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The effect of cell synchronization on the efficiency of stable gene transfer by electroporation

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We synchronized thymidine kinase deficient mouse Ltk$^-$ cells by two different methods, hydroxyurea double-block treatment or aphidicolin single-block treatment and transformed them with the cloned herpes simplex virus thymidine kinase gene at various time intervals by the electroporation technique. Marked enhancement of stable transformation efficiency was observed at the time corresponding to the peak of G_2/M phase. These results suggest that the G_2/M phase is the most efficient period for stable gene transfer by electroporation.

Cell synchronization; Transformation; Hydroxyurea; Aphidicolin; Electroporation; (Ltk$^-$ cell)

1. INTRODUCTION

In recent years, gene transfer into mammalian cells has been used extensively in the field of molecular biology. However, the precise mechanism of exogenous DNA integration into recipient cell genomes is still unknown. It has been shown that transformants are not highly transformable variants; rather, the state of the cells seems to be the critical factor [1,2]. For electroporation, it is known that the transformation efficiency of growth arrested cells is relatively low [3]. Using electroporation, we investigated the relationship between the cell cycle phase of recipient cells and the efficiency of stable transformation. For this purpose we synchronized thymidine kinase-deficient mouse Ltk$^-$ cells by two different methods, hydroxyurea treatment or aphidicolin treatment, and transformed them with the cloned herpes simplex virus thymidine kinase gene at various time intervals.

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2. MATERIALS AND METHODS

2.1. *Cell culture and synchronization technique*

A thymidine kinase-deficient mouse fibroblast cell line (Ltk$^-$) was routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. For the asynchronous cell experiments, cells were plated at a density of 5×10^5 cells per 10 cm dish and cultured for 24 h. Then the cells were harvested by trypsinization. For the synchronization by the hydroxyurea double-block treatment [4,5], cells were plated at a density of 4×10^5 cells per 10 cm dish. After 24 h, hydroxyurea was added to 0.5 mM/ml. After 24 h of hydroxyurea treatment, the cells were washed twice with PBS and cultured for 10 h in fresh medium; then hydroxyurea was added again to 0.5 mM/ml and cultured for another 24 h. The cells were washed twice with PBS and re-fed with fresh medium. For the synchronization by the aphidicolin single-block treatment [6], cells were plated at a density of 5×10^5 cells per 10 cm dish. After 24 h, aphidicolin was added to 0.5 μg/ml and the cells were cultured for another 24 h. Then the cells were washed twice with PBS and re-fed with fresh medium. After each synchronization, the cells were harvested at intervals by trypsinization and processed for cell cycle analysis and electroporation.

2.2. *Plasmid DNA*

The plasmid, pHSV106, contains the herpes simplex virus thymidine kinase gene cloned into the *Bam*HI site of pBR322. This plasmid was linearized with *Hin*dIII and used for transformation.

2.3. *Electroporation*

Cells, 2×10^6, were suspended in 0.5 ml of electroporation buffer (137 mM NaCl, 5.4 mM KCl, 1.1 mM

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$Na_2HPO_4 \cdot 12H_2O$, 1.47 mM KH_2PO_4, 0.5 mM $MgCl_2 \cdot 6H_2O$, 9 mM $CaCl_2$, 6.1 mM glucose, pH 7.14) [7]. Then 3 μg of linearized plasmid DNA was added, and after incubation on ice for 5 min, an exponentially decaying electrical pulse (initial strength 1500 V/cm, $T_{1/2}$ = 1.7 ms) was applied once on ice. The mixture was left on ice for 15 min; then 2 ml of fresh medium was added and the cells were counted. For the determination of transformation efficiency, 1×10^6 electroporated cells were plated in a 10 cm dish with 8 ml of medium, and 48 h later, 2 ml of medium containing 5 × HAT (5×10^{-4} M hypoxanthine, 2×10^{-6} M aminopterine, 8×10^{-5} M thymidine) was added. The culture medium was replaced with fresh DMEM containing 1 × HAT every 6th day. After 14 days of HAT selection, cells were stained with Giemsa solution, and colonies were counted with a low power binocular microscope. For the determination of cell viability, 1×10^5 electroporated cells were plated in a 6 cm dish and cultured for 96 h. Then they were trypsinized, and viable cells were counted by the trypan blue dye exclusion method. As a control for each viability testing, 1×10^5 cells treated in the same manner except for electroporation were plated and viable cells were counted 96 h later.

2.4. *Cell cycle analysis*

Cells were washed twice with PBS and fixed in 70% ethanol for at least 1 h. Fixed cells were stained with propidium iodide, as described by Krishan et al. [8], and analyzed for DNA contents with a FACS440 cell sorter. The fraction of cells in each cell cycle phase was estimated as described by Grdina et al. [9].

3. RESULTS AND DISCUSSION

By keeping the condition of electroporation constant, we could obtain a more constant efficiency of stable transformation by electroporation than by other methods. For asynchronous cells, three separate experiments gave an efficiency of 116 ± 13 transformants/10^6 treated cells (fig.1A). When *Hin*dIII linearized pBR322 was used as a negative control, no transformants were obtained. At 1500 V, the viability of the cells measured by our method was 87.5 ± 17.5% (mean ± SD) (fig.1B).

After hydroxyurea treatment, most of the cells were synchronized in the early S phase (or G_1/S boundary). G_2/M phase cells began to increase 4 h after re-feeding and reached a significant peak at 6 h, then began to decrease gradually (fig.1C). Cell viability after electroporation remained relatively stable (65–100%) throughout the cycle (fig.1B), while transformation frequency reached a sharp peak at 6 h, corresponding to the peak of the G_2/M phase of the cell cycle (fig.1A). The maximal transformation efficiency was 7–8 times higher than that of asynchronous cells. When cells were synchronized with aphidicolin, most of them accumulated in the mid-S phase. The fraction of the

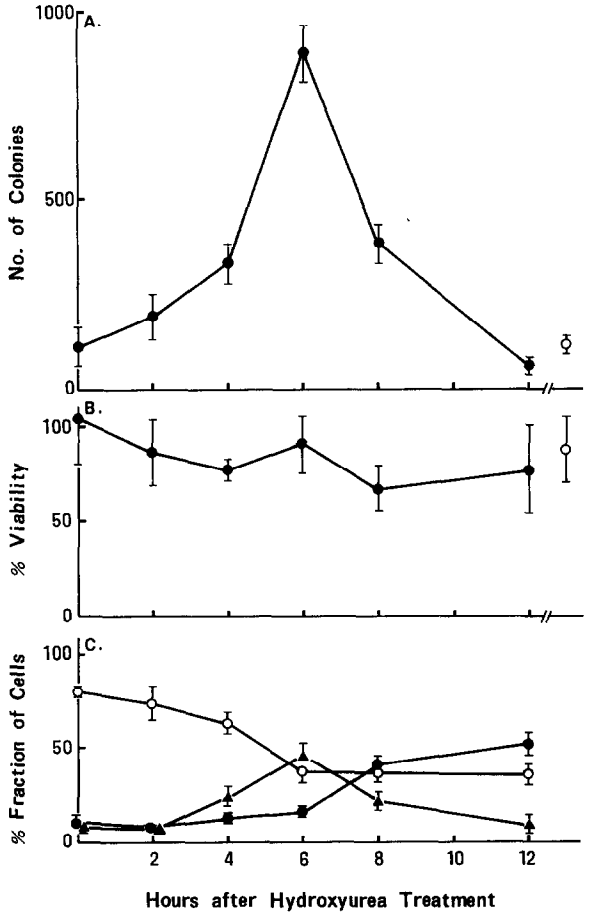

Fig.1. (A) Frequency of stable transformation after synchronization by the hydroxyurea treatment. Closed circles represent HAT-resistant colonies per 10^6 treated cells at the indicated times. The open circle on the right shows transformation efficiency for asynchronous cells. Each point is the average of three experiments and range bars indicate SD. (B) Viability after electroporation of cells synchronized by the hydroxyurea treatment. % viability = viable cells among electroporated cells × 100/viable control cells (see section 2). Closed circles represent % viability of cells at the indicated times after hydroxyurea treatment. The open circle on the right represents % viability of asynchronous cells. Each point is the average of three experiments and bars indicate SD. (C) Fraction of cells in each cell cycle phase following synchronization by the hydroxyurea treatment. Cells were harvested at the indicated times after hydroxyurea treatment and analyzed for DNA contents. Closed circles, open circles and closed triangles represent G_1 phase, S phase and G_2/M phase cells, respectively. Each point is the average of three separate experiments and bars indicate SD.

G_2/M phase cells reached its peak at 4–6 h after re-feeding (fig.2C), and again the peak of the transformation efficiency coincided with that of

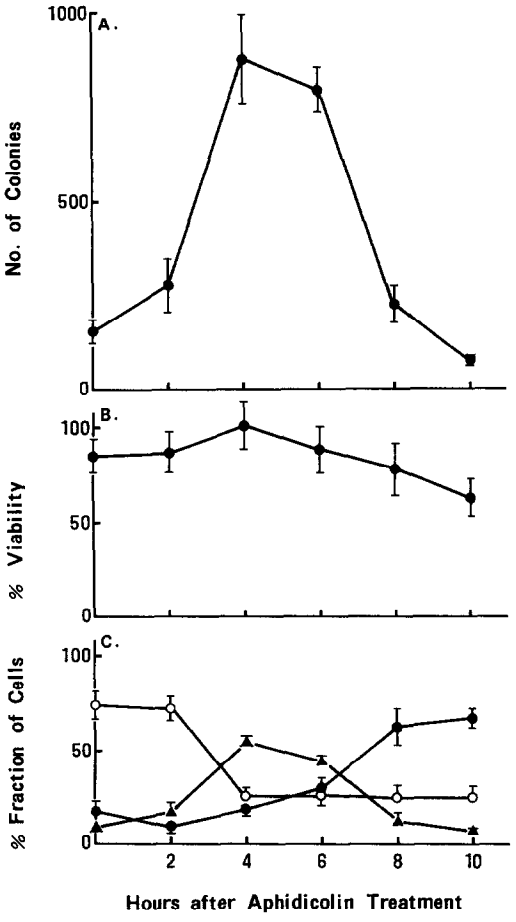

Fig.2. (A) Frequency of stable transformation after synchronization by the aphidicolin treatment. (B) Viability after electroporation of cells synchronized by the aphidicolin treatment. (C) Fraction of cells in each cell cycle phase following synchronization by the aphidicolin treatment. Symbols and the definition of terms are the same as in fig.1. Each point is the average of three experiments and bars indicate SD.

the G_2/M phase cells (fig.2A). Cell viability remained relatively stable throughout the cycle (fig.2B).

These results suggest that the G_2/M phase is the most efficient period for stable transformation by electroporation. Two different synchronization methods gave similar results, indicating that the effect is not specific for each DNA synthesis inhibitor. In addition, our results are inconsistent with those by Okada et al. who showed, using cell synchronization by the aphidicolin treatment, that the M phase is the most efficient period for gene transfer into plant protoplasts [10].

The reason for this apparent cell cycle dependency is unknown. It may simply be due to the loss of the nuclear membrane at the M phase. Recently, however, it has been suggested that DNA topoisomerase II is responsible for illegitimate recombination in mammalian cells [11], and it is known that the activity of DNA topoisomerase II in cells is highest at the G_2/M phase [12]. Our result may support this hypothesis.

REFERENCES

[1] Wigler, M., Sweet, R., Sim, G., Wold, B., Pellicer, A., Lacy, E., Maniatis, T., Silverstein, S. and Axel, R. (1979) Cell 16, 777–785.
[2] Graf, L.H., Urlaub, G. and Chasin, L.A. (1979) Somatic Cell Genet. 5, 1031–1044.
[3] Gilbert, C., Hayakawa, H. and Berg, P. (1987) Nucleic Acids Res. 15, 1311–1326.
[4] Pfeiffer, S.E. and Tolmach, L.J. (1967) Cancer Res. 27, 124–129.
[5] Strain, A.J., Wallace, W.A.H. and Wyllie, A.H. (1985) Biochem. J. 225, 529–533.
[6] Ayusawa, D., Shimizu, K., Koyama, H., Takeishi, K. and Seno, T. (1983) J. Biol. Chem. 258, 12448–12454.
[7] Hama-Inaba, H., Takahashi, M., Kasai, M., Shiomi, T., Ito, A., Hanaoka, F. and Sato, K. (1987) Cell Struct. Funct. 12, 173–180.
[8] Krishan, A. (1975) J. Cell Biol. 66, 188–193.
[9] Grdina, D.J., Meistrich, M.L., Meyn, R.E., Johnson, T.S. and White, R.A. (1984) Cell Tissue Kinet. 17, 223–236.
[10] Okada, K., Takebe, I. and Nagata, T. (1986) Mol. Gen. Genet. 205, 398–403.
[11] Bae, Y.-S., Kawasaki, I., Ikeda, H. and Liu, L.F. (1988) Proc. Natl. Acad. Sci. USA 85, 2076–2080.
[12] Chow, K. and Ross, W.E. (1987) Mol. Cell. Biol. 7, 3119–3123.