Transfection by Cationic Liposomes Using Simultaneous Single Cell Measurements of Plasmid Delivery and Transgene Expression*

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Cationic liposomes are potentially important gene transfer vehicles, although their application has been limited by relatively low efficiency of transgene expression. Single cell quantitative methods, such as those used in this study, should permit a more detailed understanding of the relationships between delivered plasmid and transgene expression. Intracellular plasmid delivery and transgene expression were measured simultaneously using photoconjugated ethidium monoazide as an intracellular plasmid delivery marker and green fluorescent protein (GFP(S65T)) as a transgene expression marker. Quantitative flow cytometry was used to estimate plasmid copy number and GFP(S65T) molecules in single cells. The plasmid was delivered to HeLa cells with a cationic liposome vehicle containing 1,2-dioleoyl-oxy-3-trimethylammonium-propane and dioleoylphosphatidylethanolamine (1:1 mol/mol). Treatment was carried out continuously for 24 h. Flow cytometry measurements on 20,000 cells were performed during treatment and for 48 h post-treatment. On a single cell basis, transgene expression efficiency and average GFP(S65T) expression level increased with intracellular plasmid copy number. After 3-h exposure to the liposomal vector, more than 95% of the cells were positive for plasmid entry, but none had detectable transgene expression. Maximum transgene expression was achieved at 24 h and remained unchanged at the 72-h measurement. At 24 h, the average positive cell contained 1.6 × 10^5 plasmid copies and 2.3 × 10^6 GFP(S65T) molecules. Importantly, the measurement strategies revealed that transgene expression varied widely within the entire cell population. Although only 30% of all cells expressed transgene, the subpopulation of cells that rapidly incorporated the vector demonstrated 100% efficiency in transgene expression. This study identifies parameters that modulate highly efficient transgene expression from plasmid delivery by cationic liposomes.

Gene delivery has been achieved by a variety of techniques, but much current effort is focused on either a viral or a cationic liposomal vector (1–3). Liposomes possess many physical characteristics that make them attractive candidates as gene delivery vectors (4). The safety of cationic liposomal vectors, for example, has been demonstrated in both single use and repeated dose protocols (5, 6). In addition to clear prospects for in vivo therapeutic use, cationic liposomal vectors are also an appropriate ex vivo transfection method. A major limitation of cationic liposomal vectors is low transgene expression efficiency.

Significant efforts to improve liposomal vector performance are underway in a number of academic and industrial laboratories. The search for improved transgene expression is primarily focused on the development of new liposome formulations (7–10) and the synthesis of new cationic lipids (11–15). The fundamental mechanisms and rates of liposomal gene delivery and subsequent intracellular processing of the delivered transgene are, however, not well understood. Optimization of liposomal vector performance is currently performed empirically using transgene protein in the final cell lysate as the primary indicator of efficiency. Unfortunately, interpretation of these studies rarely considers the biophysical interactions between liposomal vector and cells. Early reports (16–18) examined the entry of cationic liposomal vectors only qualitatively. A quantitative and systematic analysis of the delivered plasmid characteristics and transgene expression on a cellular basis has not been described.

The objective of this study is to quantify relationships between delivered plasmid and transgene expression in a cell population. Single cell fluorescence techniques were developed to examine plasmid delivery and intracellular processing kinetics as a function of DNA dosage across a population of clonal cells. Two independent fluorescent probes were used in this technique, ethidium monoazide (EMA)-conjugated plasmid and the transgene product, a mutated Aequorea victoria green fluorescent protein (GFP(S65T)) (19). The plasmid contains the reporter sequence for GFP(S65T), a protein which is fluorescent after synthesis and folding (20). GFP(S65T) fluorescence was used to obtain single cell measurement of transgene expression. EMA, a fluorescent intercalating DNA probe, spectrally similar to ethidium bromide, was used to label plasmid by covalent bonding after photoactivation. EMA fluorescence was used to estimate the plasmid copy number associated with each cell. Flow cytometry provided single cell measurements of plasmid copy number and transgene expression by simultaneous application of two independent fluorescent markers.

EXPERIMENTAL PROCEDURES

Plasmid Amplification and Labeling—The pGreenLantern-1 plasmid (Life Technologies, Inc.; pGL1) is 5.0-kb long with a cytomegalovirus promoter. This plasmid contains a humanized sequence (21) coding for GFP(S65T), a protein which is fluorescent after synthesis and folding (20). GFP(S65T) fluorescence was used to obtain single cell measurement of transgene expression. EMA, a fluorescent intercalating DNA probe, spectrally similar to ethidium bromide, was used to label plasmid by covalent bonding after photoactivation. EMA fluorescence was used to estimate the plasmid copy number associated with each cell. Flow cytometry provided single cell measurements of plasmid copy number and transgene expression by simultaneous application of two independent fluorescent markers.

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1 The abbreviations used are: EMA, ethidium monoazide; GFP, green fluorescent protein; GST, glutathione S-transferase; FL1, FL2, and FL3, fluorescence emissions centered at 530, 580, and 610 nm, respectively; ANOVA, analysis of variance; CMF, calcium- and magnesium-free; PBS, phosphate-buffered saline.

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ethidium bromide. Unlike ethidium bromide, EMA forms covalent bonds with DNA bases after photoactivation, providing a stable fluorescent reporter of the bound DNA/RNA. The labeling of plasmid with EMA by photoactivation was carried out as described previously (23). The EMA-conjugated, fluorescent pGL1 is denoted as EMA-pGL1 in this study.

Liposome Preparation—A total mass of 20 mg of 1,2-dioleoyloxy-3-trimethylammonium-propane (50 mol %) and dioleoylphosphatidylethanolamine (50 mol %; Avanti Polar Lipids) was dissolved in a 100-mM chloroform flask containing 5 ml of chloroform. The cationic liposomes containing 1,2-dioleoyloxy-3-trimethylammonium-propane and dioleoylphosphatidylethanolamine were prepared by the film hydration method, followed by extrusion to yield unilamellar liposomes of 0.1 μm as described previously (23).

Transfection—The transfection reagent contained a constant total dose of 2000 ng of DNA per 35-mm Petri dish of cultured HeLa cells. The total DNA dose (total pGL1) was composed of EMA-pGL1 and unlabeled pGL1. EMA-pGL1, covalently labeled with EMA, cannot participate in transgene expression because the EMA-DNA conjugate bonds prevent transcription. The EMA-pGL1 plasmid offers detection of plasmid entry across the cell membrane, and the unlabeled pGL1 plasmid provides measurement of transgene expression. Both the transgene expression and plasmid copy number can be determined simultaneously in individual cells by this technique. Combinations of EMA-pGL1 and pGL1 plasmids were used to examine the copy number. Independent series of experiments were carried out with unlabeled pGL1 plasmid of 1000, 500, 100, or 10 ng with sufficient EMA-pGL1 plasmid to provide a total plasmid dose of 2000 ng in all experiments. For each 35-mm Petri dish of HeLa cells, EMA-pGL1 and pGL1 plasmid were well mixed in 100 μl of Opti-MEM medium (Life Technologies, Inc.) before the addition of 12 μg of DNA per 35-mm Petri dish at 20,000 cells/cm2.

Cell line; ATCC-CCL-2) were maintained at 37 °C, 100% humidity in T-175 flasks with regular medium changes over 3–4 days supplemented with 10% calf serum (Life Technologies, Inc.), 1% glutamine, and was maintained until the end of the experiments. Three 35-mm Petri dishes were harvested at each desired incubation time (control, 0.5, 3, 6, 12, 24, 48, and 72 h) for analysis.

Cell Culture, Harvest, and Fixation—HeLa cells (a human epithelial cell line; ATCC-CCL-2) were maintained at 37 °C, 100% humidity in complete medium containing Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum (Life Technologies, Inc.), 1% glutamine, and 1% antibiotics of penicillin-streptomycin-amphotericin. Cells were maintained in T-175 flasks with regular medium changes over 3–4 days and were split after achieving complete confluence. Cells were harvested from the T-175 flasks 16–18 h before transfection and seeded onto 35-mm Petri dishes at 20,000 cells/cm2.

At the conclusion of a sampling interval, the cells were washed with cold phosphate-buffered saline (PBS, pH 7.2) twice. Cell harvest was done by incubation with 1 ml of 0.15% trypsin, 1 mM EDTA. After incubation, 1 ml of complete medium was added, and cells were fixed in the presence of 1% paraformaldehyde for 5 min at room temperature. The fixed cells were then washed three times with CMF-PBS containing 1% paraformaldehyde and stored at 4 °C for flow cytometric analysis.

Flow Cytometric Analysis—Cell samples were analyzed by a FACScan (Becton-Dickinson) flow cytometer that has an argon laser exciting at a wavelength of 488 nm. For each sample, 20,000 events were collected by list-mode data, which consisted of side scatter, forward scatter, and fluorescence emissions centered at 530 nm (FL1), 580 nm (FL2), and 610 nm (FL3). No electronic compensation was performed. Software-based compensation, however, was used as described below. Winlist (Verity Software House) software was employed to analyze the list-mode data.

EMA and GFP Calibration Reagent—Flow cytometric measurement of EMA and GFP(S65T) was calibrated using polystyrene beads with a known degree of adsorbed fluorochrome. Polystyrene beads (Polysciences, Inc.) were washed with CMF-PBS three times. A final concentration of 4 × 106/ml beads was incubated with 0.18 mg/ml EMA-pGL1 at room temperature for 18 h with gentle mixing. The coated beads were washed twice with CMF-PBS and resuspended in PBS containing 0.1% Tween 20, 0.1% bovine serum albumin, and 0.1% sodium azide at 4 °C.

The mutated green fluorescent protein was prepared in our laboratory for calibration through a previously described technique (24). Mutated GFP(S65T) was fused with glutathione S-transferase (GST) and amplified in E. coli. The fusion protein (GST-GFP(S65T)) was purified through a GST affinity column. The humanized, mutated GFP plasmid used in this study codes for a GFP with the same structure as the mutated GFP(S65T). The humanized GST–GFP(S65T) has the same fluorescence intensity as the GFP(S65T) when excited at the same wavelength (24). GST-GFP(S65T) adsorption onto polystyrene beads was carried out as described for EMA-pGL1 coating but with a final concentration of 23.5 μg/ml GST-GFP(S65T) per 4 × 106/ml beads.

The amount of the fluorophore that remained in the supernatant after coating was determined spectrophuorometrically for both EMA-pGL1 and GST-GFP(S65T) coated bead preparations. The amount of fluorophore removed from the fluid phase during coating was presumed to be adsorbed onto all the beads uniformly. The coated bead concentration was measured with an electronic particle counter. Molecules of equivalent soluble fluorochrome per bead was calculated by dividing the total adsorbed molecules of fluorophore by the total number of beads.

Autofluorescence, EMA, and GFP Signal Compensation—Control HeLa cells excited at 488 nm were autofluorescent in FL1, FL2, and FL3. EMA fluorescence was significant in FL2 and FL3, but not detectable in FL1. GFP(S65T) fluorescence occurs primarily in FL1 but is also detectable in FL2 and FL3. Compensation for the spectral overlap among cellular autofluorescence, GFP(S65T) and EMA fluorescence is based on fluorescence intensity linear superposition in each spectral region (25).简而言之，本系统中无EMA可检测到。EMA fluorescence intensity in FL1, FL2 and FL3 measured for each of the three calibration samples. In this system, no EMA is detectable in FL1, forcing E1 = 0. Calibration of cellular autofluorescence in FL2 and FL3 is relative to the measurement in FL1, forcing A1 = 1. GFP(S65T), EMA, and autofluorescence are estimated in experimental samples from the measured FLi by premultiplying both sides of the compensation equation with the inverse of the compensation coefficient matrix.

Plasmid and GFP Quantification—Fluorescence intensities of the GFP(S65T) and EMA fluorophores were obtained from the intensities of coated beads divided by their respective molecules of equivalent soluble fluorochrome in each experiment. The applied gene dosage consisted of a controlled combination of EMA-pGL1 and unlabeled pGL1 plasmid. The EMA-pGL1 and pGL1 plasmid associate with cells at similar rates after conjugation with cationic liposomes (16). The copy number of EMA-pGL1 plasmid associated with each cell was estimated from dividing the intensity of the compensated EMA fluorescence by the intensity of each EMA molecule. The total copy number of plasmid and the copy number of pGL1 plasmid associated with each cell was estimated from the known fraction of EMA-pGL1 plasmid. Similarly, the number of GFP(S65T) molecules within each cell was obtained by dividing the intensity of the compensated GFP(S65T) fluorescence by the intensity of each GFP(S65T) molecule.

Determination of Positive Events—Determination of GFP(S65T) positive events was performed by a standard gating technique. Briefly, the control sample was displayed as a dot plot of EMA versus GFP(S65T) in terms of compensated signals. The gate was drawn along a line of maximum detected GFP(S65T) intensity for control samples. To improve reproducibility and sensitivity, the drawn gate included 0.5% of the total events in the control sample. The gate was held unchanged through the analysis of all measurements in the same experiment. The percentage of positive events was calculated as the events within the gate divided by total number of events.

RESULTS

Plasmid Delivery—Cellular fluorescence in FL2 and FL3 increased during plasmid treatment due to cellular incorporation of the EMA fluorophore (Fig. 1). Cells exposed to the
The intracellular plasmid copy number increased during exposure to the liposomal vector and decreased after the vector removal. HeLa cells were incubated with a cationic liposomal vector for 24 h. Fluorescent EMA-pGL1 plasmid entry was detected by flow cytometry. Panel A shows increasing fluorescence intensity distribution during exposure to the liposomal vector. Panel B shows decreasing fluorescence intensity distribution after removal of the liposomal vector at 24 h, presumably due to EMA fluorescent label dilution resulting from cell division.

The liposomal vector consistently produced a log normal unimodal population of fluorescence intensity in FL3. The unimodal population shifted toward increased fluorescence intensities in cell samples that were incubated with the liposomal vector for more than 3 h (Fig. 1A). Cellular fluorescence decreased beyond 24 h, after removal of the vector from the medium (Fig. 1B). This decrease is consistent with dilution of EMA fluorescent label expected following cell division.

As shown in Fig. 2, total intracellular plasmid delivery was irrespective of EMA-pGL1 fraction while holding total administered plasmid copy number constant (ANOVA, \( p < 0.05 \)). This result is consistent with an earlier study (16) that reports that EMA labeling does not significantly alter the rate of transmembrane transport. Kinetic trends of plasmid incorporation include rapid DNA uptake immediately after the liposomal vector was applied to the cells. After 0.5 h, each cell contained \( 2 \times 10^6 \) plasmid molecules on average; the average intracellular plasmid copy number reached \( 1.5 \times 10^6 \) per cell after 24 h incubation. The rate of plasmid entry decreased 10-fold from 0.5 to 24 h. After the liposomal vector was replaced with complete medium at 24 h, the average plasmid copy number per cell decreased with an average half-life \( (t_{1/2} \approx 32 \text{ h}) \).

Transgene Expression—Detection of GFP(S65T) offers a direct measurement of transgene expression within many single cells using flow cytometry. The fluorescence of the transfected cell population is composed of the pretransfection cellular autofluorescence with the appearance of highly fluorescent subpopulation, which represents the cells synthesizing GFP(S65T) (Fig. 3) (26). A region of elevated FL1 fluorescence, presumed to represent intracellular GFP(S65T), was identified through flow cytometric gating for subsequent analysis. Cells which possessed sufficient FL1 fluorescence (i.e. within the gate) were designated as positive for GFP(S65T) synthesis. This gating was used to produce two histograms from each cell population, negative cells that had FL1 fluorescence equivalent to the cellular autofluorescence of untreated controls and GFP(S65T)-positive cells that had significantly increased FL1 fluorescence. These populations are identified in Fig. 3 with pGL1 dose as a parameter. The fluorescence intensity of the GFP(S65T)-positive cell population had a maximum of \( 10^4 \) units compared with 26 units in the untreated control cell population and was a function of applied pGL1 plasmid. Cells exposed to higher concentration of pGL1 plasmid had greater average intracellular GFP(S65T) levels. Increasing the pGL1 plasmid level also produced a distribution with a longer tail, suggesting that greater pGL1 plasmid entry resulted in increased heterogeneity in transgene expression.

The fraction of all cells that were positive for GFP(S65T) expression increases sharply after 6 h of liposomal vector incubation and achieves a maximum of 30% after 48 h (Fig. 4). After 12 h, the GFP(S65T)-positive percentages were a significant function of applied pGL1 plasmid at each sampling time (ANOVA, \( p < 0.05 \)). A positive population was detected in the 1000 ng/2000 ng and 500 ng/2000 ng treatments after 12 h. The positive percentages reached a plateau under these conditions after 24 h. Treatment with 100 ng/2000 ng delayed the detection of a significant positive population until 24 h. For the 10 ng/2000 ng treatments, the GFP(S65T)-positive percentages never exceeded 3% and were indistinguishable from the control samples, suggesting that undetectable transgene expression occurred at this level of pGL1 plasmid exposure.

The maximum fraction of GFP(S65T)-positive cells responded to pGL1 plasmid level in a dose-dependent fashion (Fig. 5). As the initially applied pGL1 plasmid increases from...
100 to 1000 ng in the treatments, the maximum positive percentage increases from 12 to 30%. The fraction of cells positive for GFP(S65T) is a nonlinear function of the applied pGL1 plasmid dosage.

The average number of GFP(S65T) molecules contained in each positive cell increased during the 24-h liposomal vector incubation (Fig. 6). Cell division in the absence of plasmid, which occurred after the removal of liposomal vector at 24 h, reduces the GFP(S65T) level per positive cell measured at 48 and 72 h. A correction for cell division, shown as the dashed line for the 1000 ng/2000 ng treatment, suggests that GFP synthesis continues for at least 72 h. Similar continuation of transgene expression after liposomal vector removal was also estimated for other treatments (not shown). After fluorescence intensity compensation, the GFP(S65T) detection limit was about $2 \times 10^5$ molecules/cell, equivalent to 80 nM for a cell size of 20 \( \mu \)m.

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**FIG. 3.** GFP(S65T) expression distribution is a function of pGL1 plasmid dosage. HeLa cells were harvested and fixed after 24-h incubation with the cationic liposomal vector. Dotted lines in each graph show the cellular autofluorescence background that is a logarithmically normal distribution. Dark solid lines represent the cell population expressing GFP(S65T). The range and average GFP(S65T) expression decrease with decreased pGL1 plasmid dosage. Treatments consist of 1000 ng/2000 ng (panel A), 500 ng/2000 ng (panel B), and 100 ng/2000 ng (panel C).

**FIG. 4.** The fraction of cells which express GFP(S65T) increases with pGL1 plasmid dosage and time in culture. GFP(S65T) expression rises sharply 6 h after DNA-liposome exposure. 30% of the cells treated with the highest pGL1 plasmid dosage (1000 ng/2000 ng) express significant levels of GFP(S65T) after 72 h. GFP(S65T) expression in the lowest pGL1 plasmid dosage (10 ng/2000 ng) was less than 3% at all times. The percentage of cells which are GFP(S65T) positive is significantly different among plasmid dosages after 12 h (ANOVA; \( p < 0.05 \)). Treatments consist of 1000 ng/2000 ng (●), 500 ng/2000 ng (■), 100 ng/2000 ng (▲), and 10 ng/2000 ng (▲) (pGL1/total-pGL1; \( n = 5 \), mean ± S.E.).

**FIG. 5.** GFP(S65T) expression is modulated by the pGL1 plasmid dosage. The subpopulation of treated cells which express GFP(S65T) increases logarithmically as a function of pGL1 dose. The GFP(S65T) response is a significant function of both pGL1 plasmid dose (except at 10 ng) and time (ANOVA; \( p < 0.05 \)) (except for the 48–72 h comparison). Responses at 48 and 72 h are not different, and only the 48-h results are shown: 12 h (●), 24 h (■), and 48 h (▲). (\( n = 5 \), mean ± S.E.).

number versus intracellular plasmid copy number (Fig. 8), each of the 128 EMA channels (one example is shown as a rectangle in Fig. 8) was used as a subpopulation in which the positive cell percentage was calculated. Each channel represents a different EMA fluorescence intensity, which is associated, by calculation, with intracellular plasmid copy number. The following results were obtained by dividing each result of five experiments into 128 channels and averaging the response across experiments in each channel.
The fraction of cells that express transgene increased with intracellular pGL1 plasmid copy number in a dose-dependent fashion (Fig. 9). This behavior is conserved at all incubation times. The highest achievable GFP(S65T)-positive percentage increased with liposomal vector exposure time. Each curve represents the GFP(S65T)-positive cellular distribution of its corresponding population average for 1000 ng/2000 ng treatment (Fig. 5). After 12 h of incubation, less than 50% of the cells with the highest intracellular plasmid copy number were GFP(S65T)-positive. After 24 h, however, subpopulations composed of 100% GFP(S65T)-positive cells were found at the highest intracellular plasmid levels of greater than 10^6 copies of plasmid per cell. The absolute number of such cells, however, is low as shown in the intracellular plasmid copy number histogram (Fig. 9). The average fraction of GFP(S65T)-positive cells for the entire population is identified in each curve of Fig. 9 with a hollow symbol. These complete population averages reflect the skewed distribution of cell number as a function of intracellular plasmid content. The same effect of delivered pGL1 plasmid on GFP(S65T)-positive percentage was found in other treatments, but not shown.

**DISCUSSION**

A common indicator for evaluating transfection efficiency is the amount of transgene product in the cell lysate, typically reported as units/mg of total cell protein. In this study, simultaneous fluorescence measurements of EMA-pGL1 plasmid and GFP(S65T) expression by flow cytometry were used to establish quantitative relationships in individual mammalian cells among the applied gene dosage, the delivered plasmid copy number, and the transgene expression.

Greater than 95% of the treated cells contain significant plasmid after a 3-h exposure to liposomal vector (Figs. 1 and 7). Transgene expression, however, is delayed relative to plasmid delivery and achieves a maximum of 30% after 48 h (Figs. 4 and 7). These results are consistent with previous evidence (16, 23) indicating that the transmembrane transport of cationic liposomal vectors is not a limiting step in transgene expression. Nevertheless, intracellular plasmid copy number is strongly correlated with transgene expression percentages and levels (Figs. 5, 6, and 9). Specifically, subpopulations with nearly complete transgene expression were found within the whole...
liposome-mediated gene delivery. The percent of GFP(S65T)-positive population for cells containing the greatest intracellular plasmid copy number (\(>10^6\) plasmid copies/cell) is relatively small as shown by the intracellular plasmid population distribution (solid line). The same data, but averaged for the entire population, reveal that only 30% of the total population is GFP(S65T)-positive (Fig. 4). Open symbols represent the total population averages shown in Fig. 4. Only plasmid levels greater than 20 total events summed over five experiments (1000 ng/2000 ng treatment) are shown: 12 h (●), 24 h ( ■), and 48 h (▲).

The transmembrane efficacy, defined as the ratio of total intracellular plasmid copy number in an entire population may be obtained by integration of the plasmid distribution histogram multiplied by the actual cell number as a function of time. Since the total intracellular plasmid copy number is 7.4 \( \times 10^{10}\) (72 h) copies. These results are shown in Fig. 2 and are scaled for actual cell number as a function of time. Since the total intracellular plasmid copy number is 7.4 \( \times 10^{10}\) and the applied plasmid copy number in a 1000 ng/2000 ng treatment is 3.7 \( \times 10^{11}\), the transmembrane efficacy of this cationic liposome vector is 20% at 24 h incubation.

The flow cytometry strategy employed here has significant advantages over existing methods. For example, a common strategy for optimizing liposomal vectors is to search for the highest transgene expression level in the cell lysate. At least two potential difficulties are poorly addressed by this simple strategy. The positive percentage of cells expressing transgene and the level of transgene expression are optimized at high plasmid doses (Figs. 4 and 6). Transgene expression heterogeneity, however, also increases with plasmid dose (Fig. 3). Metabolic engineering has shown that cellular metabolism can be altered by inducing abnormally high enzyme activity. Overexpression of heterologous genes in E. coli, for example, induces misincorporation of amino acids during translation (27). The cellular and molecular effects of transgene overexpression in gene therapy remain in question. The effect of expression heterogeneity on overall gene therapy effectiveness has been overlooked to date. The functional effectiveness of transfection formulations might be improved by seeking a more uniform expression population with moderate expression level yet a high percentage of cellular participation. Cell damage resulting from cationic liposomal vector treatment has been observed in...
this work at the elevated concentration of 2000 ng/4000 ng.
Simply increasing the plasmid dosage in an effort to improve transgene expression is limited by cell damage at these extreme conditions. Although the reduction in transgene expression induced by cell damage can be detected by bulk measurement techniques, more explicit recognition of this problem may lead to minimization of cell damage as an additional dimension for functional optimization of gene delivery systems.

Transgene expression level measured in the cell lysate, as reported by others, is the product of average transgene expression and the transgene-positive percentage. Transgene expression in single cells cannot be extracted from such a bulk measurement. The transgene expression histograms determined in this work, however, can be integrated to obtain the total GFP(S65T)-positive cells per 20,000 cells, a measurement equivalent to the single value obtained from a cell lysate (a combination of Figs. 4 and 6 shown as Table I). Populations treated with 500 ng/2000 ng and 100 ng/2000 ng have a similar percent of GFP(S65T)-positive cells. The average number of GFP(S65T) molecules within each positive cell, however, differs by a factor of 1.5. The 1000 ng/2000 ng treatment produced 1.8 times greater GFP(S65T)-positive percentage than the 100 ng/2000 ng treatment but with 3.3 times the average GFP(S65T) molecules per positive cell. Since transgene expression level is a stronger function of intracellular plasmid copy number than the GFP(S65T)-positive percentage, cell lysate techniques provide a skewed focus on transgene expression level at the expense of the cell fraction that is expressing transgene. The differences among cationic liposome formulations and gene dosages might be masked by single, averaged measurements of transgene expression in the cell lysate, which cannot independently evaluate gene transfer efficacy in terms of the expression percentage and the heterogeneity of cellular expression level.

Plasmid delivery heterogeneity, measurable by flow cytometry, plays a significant role in transgene expression heterogeneity and inefficiency. Techniques that report a single measure of gene delivery and/or transgene expression based on a population average fail to detect the important effects of cell heterogeneity shown here. Every cell is capable of incorporating the cationic liposomal vector, but not every cell is capable of transgene expression using this delivery system. Our study suggests that complete transgene expression is achievable when cells contain high levels of intracellular plasmid. These results, however, also suggest that transgene expression is not a single deterministic function of intracellular plasmid copy number.

Modulation of transgene expression by the rate of plasmid entry is one hypothesis consistent with these observations. Competition between plasmid delivery and intracellular plasmid inactivation may play an important role in controlling transgene expression. Conditions which permit the plasmid entry flux to exceed the intracellular DNA degradation rate presumably enhance transgene expression efficacy. Increasing the applied plasmid dose results in a higher average transgene expression but wider expression level heterogeneity. Gene therapy strategies might be more functionally optimized by selecting liposomal transfection formulas designed to obtain high percentage expression with a relatively uniform transgene expression level at low cytotoxicity instead of optimizing total transgene expression in the cell lysate. Simultaneous measurements of transgene expression and delivered plasmid by flow cytometry provide a useful tool to evaluate different liposomal gene delivery methods.

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