

High Yields of DNA-Transfer into Mouse L-Cells by Electroporation

H. Stopper, U. Zimmermann

Lehrstuhl für Biotechnologie, Universität Würzburg,
Röntgenring 11, D-8700 Würzburg,
Bundesrepublik Deutschland

and

E. Wecker

Institut für Virologie und Immunbiologie,
Universität Würzburg, Versbacher Straße 7,
D-8700 Würzburg, Bundesrepublik Deutschland

Z. Naturforsch. **40c**, 929–932 (1985);
received October 17, 1985

Gene Transfer, Electric Field, Stable Transformation,
Neomycin Resistance

DNA transfection in mouse L-cells was performed by means of the electroporation technique (U. Zimmermann, G. Pilwat, and F. Riemann, Z. Naturforsch. **29**, 304 (1974)). The plasmid *pSV 2-neo* used leads to neomycin-resistance in stably transfected L-cells. Optimized conditions resulted in high yields of clones at relatively low DNA concentration. The influence of temperature during pulse application and during the subsequent resealing process as well as the field parameters and medium composition are discussed.

Introduction

Introduction of foreign substances into living cells without deterioration of cellular and membrane functions can be achieved with a reversible electrical breakdown technique pioneered by Zimmermann *et al.* [1–7]. In this procedure the cell membrane is subjected to a field pulse of high intensity (kV/cm range) and of very short duration (range nano- μ sec). Once the breakdown voltage of the membrane of the order of 1V is exceeded in response to the external field, the membrane locally breaks through resulting in an increase of the overall membrane permeability dependent on the field intensity.

Electroporation of the membrane is reversible provided that the exposure time of the cell in the field is short enough. After a given time, which depends on the temperature during the resealing period and on the field strength of the applied pulse, membrane impermeability and resistance is restored.

By these means it is possible to incorporate dyes [3, 4], drugs [8–12], albumin [2, 8, 13], enzymes [14], latex particles [15, 16], DNA [17] and even

whole cells [18, 19] into various host cells as was demonstrated almost one decade ago.

Recently this method was again applied by several laboratories [20–22] for DNA transfection because of the apparent limitations of the conventional DNA transfer techniques. The system parameters required for a successful electroporation are to some extent different from those developed for electrofusion [6]. In this communication we report on electric field induced DNA transfection experiments of mouse L-cells by modifications of our previously described electroporation procedure, leading to greatly increased yields of transfection and stable gene expression.

Materials and Methods

Mouse L-cells were grown to confluency in RPMI 1640 medium (Boehringer, Mannheim, FRG) supplemented with 5% fetal calf serum (FCS).

For transfection, the RPMI medium was decanted and replaced by isotonic solutions of low ionic strength (280 mM inositol and 1.1 mM phosphate buffer). The cells were scraped off the surface of the culture vessels and centrifuged at $140\times g$. The cell pellet was suspended in the low ionic strength solution to which 0.1 mg/ml dispase was added (6 U/mg, grade I, Boehringer, Mannheim). Thereafter the cells were washed with a medium in which the field pulse application was performed: 30 mM KCl, 220 mM inositol and 1.1 mM phosphate buffer, pH 7.2.

For field pulse application at 4 °C the cells were suspended in the above medium at a density of 2×10^5 to 2.3×10^6 cells/ml. The DNA was added before field exposure to the suspension in a final concentration of 1 μ g/ml if not stated otherwise.

The circular form of a neomycin resistance gene carrying plasmid *pSV 2-neo* [23] was used in most of the experiments. For linearization 50 μ g of the plasmid-DNA were digested for 3 h at 37 °C with 3×50 units of *Eco* R 1 added after 0, 1, and 2 h. After 2 cycles of phenol/chloroform-extractions the DNA was precipitated in 2 volumes of ethanol. The plasmid was isolated from *E. coli*.

For transfection of the plasmid DNA the discharge chamber technique was used as introduced by Zimmermann *et al.* [8, 24, 25] for field mediated cell encapsulation. Briefly, the discharge chamber used consisted of 2 flat, parallel platinum electrodes

Reprint requests to Prof. Dr. E. Wecker.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/85/1100–0929 \$ 01.30/0

(1 × 3 cm), 1 cm apart, mounted in a rectangular well of a plexiglass chamber. Cell suspension was filled into the well up to the upper edge of the electrodes. Depending on the discharge chamber used, 0.5 or 2 ml of cell-free medium were then layered over the cell suspension in order to cover the well of the chamber. The duration of the pulses was adjusted to 5 μs. Field application was performed at 4 °C. The suspension was kept for 1–2 minutes at this temperature. Then the solution was removed by micropipette and transferred into 15 ml resealing medium which was preheated to 37 °C (120 mM NaCl, 3.5 mM KCl, 8.5 mM K₂HPO₄, 3 mM KH₂PO₄, 0.5 mM Mg-acetat, 0.1 mM Ca-acetat and 10 mM glucose). The cells were kept for a further 20 min in the resealing medium at 37 °C. After completion of the resealing process the cells were centrifuged and transferred in RPMI 1640 medium supplemented with 5% FCS to Falcon culture flasks (75 cm² growth area). In an aliquot of the suspension the number of total cells was counted using a Neubauer chamber. After 48 h of incubation the medium was removed and selection medium was added (RPMI 1640 containing 5% FCS and 500 μg/ml of Gibco-G-418). Dead cells were removed by exchange of the selection medium every 3–4 days. Stable transformants were enumerated by counting the colonies developing within 12–18 days after the addition of the selection medium. Results are expressed as colonies per 10⁶ cells originally counted after the resealing period.

Results and Discussion

Table I summarizes the number of stable transformants obtained by electropermeabilization under various field and resealing conditions using circular

Exp. no.	Field intensity [kV/cm]	Number of 5 μs-pulses	Number of cell-clones	Clones per 10 ⁶ cells	Clones per 10 ⁶ cells and 1 μg DNA	DNA concentration [μg/ml]
8-1	8	1	320	71.7	23.9	1
8-5	10	1	243	71.9	24.0	1
13-1	10	1	72	122	40.7	1
10-1	10	1	105	128	42.7	1
8-2	15	1	358	65.6	21.7	1
8-3	20	1	306	63.6	21.2	1
8-6	10	2	397	58.8	19.6	1
13-2	10	3	93	88	29.3	1
8-7	10	3	504	86.8	28.9	1
Average clone number:			266	84	28	
9-1	10	1	65	8.9(130)	29.7	0.1
9-2	10	1	240	41.4(9.6)	2.8	5
9-3	10	1	240	44.4(48)	14.7	1

DNA. A field strength of 8 kV/cm was sufficient to obtain good yields of clones although 10 kV/cm gave in most of the experiments better and more reproducible results. Increasing the field strength up to 20 kV/cm or multiple pulse application (3 pulses in an interval of 20 sec at 10 kV/cm) did not result in correspondingly increased yields of stable transformants. Table I also shows that a concentration of 1 μg/ml circular DNA seems to give optimal results if the absolute number of drug resistant cells is considered. If one expresses yield per 1 μg circular DNA (Table I, experiments 9-1 to 9-3), the relative yield is higher with 0.1 μg/ml DNA than with 1 μg/ml and further decreases with 5 μg/ml of DNA. On average, 84 stable transformants per 10⁶ treated cells were obtained. Gene transfer by the calcium phosphate technique yields between 1–5% of transfected cells [26–28]. Using liposomes as carriers, Schaefer-Ritter *et al.* [29] found with 10% of total cells transfected yields of 200 stable transformants per 10⁶ cells. On this basis, about 5% of total cells should have been successfully transfected in our experiments. However, Neumann *et al.* [20], also using the electric field technique for gene transfer into cells, found at least a threefold higher yield of colonies when using linear rather than circular DNA. We obtained an increase of up to 20-fold of stable transformants when linear rather than circular DNA was used under otherwise comparable experimental conditions (Table II). Now the yield was above 400 colonies per 10⁶ transfected cells which suggests that about 20–25% of total cells had been transfected originally.

It should be noted that sometimes variations in the yield of clones were observed between comparable experiments (not all data shown). More reproducible

Table I. Yield of stably transfected clones under various field conditions, obtained with circular plasmid DNA. If not otherwise stated the DNA concentration was 1 μg/ml. Numbers in brackets refer to the number of clones per 1 μg DNA. The number of cells varied between 6 × 10⁵ and 7 × 10⁶ per experiment. In control experiments, in which cells were subjected to the same experimental procedure without pulse application, either no or only a single clone was found.

Table II. Comparison between the yield of stable transformants obtained with circular and linearized plasmid DNA. The linearized DNA was obtained from the circular form by digestion with *Eco* R 1 (see Materials and Methods). The results were obtained in parallel experiments using 1 μg/ml of DNA and 1.3 and 2.4 × 10⁶ cells/ml for the circular and linear form of DNA respectively.

Form of DNA	Field intensity [kV/cm]	Number of 5 μs-pulses	Number of cell clones	Clones per 10 ⁶ cells	Clones per 10 ⁶ cells and 1 μg DNA
circular	10	1	112	22	5.5
linear	10	1	4153	439	109.8

results can be expected if the manual steps involved in the procedure can be standardized by the development of more sophisticated apparatus.

In discussing the experimental conditions applied by other groups for field mediated transfer, several differences to our procedure need to be pointed out.

Neumann *et al.* [20] used trypsin rather than dispase to disperse the cells. This leads to long-lasting changes of membrane properties as revealed by the electro-rotation technique (see review [30]). They also applied the electric pulses in solutions containing high concentrations of NaCl in the absence of K-ions. Because of leakiness of K-ions through the permeabilized membranes, the viability of the cells is likely to be greatly diminished as shown for many cells by Zimmermann and coworkers [3]. Neumann *et al.* [20] furthermore applied field pulses at 20 °C. At this temperature the life span of the high permeabilization state of the membrane is very short. Even small molecules like sucrose are taken up only in very small amounts at this temperature as compared to uptake at 4 °C. This may be the reason why Neumann *et al.* [20] obtained optimal results with DNA concentrations of up to 50 μg/ml and very poor yields at lower DNA concentrations. With the calcium phosphate technique such high DNA concentrations are toxic for cells. Indeed, we found that already 5 μg/ml of DNA caused a reduction of the number of colonies and, on the basis of colonies per μg of DNA, best results were actually obtained with DNA concentrations of 0.1 μg/ml. Therefore it seems that the efficiency of transferring DNA into the cells is superior under our conditions. The rapid resealing process of the membrane at 20 °C also explains why Neumann *et al.* [20] reported that at least 3 pulses had to be applied in order to get gene transfer. As shown in Table I, under optimal conditions one pulse is enough, even though three pulses may sometimes be of advantage. With respect to the

other field conditions, however, Neumann *et al.* [20] now confirm our previous results in field induced loading experiments.

Potter *et al.* [21] also found a 50-fold higher yield of transfectants when using linear rather than circular DNA. With linear DNA optimal yields were about 200–300 transfectants per 10⁶ cells. However, these figures are based on viable cells and would therefore have to be reduced by about one half if total cells had been counted as in our case. The field application was carried out at 0 °C by Potter *et al.* [21]. A field strength of 4–8 kV/cm used by these authors is too low at this temperature because below 4 °C the breakdown voltage is further increased dramatically and also irreversible changes in the membrane are observed under the influence of the breakdown pulse [31, 32]. The DNA concentration used by Potter *et al.* [21] (20 μg/ml) also indicates a relatively low efficiency as discussed above for the work of Neumann *et al.* [20].

The methods described here were designed to avoid the shortcomings discussed above. If executed properly, the electric field pulse technique is a highly efficient method for DNA transfer, in particular when linear forms are used. Further developments and applications of this technique to other cells are certainly required. However, the bulk of literature being available on reversible electrical breakdown, field mediated transfer of foreign substances into cells and electrofusion pave the way for further optimization of the electro transfection technique.

Acknowledgements

We are grateful to Dr. G. Pilwat, KFA Jülich, for his help and stimulating suggestions and Ms. R. Schmitt for expert technical assistance. Grants of the DFG (SFB 176 and 165) to U. Z. and E. W. are greatly appreciated.

- [1] U. Zimmermann, J. Schulz, and G. Pilwat, *Biophys. J.* **13**, 1005 (1973).
- [2] U. Zimmermann, G. Pilwat, and F. Riemann, *Z. Naturforsch.* **29**, 304 (1974).
- [3] U. Zimmermann, J. Vienken, and G. Pilwat, *Bioelectrochem. Bioenerg.* **7**, 553 (1980).
- [4] U. Zimmermann, P. Scheurich, G. Pilwat, and R. Benz, *Angew. Chemie, Int. Ed. Engl.* **20**, 325 (1981).
- [5] U. Zimmermann and J. Vienken, *J. Membrane Biol.* **67**, 165 (1982).
- [6] U. Zimmermann, *Biochim. Biophys. Acta* **694**, 227 (1982).
- [7] U. Zimmermann, K.-H. Büchner, and W. M. Arnold, in: *Charge and Field Effects in Biosystems* (M. J. Allen and P. N. R. Usherwoold, eds.), p. 293, Abacus Press 1984.
- [8] U. Zimmermann, G. Pilwat, and F. Riemann, German Patent no. 2405119, filed February 2, 1974; British patent no. 1481480; U.S. Patent no. 4081340 and French patent no. 7502743.
- [9] U. Zimmermann and G. Pilwat, *Z. Naturforsch.* **31c**, 732 (1976).
- [10] U. Zimmermann, G. Pilwat, and J. Vienken, in: *Recent Results in Cancer Research* (G. Mathe and F. M. Muggia, eds.), **Vol. 75**, p. 252, Springer Verlag, Berlin, Heidelberg 1980.
- [11] U. Zimmermann, in: *Targeted Drugs* (E. Goldberg, ed.), p. 153, John Wiley & Sons Inc., New York 1983.
- [12] K. Kinoshita jr. and T. Y. Tsong, *Nature* **272**, 258 (1978).
- [13] U. Zimmermann, G. Pilwat, and F. Riemann, *Biochim. Biophys. Acta* **375**, 209 (1975).
- [14] U. Zimmermann, F. Riemann, and G. Pilwat, *Biochim. Biophys. Acta* **436**, 460 (1976).
- [15] J. Vienken, E. Jeltsch, and U. Zimmermann, *Cytobiology* **17**, 182 (1978).
- [16] W. Schüssler and G. Ruhenstroth-Bauer, *Blut* **49**, 213 (1984).
- [17] D. Auer, G. Brandner, and W. Bodemer, *Naturwissenschaften* **63**, 391 (1976).
- [18] U. Zimmermann, *Trends in Biotechnology* **1**, 149 (1983).
- [19] U. Zimmermann, J. Vienken, and G. Pilwat, in: *Investigative Microtechniques in Medicine and Biology* (J. Chajen and L. Bitensky, eds.), **Vol. 1**, p. 89, Marcel Dekker Inc., New York and Basel 1984.
- [20] E. Neumann, M. Schaefer-Ritter, Y. Wang, and P. H. Hofschneider, *EMBO J.* **1**, 841 (1982).
- [21] H. Potter, L. Weir, and P. Leder, *Proc. Natl. Acad. Sci. USA*, **81**, 7161 (1984).
- [22] I. Karube, E. Tamiya, and H. Matsuoka, *FEBS Letters* **182**, 90 (1985).
- [23] P. J. Southern and P. Berg, *J. Med. Appl. Genetics* **1**, 327 (1982).
- [24] U. Zimmermann, G. Pilwat, and F. Riemann, *Biophys. J.* **14**, 881 (1974).
- [25] F. Schneideweiß, U. Zimmermann, and M. Salemu-deen, *Biochim. Biophys. Acta* **466**, 373 (1977).
- [26] F. L. Graham and A. J. Van der Eb, *Virology* **52**, 456 (1973).
- [27] A. Pellicier, D. Robins, B. World, R. Sweet, J. Jackson, I. Lowry, J. M. Roberts, G. K. Sim, S. Silverstein, and R. Axel, *Science* **209**, 1414 (1980).
- [28] J. W. Bermann, R. S. Basch, and A. Pellicier, *Proc. Natl. Acad. Sci.* **81**, 7176 (1984).
- [29] Schaefer-Ritter, Y. Wang, and P. H. Hofschneider, *Science* **215**, 166 (1982).
- [30] W. M. Arnold and U. Zimmermann, in: *Biological Membranes* (D. Chapmann, ed.), **Vol. 5**, p. 381, Academic Press, London 1983.
- [31] H. G. L. Coster and U. Zimmermann, *J. Membrane Biol.* **22**, 73 (1975).
- [32] R. Benz, F. Beckers, and U. Zimmermann, *J. Membrane Biol.* **48**, 181 (1979).