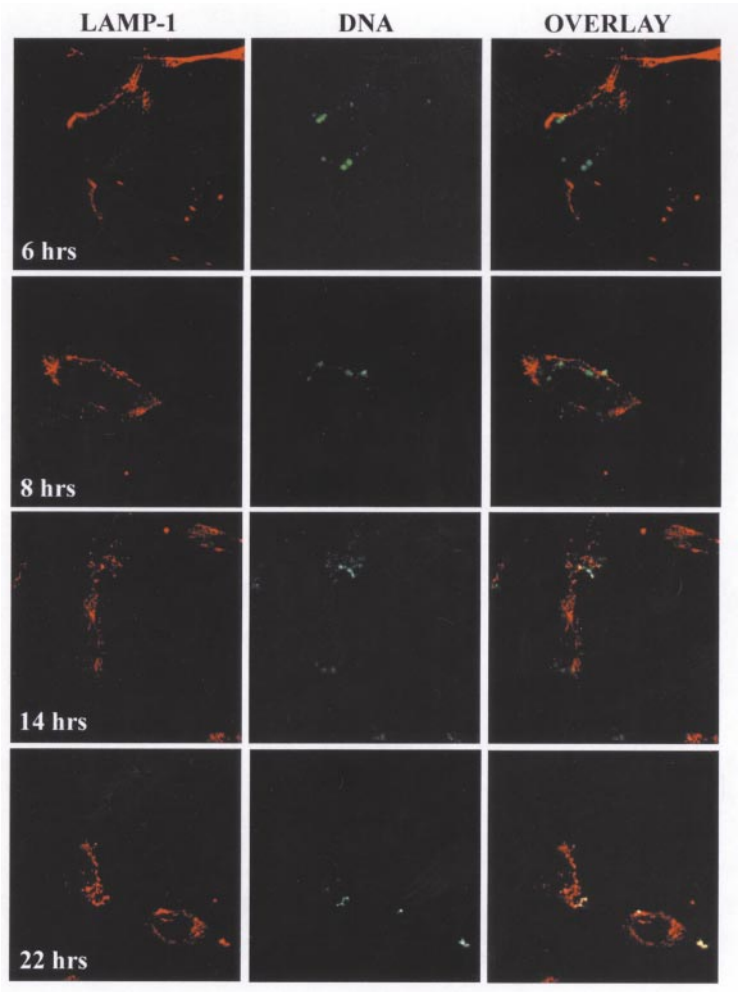

FIG. 5. Intracellular trafficking of the complexes in C2C12 myoblasts transfected with lipopolyplexes containing FITC-labeled plasmid. After the end of transfections cells were re-plated and then fixed at the indicated times. The lysosomal compartment was immunostained with and anti LAMP-1 antibody. Original magnification, ×100.

quantitated with a luminometer (TD 20/20, Turner Scientific, USA). Light emission was normalized to the protein concentration of each sample, determined by the Bradford assay. The relationship between light units and luciferase concentration was determined by establishing a standard curve for the activity of purified firefly luciferase (Sigma).

Statistical analysis (*t*-test for independent samples after Levene homogeneity of variance test) was performed by using SPSS statistical software package.

BrdU incorporation assays. Cells were grown on gelatin-coated coverslips and transfected as described above. At the desired time points, BrdU was added to the culture medium to a final concentration of 100 μM and after 1 hour cells were rinsed and fixed in paraformaldehyde (2% w/v in PBS) at room temperature for 15 minutes. The coverslips were then treated with 50 mM NH_4Cl at 37°C for 20 minutes to quench autofluorescence, and then with Triton X-100 (0.5% in PBS) at room temperature for 15 minutes for membrane permeabilization. Fragmentation of DNA was achieved by exposing the cells to 1 M HCl at 37°C for 20 minutes. Cells were then stained with a monoclonal antibody against BrdU (Roche Diagnostic), at the dilution of 1:5, with standard techniques. Nuclei were counter-stained with DAPI. The same procedure was applied to the time-matched, non-transfected controls. The percentage of BrdU incorporation was calculated by counting the ratio of BrdU positive versus total nuclei, randomly choosing four fields per well (three wells per condition were used).

Cytoplasmic microinjection. Microinjections were performed essentially as described [10], with a semi-automatic injection system (Eppendorf Transjector 5246) attached to a micromanipulator (Eppendorf 5171). Borosilicate thin-walled glass pipettes with filament (I.D. 0.78 mm, O.D. 1 mm) were made with a pipette puller (P97 Sutter Instrument). Plasmid DNA (0.2 mg/ml) and tetramethilrhodamine (TRITC)-dextran (0.5 mg/ml, MW 70 kDa, lysine-fixable, Molecular Probes) were microinjected in KG-medium (110 mM K^+-glutamate, 30 mM NaCl, 2 mM $MgCl_2$, pH 7.2) at 150 kPa over 0.2 seconds. Injections, performed at room temperature in bicarbonate-free minimal essential medium (α-modification, α-MEM) supplemented with 10 mM Na-Hepes, pH 7.3, delivered approximately 7500 copies of plasmid DNA in an injection volume of approximately 280 ± 25 fl. We routinely microinjected 100–150 cells on each laminin-coated coverslip over a 15- to 30-minute period. Immunofluorescence photographs were taken with a Contax camera on Kodak EpH 1600 film using either a fluorescein or a rhodamine filter set (Zeiss). Exposure time, scanning, and image processing parameters were kept identical within individual experiments.

Intranuclear microinjections. The pL018 plasmid was injected in the nuclei at the concentration of 0.01 mg/ml (in KG medium) at 100 kPa over 0.1 second. Borosilicate thin-walled glass pipettes with filament (I.D. 0.78 mm, O.D. 1 mm) were made with a pipette puller (P97 Sutter Instrument). The shape of the pipette and the injection parameters were modified to optimize cell viability after injection. Survival rate after nuclear injection was determined by injecting the cells with FITC-dextran (70 kDa, Molecular Probes) and counting them at 2 and 4 hours postinjection. For the evaluation of expression efficiency, exactly 100 successful injections were performed on each coverslip. After 2 or 4 hours of incubation cells were rinsed twice in PBS and then lysed with 100 μl of cell lysis buffer (Promega). Cell lysate (20 μl) was mixed with 100 μl luciferase assay substrate (Promega) and the light emission measured as described above. Luciferase expression data were then normalized considering the different survival rates in the two cell lines, as evaluated with the FITC-dextran injections. Such rates were found to be approximately 80% (78 ± 2.9) in C2C12 and 60% (62 ± 5.5) in primary myoblasts. The same injection conditions were also applied to evaluate the volume of injection by delivering purified luciferase protein (Sigma) at the concentration of 2.5 mg/ml and performing a luciferase assay on the cell lysate immediately after injection. By comparing the cell readings with the light emission obtained from a known amount of luciferase solution, the injection volume was calculated to be 359 ± 38 fl in C2C12 and 233 ± 11 fl in primary myoblasts.

Quantitative single-cell fluorescence video-image analysis. Both microinjected and transfected cells were observed with a Zeiss Axiovert 100 inverted fluorescence microscope equipped with a 63xNA 1.4 Planachromat objective using a quartz halogen illumination system. For quantitative evaluation of the fluorescence associated with single cells, images were captured with a cooled-CCD camera (Princeton Instruments, USA). Image acquisition was controlled by the Metafluor software (Universal Imaging Corp.), operating on a PC. The integrated fluorescence intensity of fluorescence signals, derived from approximately 100 injected cells per condition, were calculated from background-subtracted images.

Immunocytochemistry. Immunocytochemistry was performed by standard techniques as described [10]. In primary myoblasts, lysosomes were stained with mouse monoclonal antibodies raised against lysosome-associated membrane protein LAMP-1, LAMP-2, and CD 63 (Hybridoma Facility, John Hopkins University), early endosomes were identified with a rabbit polyclonal antibody against the endosomal marker Rab5B (Santa

Cruz). Late endosomes were labeled with TRITC-dextran (7 mg/ml, MW 70 kDa, lysine-fixable, Molecular Probes). After the internalization of TRITC-dextran for 20 minutes at 37°C, cells were chased in dextran-free medium for 15 minutes at 37°C, washed thoroughly with ice-cold dextran-containing medium (300 mg/ml) and fixed with 3% paraformaldehyde. For standard transfections, the labeling step was performed during the last 20 minutes of exposure to the lipopolyplexes. For the "short time co-localization" experiments, cells were instead exposed simultaneously to the complexes and dextran for 20 minutes, chased in dextran-free medium for 15 minutes, washed thoroughly with unlabeled dextran (300 mg/ml in full medium) and fixed. In C2C12 cells lysosomes were identified with a goat polyclonal antibody against the lysosome-associated membrane protein LAMP-1 (Santa Cruz Biotechnology Inc.). Colocalization of FITC-labeled DNA and subcellular organelles were performed with a Zeiss 510 laser fluorescence confocal microscope, equipped with a ×100 objective.

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