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NOTE

Evaluation method for gene transfection by using the period of onset of gene expression and cell division

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Using single-cells time-lapse analysis, we investigated the mechanism of gene expression using nine transfection reagents. Although onset of gene expression occurred after cell division by all reagents, 91.6% periods, which depended on onset and cell division, had statistical significance. Evaluation of those periods is useful for elucidating mechanism of transfection.

[**Key words:** Cell cycle; Single-cell analysis; Transfection reagent; Time lapse imaging; Gene expression]

The ability to deliver genes with high efficiency and specificity using non-viral vectors will enable a wide range of biomedical efforts, from basic research to clinical translation. Although the potential benefits of gene therapy for the treatment of acquired and inherited genetic diseases have been demonstrated through pre-clinical studies, the results of human gene therapy have been less than satisfactory. One reason for the poor efficacy is the low gene transfer efficiencies of non-viral vectors, which despite advances in their development, are unable to achieve the gene transfer efficiencies of viral vectors (1).

To determine the cause and improve gene transfer inefficiency by non-viral vectors, a number of studies have attempted to elucidate the biological steps of mechanisms of gene expression (2–5). It is generally accepted that transfection activity is rate limited by several intracellular processes, such as cellular uptake, endosomal escape, nuclear transfer, and transcription (6), which can be divided into the following stages: (i) cell binding; (ii) cell entry/endocytosis; (iii) endosomolysis; (iv) cytosolic transit toward the nucleus; and (v) nuclear entry (7). An additional three steps are necessary to express the delivered gene by the host cell: (vi) transcription, (vii) translation, and (viii) post-translation modification and protein folding. Several reports have suggested that nuclear entry may represent the greatest barrier to efficient gene expression (8–10) and our previous study that the onset of gene expression is highly dependent on cell division (11), also strongly suggested that the rate limiting step of onset of gene expression is cell division. Mintzer et al. reported that dividing cells often exhibit higher transfectability than nonmitotic cells (12). Although the main limiting step of transfection may be nuclear transport, the exact rate-limiting process has yet to be clarified, and further studies are required to elucidate the process controlling gene transfer efficiency.

In this study, we addressed to elucidate the rate-limiting process by using a same vector with different types of transfection reagents. By conducting gene transfection investigations using a vector encoding the identical promoter and gene, it is expected that the transfected gene will be transcribed, translated, and post-translated (i.e., steps vi, vii, and viii) at nearly identical rates. There are several types of gene transfection reagents that were made by cationic lipid, cationic polymer, cationic sugar, lipopolyamine based reagents, and inorganic reagents. By using these types of reagents, it is expected to elucidate the reagent dependent mechanisms. If the rate limiting step is nuclear entry, the correlation between cell division and the onset of gene expression would not be affected by the transfection reagent.

Here, using single-cell time lapse imaging of human cells transfected with a vector expressing a yellow fluorescent protein variant (Venus) gene under control of the cytomegalo virus (CMV) promoter using nine different transfection reagents, we investigated the difference of the onset of gene expression. The vector of pCMV-Venus was constructed by using pCMV-EGFP expression vector (BD Biosciences Clontech, Tokyo, Japan). For all experiments, HeLa were obtained from RIKEN BRC cells (RCB0007) and were routinely cultivated in an incubator at 37°C with an atmosphere of 5% CO_2 in air. For the Venus-reporter assay, HeLa cells were seeded at 2×10^4 cells per well in 24-well culture plates. Following transfection, phase-contrast and fluorescent images of cells were recorded at 10-min intervals for 24 h with exposure times of 300 ms by programmable cellular image tracer, which had been co-developed with Olympus (Olympus, Tokyo, Japan). Using the final obtained image, we selected and numbered cells that exhibited fluorescence, and the fluorescent images were then examined and tracked back in order to determine when the onset of gene expression occurred. The timing of

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cell division was determined from phase-contrast images. During the M phase, since a parent cell detached from the culture dish before dividing into two daughter cells, it was relatively easy to distinguish dividing from non-dividing cells. For transfection of HeLa cells with pCMV-Venus, we used nine different transfection reagents and they were divided into four types. The type of cationic liposome: Lipofectamine™ LTX (Invitrogen, Tokyo, Japan), Lipofectamine™ 2000 (Invitrogen), FuGene® HD (Roche, Tokyo, Japan), and FuGene® 6 (Roche), cationic polimer: jetPEI™ (Polyplus-transfection, Illkirch, France), and Nanojuice™ (Merck, Tokyo, Japan), cationic sugar: Sugarfect® (MedGEL, Kyoto, Japan), inorganic transfection reagent: Calcium Phosphate Transfection kit (Invitrogen), liopopolyamine based reagent: DreamFect™ (OZ Bioscience, Marseille, France). HeLa cells were transfected with pCMV-Venus following the protocols supplied by each manufacturer. Initially, the average and standard deviation (σ) of onset of gene expression and timing of cell division were calculated for each transfection condition. *Dif* was defined as difference between timing of cell division and onset of gene expression. Dif_{ave} and Dif_{std} calculated the average of *Dif* and standard deviation, respectively. To remove outliers, we recursively removed data that was larger/smaller than the average plus/minus 2σ, and correlation coefficients and regression lines were then calculated. To evaluate the relationship between cell division and onset of gene expression, we applied a linear regression model to the entire data set, and calculated Pearson's *r* correlation. Statistical significance was assessed by Welch's test ($P<0.01$). All statistical analyses were performed using NAG Statistical Add-Ins for Excel (NAG®, Oxford, UK).

Fig. 1 shows typical time-lapse images of dividing HeLa cells transfected with Venus. Most cells began to express the transfected gene after cell division, with all transfection reagents resulting in the identical tendency. This finding consists the results of our previous study (11), and suggests that the onset of gene expression is independent of transfection reagents.

To more accurately evaluate the initiation of gene expression following cell division, we digitalized the fluorescence and phase-contrast data for HeLa cells transfected with each of the nine transfection reagents. After removing the outlier data, scatter plots of the onset of gene expression and timing of cell division were generated for cells transfected with each of the nine examined transfection reagents (Fig. 2). Using any of nine reagents, strong correlations between onset of gene expression and cell division were observed. These findings suggest that transfected genes were able to be delivered into cytosol but not be delivered into nucleus and, at least 9 transfection reagents, all reagents probably deliver genes into nucleus at the timing of nuclear envelope breakdown. Because our method measured events of cell division and the fluorescent intensity of Venus, there is the time lag between nuclear envelope breakdown and cell division. Additionally, in this study, because we recorded every 10 min and we did not regulate the amount of transfected gene, it is necessary to further investigate to elucidate the precise onset of gene expression.

Table 1 shows the correlation coefficients and coefficient of regression lines for each transfection reagent. The average correlation coefficient and standard deviation are 0.995 and 6.47×10^{-3}, respectively. The high observed correlation suggests that the initial expression of transfected genes was highly dependent on the cell division. Sergio et al. reported diameter of DNA complex is more than 100 nm (13); however, small particles, which diameter is up to 9 nm, are possible to enter the nucleus by passive diffusion and up to 25 nm is possible to enter the nucleus by active transport (10,14). These reports suggest that most of all DNA complexes cannot enter the nucleus through nuclear pore. Although strong correlations were commonly observed using any transfection reagents, almost all combinations (91.6%) of two reagents of Dif_{ave}, which is the average of time period between cell division and onset of gene expression, have the statistical significance ($P=4.84\times10^{-5}$) except combinations of (Nanojuice and Fugene6; $P=0.010$), (Sugarfect and Fugene6; $P=0.014$), and (FugeneHD and Fugene6; $P=0.783$) and the intercepts of the plotted regression lines and deviation (Dif_{std}) suggest influenced by the transfection reagents. The difference of intercepts indicates that the onset of gene expression following nuclear entry of the transfected gene (i.e., steps vi, vii, and viii) varies depending on the transfection reagent used. As mentioned above, because we used the identical transfected target gene, which encoded Venus as the reporter protein, and vector in this study, the duration of steps vi, vii, and viii should have been identical. Godbey et al. reported that in the case of using Poly(ethylenimine) for transfection, polyplex did not dissociate from DNA after nuclear entry

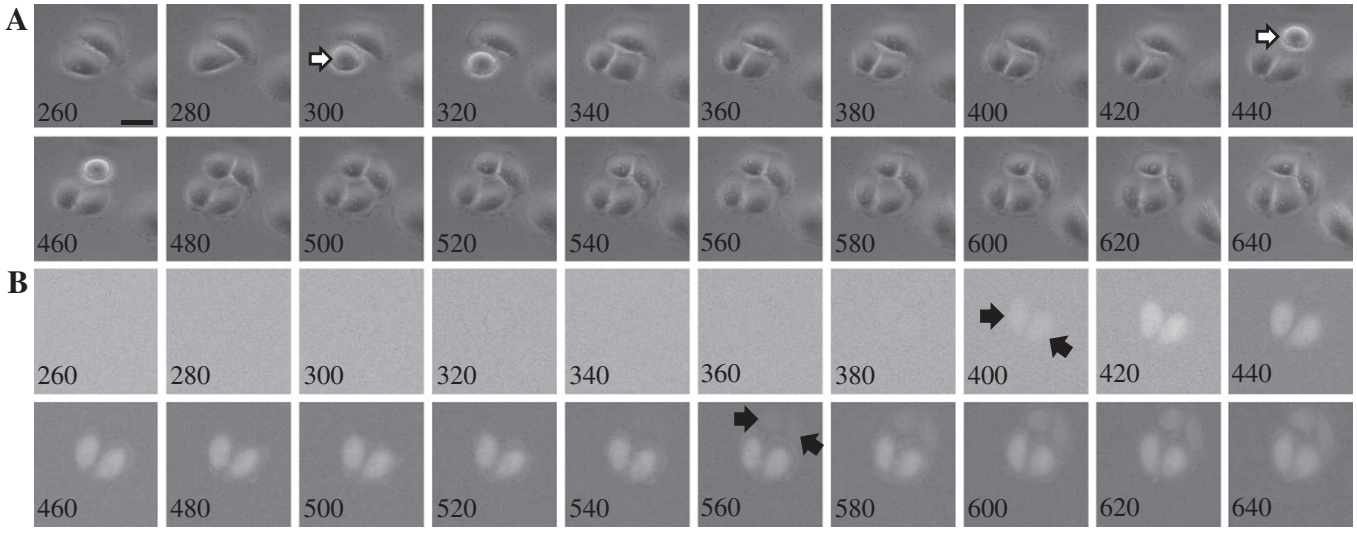

FIG. 1. Representative time-lapse images of HeLa cells expressing Venus after cell division. Time lapse phase-contrast (A) and fluorescent images (B) of HeLa cells transfected by jetPEI™ with Venus under control of the CMV promoter. White arrows represent the onset of cell division (at 300 min, and 440 min), and black arrows represent the onset of recognition of fluorescent intensity (at 400 min, and 560 min). Images were recorded every 10 min for 24 h (images shown were obtained from 260 to 640 min, recorded every 20 min). t = time in minutes; scale bar = 25 μm.