Quantitative Analysis of Correlation between Number of Nuclear Plasmids and Gene Expression Activity after Transfection with Cationic Liposomes

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Received November 20, 2001; accepted January 4, 2002

Purpose. A quantitative understanding of the intracellular trafficking of plasmids delivered by nonviral vectors is essential for optimizing vector functions to increase their transfection efficiency. In this study, quantitative methods were developed to measure plasmids delivered to the nucleus, and the relationship between transfection activity and the number of plasmids in the nucleus were analyzed.

Methods. AH130 cells were transfected with plasmids in cationic liposomes at various doses. The nuclear fraction was isolated after NP-40 lysis, and the unincorporated plasmids were enzymatically degraded and washed away. Intranuclear plasmids were amplified by quantitative PCR, and the number of plasmids was determined. Plasmid amounts in the nucleus were also measured by Southern analysis to confirm the quantification.

Results. Both methods led to similar results in measuring the nuclear plasmids within the same order of magnitude. A remarkable saturation was found for transfection activity vs. number of plasmids in the nucleus, whereas no saturation was observed in nuclear-delivered plasmids vs. dose.

Conclusions. These results clearly demonstrate the importance of the quantitative measurement of intracellular trafficking of plasmids after transfection. The findings herein described suggest that efficient transgene expression as well as enhanced nuclear delivery is required in order to achieve the maximal transfection activity of nonviral vectors.

KEY WORDS: intracellular trafficking; cationic liposome; gene delivery; plasmid quantification; nuclear delivery.

INTRODUCTION

In the development of gene delivery systems, cationic liposomes, which are characterized by their low immunogenicity and toxicity, represent a prospective tool for use as a nonviral vector. However, their transfection efficiency, a major limitation of nonviral vectors, is much lower than that of viral vectors. Although many efforts have been made to improve the transfection efficiency (1–4), its level is not yet sufficient for use of such liposomes in actual gene therapy. Since non-modified liposomal vectors, unlike viral vectors, have no innate, specific devices for controlling intracellular gene trafficking, it is necessary to optimize their intracellular trafficking. Thus far, the modification of liposomal vectors has been an empirical process, and the degree of liposomal transfection has been evaluated only by measuring the enzymatic activity of the final product of a reporter gene such as luciferase or chloramphenicol acetyltransferase. However, it will be difficult to completely understand the underlying mechanisms of liposomal gene delivery, and to optimize the liposomal vector without opening the "black box".

Recent studies have indicated that the nuclear membrane is a serious barrier to the expression of exogenous gene (5–7); and, thus, many attempts have been made to overcome this barrier. The nuclear localization signal (NLS, Ref.8) and a small sequence of simian virus 40 (SV40, Ref.9) have sequence-dependent activity for the transport of DNA to the nucleus, and the ligation of these sequences to reporter plasmids has been reported to increase the efficiency of gene expression (10,11). However, the mechanism of nuclear transport of the delivered gene, as well as the relationship between the amounts of gene delivered to the nucleus and gene expression, is unclear at present. A quantitative understanding of intracellular gene trafficking will be required to understand the factor(s) governing the efficiency of gene expression. To achieve this goal, an accurate measurement of the amount of plasmids in various subcellular compartments is needed.

In this study, the amount of plasmid DNA delivered into the nucleus was determined by both PCR analysis and Southern analysis, and the quantitative relationship between the plasmid delivery to the nucleus and gene expression was evaluated. The results suggest that, not only the nuclear transport of plasmids, but the processes after nuclear transport of plasmids as well were important for the efficient expression of nonviral vectors.

MATERIALS AND METHODS

Materials

Taq DNA polymerase (Ex Taq) and DNA Labeling Kit (code 6045) were purchased from TaKaRa Shuzo (Otsu, Japan). [α-^{32}P] dCTP (specific activity = 111 TBq/mmol) and nitrocellulose membranes (type BA85) were purchased from NEN Dupont and Schleicher & Schuell (Dassel), respectively. The pGEM^{R}-T vector was purchased from Promega (WI, USA).

Cell Culture

For transfection studies, a rat ascites hepatoma cell line, AH130, was used. The cells were cultured in RPMI1640 medium supplemented with 10% heat-inactivated fetal calf serum unless otherwise noted and were incubated in 5% CO_2 at 37°C (15).

Preparation of Plasmids

pSV2CAT has long been used as a reporter plasmid for a positive control in transfection studies (16). However, the preparation of a large amount of this plasmid was difficult, probably due to its low replication in the host *E.coli* cells.

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Thus, the plasmid pGEM/SV2CAT was prepared as follows: the entire structural gene of chloramphenicol acetyltransferase (CAT) was amplified by PCR using HT697 (5′-CCTGGGGATCCAGACATGAT) and HT696 (5′-GAGGCAGCTGTGGAATGTGT) as primers and pSV2CAT (0.1ng/μl) as a template. The obtained PCR product was subcloned into the pGEM-T vector. Proper subcloning was confirmed from its nucleotide sequence. The resulting plasmid, referred to as pGEM/SV2CAT, was used in this study. This plasmid was easily prepared and showed essentially the same functional property as pSV2CAT (data not shown).

Cell Transfection

Cationic liposomes (TFL-3: *O, O'* - Ditetradecanoyl - *N* - (α -trimethylammonioacetyl) diethanolamine chloride / dioleoylphosphatidylethanolamine / Cholesterol = 1 / 0.75 / 0.75 [molar ratio]) were prepared as described previously (17).

Plasmid/cationic liposome complexes (20 μg / 250 nmol lipid / ml) were prepared and added to 5×10^6 cells at a final concentration of 0.4 ~6 μg / ml in serum-free medium. After incubation for 4 h in 5% CO_2 at 37°C, the cells were washed and incubated for a further 36 h. CAT activities were measured by standard procedures (18).

Isolation of Nuclei and Genomic DNA

Cells were suspended in 0.5 ml of lysis buffer (0.5% Nonidet P-40, 10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris-HCl buffer; pH 7.4) to dissolve the plasma membrane, and the nuclear fraction was then isolated by centrifugation at 1400 × g for 5 min. This treatment (washing) was repeated twice, and the final pellet was used as the nuclear fraction. To prevent the detection of extranuclear plasmids, the nuclear fraction was treated with *Eco*RI, for which a recognition site exists in the amplified region of pGEM/SV2CAT (for PCR analysis), or with *Bam*HI (for Southern analysis). We confirmed that plasmids digested by *Eco*RI were not amplified, and that no band corresponding to intact plasmids was obtained when the plasmids was digested by *Bam*HI (data not shown).

When nuclear DNA (containing plasmids) was extracted, proteinase K was first added to the nuclear fraction to give a final concentration of 0.1 mg / ml. After incubation at 37°C for 4 h, proteins were eliminated by phenol/chloroform treatment; and the DNA was precipitated by the addition of ethanol. The precipitate was dissolved in TE (1 mM EDTA, 10 mM Tris-HCl buffer; pH 8.0) and used as a DNA sample. Concentrations of DNA were determined by measurement of the absorbance at 260 nm with a Shimadzu UV-1200 spectrophotometer.

The number of nuclei in the samples was calculated from the amount of genomic DNA, based on the assumption that the nucleus of one AH130 cell contains 6×10^9 nucleotide pairs of DNA. Namely, one nucleus contains 6.6×10^{-6} μg of DNA because the average molecular weight of 1 nucleotide pair is 660.

PCR Analysis

A part of the CAT region of pGEM/SV2CAT in the DNA samples was amplified by PCR with primers RHY001 (5′-CCGTTGATATATCCCAATGGC) and RHY006 (5′-CCACTCATCGCAGTACTGTT). The reaction mixture (100 μl) for the PCR consisted of an adequate amount of template, 100 pmols of primers, 1.6 mM dNTP, 2.5 units of Taq DNA polymerase, 1.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl buffer (pH 8.3). The PCR was performed by denaturation at 94°C for 3 min, followed by 30 cycles at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. After the PCR procedure, 10 μl of the reaction mixture was subjected to gel electrophoresis, and the amplified DNA was quantified by measuring the fluorescence intensity of ethidium bromide bound to the DNA with an ATTO imaging analyzer, model AE-6911 (Tokyo).

Southern Analysis

For Southern analysis, aliquots of DNA samples (approximately 1~10 μg) were first digested with 120 units of *Hin*dIII at 37°C overnight, and then subjected to agarose gel electrophoresis. After electrophoresis, the DNA samples were transferred to a nitrocellulose membrane.

For the detection of plasmids, a DNA fragment corresponding to the open reading frame of CAT, prepared by PCR with primers RHY001 and RHY006, was radiolabeled and used as a probe.

Southern hybridization was carried out using a standard protocol (18). Briefly, membranes were first pre-hybridized at 42°C overnight in a solution consisting of 0.01 mg/ml salmon sperm DNA, 5 × SSPE, 5 × Denhardt's, 0.1% SDS, and 50% formamide and then hybridized at 42°C overnight in the same solution containing the ^{32}P-labeled probe. After hybridization, the membranes were washed 3 times with 2 × SSC containing 0.1% SDS at room temperature for 5 min, and then with 1 × SSC containing 0.1% SDS at 60°C for 60 min. After the washing, the membranes were exposed to the imaging plate of a bioimage analyzer Fuji-BAS 1500 (for quantification) or to X-ray film (for visualization).

RESULTS

Reliability of Quantification by PCR Analysis

We first examined the reliability of quantification by PCR. For this, the linearity between the signal intensity of the DNA band, determined by an image analyzer, and the amount of DNA subjected to gel electrophoresis was examined. Various amounts of plasmids were subjected to gel electrophoresis and stained with ethidium bromide, and the signal intensity of each DNA band was then converted to a 'peak area' by the image analyzer. The value of the peak area showed a linear relationship with the amount of DNA when the value of the former was less than 1,000 (arbitrary units, data not shown). The linearity between the amount of PCR products (i.e., signal intensity of amplified DNA band) and the amount of template used for PCR was then examined. The linear relationship was observed in a template range of 2×10^{-9} μg/μl to 5×10^{-8} μg/μl, as shown in Fig. 1.

Preparation of Nuclear Fraction

For quantification of the amount of reporter plasmids in nuclei, the method of preparation of nuclei from transfected cells and the degree of purity of the resulting nuclear preparation are very important issues. For the preparation of a

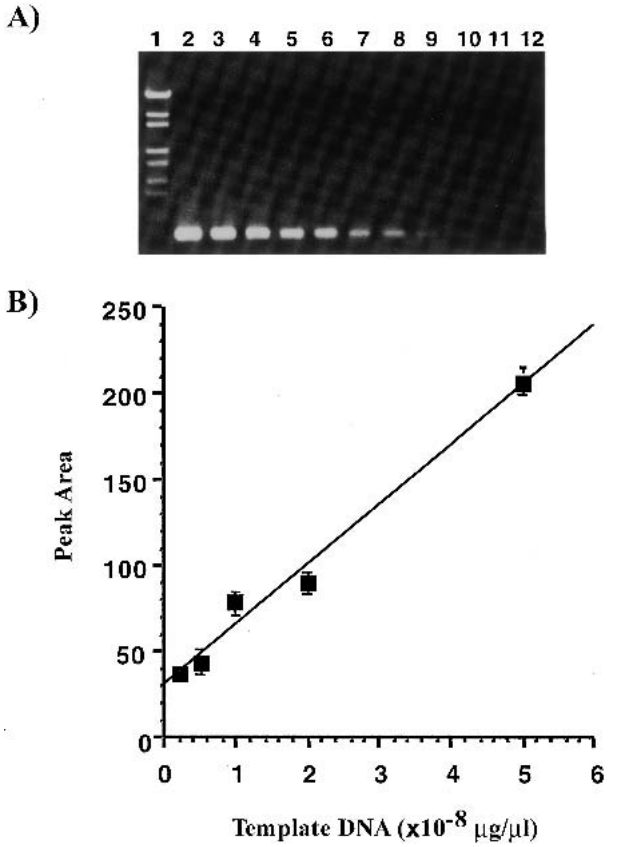

Fig. 1. Calibration curve of pGEM/SV2CAT in PCR analysis at 30 cycles Specific amounts of plasmids were amplified by PCR, and each sample was then subjected to gel electrophoresis and stained with ethidium bromide (A). λ DNA digested with *Sty*I was used as a size marker (lane 1). The amounts of plasmid used as templates were 1×10^{-6}, 5×10^{-7}, 2×10^{-7}, 1×10^{-7}, 5×10^{-8}, 2×10^{-8}, 1×10^{-8}, 5×10^{-9}, 2×10^{-9}, 1×10^{-9} μg/μl of reaction mixture for lanes 2–11, respectively. Lane 12 represents a negative control. The signal intensities of the amplified DNA bands were quantified by an image analyzer, and were plotted against the amounts of template PCR (B).

nuclear fraction, Nonidet P-40, a nonionic detergent, has been successfully used (12,13). Thus, we first lysed the transfected cells with 0.5% Nonidet P-40 and purified the nuclei by repeated centrifugation. No whole cells were observed in this nuclear fraction by phase-contrast microscopy (data not shown). The activities of marker enzymes of lysosomes and plasma membrane (acid phosphatase and 5′-nucleotidase) in this nuclear preparation were then compared with those of the original cellular lysate. The activities of acid phosphatase and 5′-nucleotidase in the nuclear fraction were the same as those of the blank (data not shown).

Measurement of Intranuclear Plasmids after Transfection

Based on these results, for determination of the amount of plasmids in samples, the samples were appropriately diluted to fit the calibration curve. Individual calibration curves were prepared for each experiment. Therefore, the number of plasmids delivered into nuclei was estimated to be $(5.84 \pm 3.23) \times 10^3$ copies/nucleus (means ± S.D.) when AH130 cells (5×10^6 cells) were transfected with 20 μg of pGEM/SV2CAT complexed with cationic liposomes ("lipoplex").

Effect of Dose of Lipoplex on the Levels of the Amount of Plasmids Delivered into Nuclei and Gene Expression

We subsequently examined the effects of the dose of lipoplex on the amounts of intranuclear plasmids and gene expression (Fig. 2A). The amount of plasmids delivered to the nucleus increased in a dose-dependent manner. On the other hand, although gene expression increased as a function of the dose at lower doses, it did not increase linearly at higher doses.

To understand the relationship between the amount of plasmids delivered to the nucleus and gene expression, we replotted the same data, as shown in Fig. 2B. Consequently, the amount of plasmids delivered to the nucleus and gene expression showed a hyperbolic relationship. Namely, gene expression increased depending on the amount of intranuclear plasmids when the copy number was less than about 4,000. However, it increased only slightly when the amount of plasmids delivered to the nucleus was in excess of 4,000 copies per nucleus.

Quantification of Plasmids Delivered into the Nucleus by Southern Analysis

In the above experiments, we used PCR analysis for the quantification of plasmids. PCR is currently regarded as the most sensitive method for the detection of DNA. By use of PCR in a quantitative manner, a trace amount of plasmids in each organelle can be quantified. We first used PCR analysis for the quantification of plasmids because it was expected that the amount of plasmids delivered to the nucleus would be very small. However, detectable amounts of plasmids, even in the case of quantification using Southern analysis, were delivered to the nuclei. PCR analysis detects not only intact plasmids but also partially degraded plasmids that contain the amplified region as well. On the other hand, Southern analysis is able to differentiate whether plasmids are intact or not and quantifies only intact plasmids. Therefore, we next confirmed the above experiments by Southern analysis.

Prior to the measurements of plasmids, the linearity between the signal intensity of the hybridization band of Southern analysis and the amount of plasmids subjected to gel electrophoresis was examined. A linear relationship between the signal intensity and the amount of plasmids was observed in a range of signal intensity smaller than 20,000 arbitrary units (as measured using a FUJI bioimage analyzer; data not shown). Thus, the linear region under a signal intensity of 20,000 was used for the quantification of plasmids. Furthermore, since a determination of the amount of plasmids in nuclei was desired, the possible effects of irrelevant DNA on the signal intensity of plasmids in Southern analysis were also examined. The effect of the presence or absence of irrelevant DNA was almost negligible under the present experimental conditions, as shown in Fig. 3.

Therefore, the number of plasmids delivered into the nuclei was estimated as $(1.34 \pm 0.18) \times 10^3$ copies/nucleus (mean ± S.D.), when AH130 cells (5×10^6 cells) were transfected with 20 μg of lipoplex. The amount of intranuclear plasmids, as determined by Southern analysis was about a quarter of that by PCR. However, this relationship was nearly constantly observed in repeated experiments.

Furthermore, as shown in Fig. 4, essentially the same

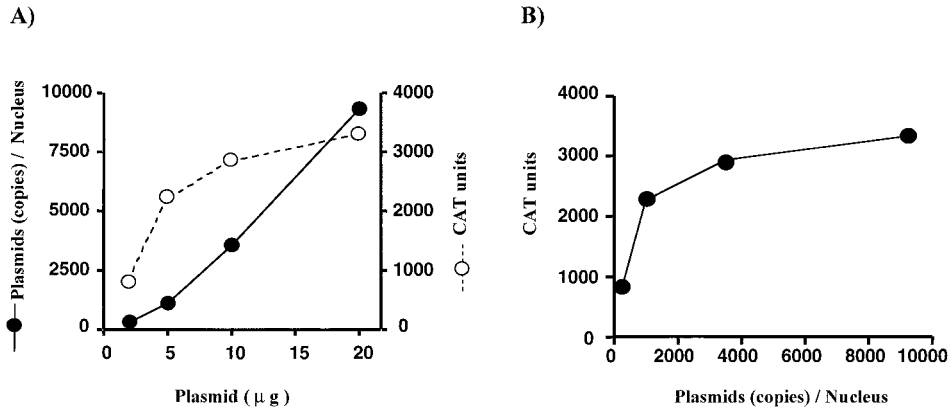

Fig. 2. Effect of dose of plasmids on the amount of intranuclear plasmids and gene expression in AH130 cells as evaluated by PCR analysis Various amounts (2 ~20 μg) of pGEM/SV2CAT were used to transfect AH130 (5×10^6 cells). The amounts of intranuclear plasmids (closed circles) and expression of the reporter gene (open circles) were then measured by PCR analysis and CAT assay, respectively (A). Results for intranuclear plasmids and gene expression in Fig. 2A were replotted (B). A typical result is shown. Similar results were obtained in 2 other independent runs.

relationship was observed by Southern analysis between the activity of the reporter plasmid product and the amount of nuclear plasmids, as obtained by PCR analysis (Fig. 2B).

DISCUSSION

Quantitative information, especially at the organelle level, is required for the optimization of gene delivery systems, as well as qualitative information. In this study, plasmids, which were delivered to nuclei by cationic liposomes, were detected and quantified by both PCR analysis and Southern analysis. Because the quantitative data by PCR analysis was higher than expected, we confirmed the results by another quantitative method, Southern analysis. However, these results did not differ in order of magnitude. The results obtained by PCR analysis under the same experimental conditions as were used for Southern analysis showed values about four times higher than those by Southern analysis. A possible explanation for this difference is as follows: PCR analysis detected not only intact plasmids but also partially degraded plasmids that contained the amplified region. Actually, the results of Southern analysis showed a relatively high background. In the case of Southern analysis, these signals were not counted. However, some of them could be detected in the PCR analysis.

To obtain the precise number of plasmids per nucleus, it

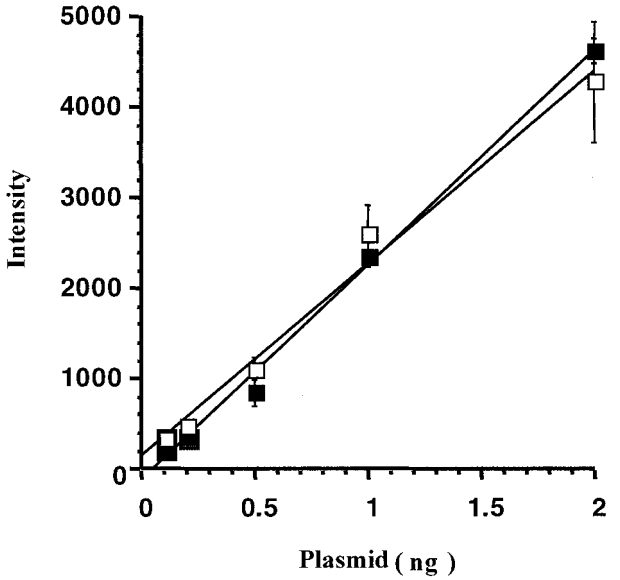

Fig. 3. Calibration curves of pGEM/SV2CAT in Southern analysis are shown. The indicated amounts of plasmids with (open squares) or without (closed squares) genomic DNA (1 μg) were subjected to gel electrophoresis and detected by Southern analysis. The signal intensities of the plasmid bands were quantified by use of a Fuji-BAS 1500 bioimage analyzer, and were plotted against the amounts of plasmids subjected to electrophoresis.

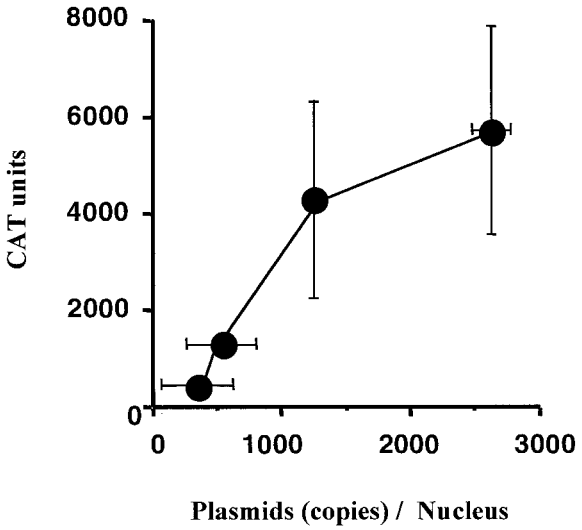

Fig. 4. Effect of dose of plasmids on the amount of intranuclear plasmids and gene expression in AH130 cells as evaluated by Southern analysis Various amounts (2 ~30 μg) of pGEM/SV2CAT were used to transfect AH130 (5×10^6 cells). The amounts of intranuclear plasmids and expression of reporter gene were then measured by Southern analysis and CAT assay, respectively. The results for intranuclear plasmids and gene expression were replotted. Data represent the mean ± S.D.

is necessary to determine the number of nuclei precisely as well as that of the plasmids in the nuclear fraction. We determined the number of the nuclei in the final samples by measuring the amount of genomic DNA. Although this is based on the assumption that the AH130 cell is diploid, this is the most reliable method for the determination of the number of nuclei in a sample.

The amount of plasmids delivered into the nucleus was quantified at the same time as the gene expression (36 h post-transfection). Probably, most plasmids and complexes in endosome-lysosome or cytosol would have been degraded by this time. To remove the extranuclear plasmids the nuclear fraction was washed and treated with restriction enzyme. However, at the shorter period, we more carefully examined the possible overestimation of plasmids derived from contamination of extranuclear plasmids in the nuclear fraction.

Because not all of the cells would be expected to be transfected by plasmids, our results represent the average number of plasmids delivered into nuclei in a large population of cells. In contrast, flow cytometric analysis provides important information based on a single cell. By using such an analysis, James and Giorgio measured the amount of plasmids delivered to the nucleus when HeLa cells were transfected with cationic liposomes (14). The average number of plasmid copies associated with the nuclei over 24 h was determined to be 1510 copies per nucleus. These results are in agreement with ours. Therefore, at least 10^2~10^4 copies plasmids could be delivered to a nucleus of a cell, for which the level of gene expression is sufficiently high.

Delivery of plasmids to the cytoplasm and nucleus are generally considered as major obstacles to gene expression. Therefore, a number of efforts have been made to overcome these barriers. For example, Zanta *et al.* succeeded in efficient gene delivery by using the NLS peptide (10). The expression of a linear reporter gene having one molecule of NLS was significantly higher than that observed with non-modified DNA. However, the expression of DNA modified by NLS was not significantly increased even when its dose was increased by 10 times. The reason why gene expression did not increase with an increase in the plasmid dose in that study remains obscure. For clarification, it is necessary to determine the quantitative relationship between gene expression and the amount of intranuclear plasmids. In this study, we also examined relationship between gene expression and the amount of intranuclear plasmids. Therefore, we observed the saturation of gene expression against intranuclear plasmids (Figs. 2B and 4). Although such a relationship would be observed if the substrate of the reporter gene product (CAT) were exhausted, our experiments on the quantification of gene expression were always carried out in the presence of a large excess of substrate (chloramphenicol). Therefore, the saturation of gene expression observed in our experiments is not the result of exhaustion of substrates in the reaction system. In addition, cell toxicity by transfection did not affect the results of plasmid number per nucleus because cell viability and cell number of each sample after 36 h of incubation were almost same ($86.3 \pm 3.99\%$, $1.577 \pm 0.187 \times 10^7$ cells [means ± SD], respectively). From these results, we conclude that gene expression is not always completely proportional to the amount of plasmids delivered into the nucleus, but shows saturation when an excess amount of plasmids are delivered into the nucleus. Thus, a more efficient system might be realized by optimizing of the process after plasmid entry into the nucleus.

ACKNOWLEDGMENTS

This study was supported by fellowships and grants from Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists and Grants-in-Aid for Scientific Research on Priority Areas from The Ministry of Education, Science, Sports, and Culture of Japan, respectively.

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