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Electroporation for the efficient transfection of mammalian cells with DNA

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ABSTRACT

A simple and reproducible procedure for the introduction of DNA into mammalian cells by electroporation is described. The parameters involving the cells, the DNA, and the electric field are investigated. The procedure has been applied to a broad range of animal cells. It is capable of transforming more than 1% of the viable cells to the stable expression of a selectable marker.

INTRODUCTION

The transfection of DNA into eucaryotic cells permits the study of a broad range of problems in molecular biology. A large number of methods are used, including those involving protoplast fusion (1,2), DEAE dextran (3), and calcium phosphate coprecipitation with either DNA (4) or recombinant bacteriophage (5,6). Variations on the DEAE dextran and calcium phosphate methods have included transfection of cells in suspension (7), and treatment with DMSO (8), glycerol (9), chloroquine (10), or sodium butyrate (11). The efficiency of transfection by these methods varies widely between different cell lines, and may be unacceptably low in many cases. This can pose a serious obstacle to experiments which depend on the use of specific cell lines corresponding to a given tissue type or particular mutation. There is a great need for methods which are capable of introducing DNA into eucaryotic cells with high efficiency and without restriction to cell type.

Electroporation involves the exposure of cells to a pulsed electric field which presumably creates pores in the plasma membrane (12). It has been used to introduce DNA into both plant (13-15) and animal cells (16-18), and has been successfully applied to a wide range of cell types which have not been accessible to other methods. Because the procedure involves a physical interaction between the electric field and cell membrane, it may be less dependent on cell type than other methods.

This paper investigates how electroporation can be optimized with respect to parameters involving the cells, the DNA, and the electric field. The procedure described is simple, reproducible, and can be highly efficient. We have applied it to a wide variety of mammalian cells from different species and tissue types, including cells from primary culture, derived from tumors, and immortalized by SV40.

MATERIALS AND METHODS

Plasmids

The plasmids pRSV-gpt, pRSV-neo, and pRSV-cat (20) are mammalian expression vectors for the bacterial genes xanthine-guanine phosphoribosyltransferase, neomycin phosphotransferase, and chloramphenicol acetyltransferase, respectively. The genes are transcribed from the Rous sarcoma virus long terminal repeat and the transcript is processed at SV40 splicing and polyadenylation signals located downstream from the bacterial segment. The plasmid pMT-hGH-SV2, a kind gift from Peter Kushner (California Biotechnology, Mountain View, CA), contains a genomic clone of human growth hormone with the 5' regulatory region replaced by a fusion of the SV40 enhancer - early promoter and the human metallothionein promoter. Plasmid DNA was prepared as described earlier (21).

Cell lines

Fibroblasts immortalized by SV40 from normal human fetal lung (WI-38, VA13 subline 2RA, ATCC CCL 75.1), and from a patient with xeroderma pigmentosum (XP12RO, Group A) were obtained from Errol Friedberg (Stanford University). Primary human fetal fibroblasts (IMR-90, ATCC CCL 186) were obtained from the American Tissue Culture Collection, Rockville, MD; human cervical carcinoma cells (HeLa) were obtained from Roger Kornberg (Stanford University); African green monkey kidney cells (CV-1) have been described previously (22); and mouse embryo fibroblasts (NIH3T3) were obtained from Richard Mulligan (M.I.T.). The cells were grown in Dulbecco's modified Eagle's medium containing penicillin and streptomycin and supplemented with 10% calf serum for NIH3T3 cells, 10% newborn calf serum for CV-1 cells, and 10% fetal calf serum for all other cells.

Electroporation

Growing cells were divided with trypsin/EDTA, spun down, and resuspended in 1 ml buffer containing DNA. Unless otherwise specified, standard conditions were: buffer of 1X HeBS, 20 mM Hepes pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose (4); cell concentration of 3 x 10^6

cells/ml; and DNA concentration of 20 µg/ml of plasmid plus 500 µg/ml of salmon sperm DNA sonicated to sizes from 1 to 6 kb. The cells were then exposed to a single voltage pulse at room temperature, allowed to remain in the buffer for ten minutes, and then plated onto tissue culture dishes.

Relative cell viability was measured by plating cells exposed and not exposed to the electric field on parallel plates, allowing them to grow for 72 hours, and then measuring relative cell number either directly by counting or indirectly by the method of alkali lysis described previously (22).

Calcium phosphate transfection

Cells which had been plated on the previous day at a density of 10^6 cells per 85 mm dish were transfected with 1 ml of a coprecipitate of 20 µg of DNA and calcium phosphate (4) followed 4 hours later by exposure to 15% glycerol for 1 minute (9).

Assays for CAT, XGPRT, and growth hormone expression and for stable transformants

Cell extracts were made 48-72 hrs. following transfection from cells which were washed, scraped from the dish with a rubber policeman, resuspended in 100 µl of 0.25 M Tris pH 8, and sonicated for 2 minutes. The cell debris was pelleted for 10 minutes at 4°C in an Eppendorf microfuge and the supernatant assayed for enzyme activity.

CAT enzyme expression was assayed with ^{14}C labelled chloramphenicol as previously reported (23). The reaction products were extracted with ethyl acetate, separated by thin layer chromatography, autoradiographed, and quantitated by counting the spots in a scintillation counter.

XGPRT enzyme expression was assayed with ^{14}C labelled xanthine as previously reported (21). The reaction products were extracted with phenol/chloroform, separated by thin layer chromatography, autoradiographed, and quantitated by counting the spots. Both CAT and XGPRT activity were expressed as picomoles of substrate converted per minute per microliter of extract (pmoles/min/µl).

Growth hormone expression was measured in the tissue culture medium with a solid phase two-site immunoradiometric assay kit under the conditions recommended by the manufacturer (Hybritech Inc., San Diego, CA). Samples were diluted into the linear range of the assay with gamma globulin free horse serum (Gibco, Grand Island, NY). The use of human growth hormone as a reporter gene for DNA transfection has been described previously (24).

Selection for XGPRT transformed cells was begun three days following transfection by adding medium containing 10 µg/ml mycophenolic acid, 100 µg/ml

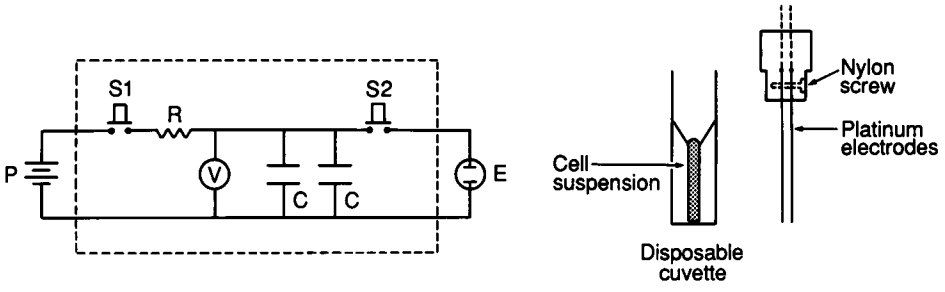


Figure 1. Electroporation apparatus.
The capacitors C were charged by the switch S1 from the power supply P through the resistor R (2000 ohms, 20 watts), which was chosen so that a standard power supply (rated to 300 volts, 150 ma) could be used. Each capacitor was 540 μF rated to 450 volts, providing a total capacitance of 1080 μF. The capacitors were discharged by the switch S2 through the electrode E which was placed in the cell suspension. The voltmeter V monitored the successful charging and discharging of the capacitors, as a safety feature, since the charged capacitors are potentially dangerous if inadvertantly discharged through the user. A gas sterilized plastic cuvette (Starstadt) was filled with 1 ml of buffer containing DNA and the cells in suspension. The platinum electrodes (0.375" by 0.007", spaced 3.8 mm apart) were fastened by a nylon screw to a plastic base which was machined to fit into the cuvette.

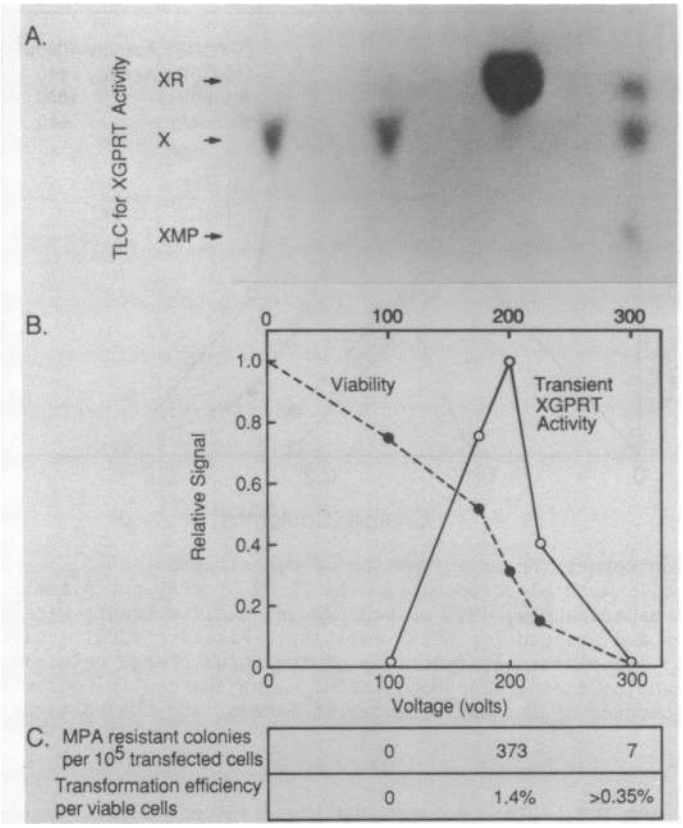
xanthine, 15 μg/ml hypoxanthine, 2 μg/ml aminopterin, 10 μg/ml thymidine, 5 μg/ml glycine, and 150 μg/ml glutamine (26). Selection for neo transformed cells was begun three days following transfection by adding medium containing 400 μg/ml G418 (27). After a suitable period of growth in selective medium, the cells were washed, fixed with methanol, and stained with 10% Giemsa to count the colonies of transformed cells.

RESULTS

Electroporation apparatus

The basic design of the electroporation apparatus is shown in Figure 1. The circuit allowed the capacitors to be charged from a power supply and then discharged through the cell suspension via the platinum electrode. The electrical pulse delivered to the cells had a voltage profile with a rise time of less than 0.5 msec. followed by an approximately exponential decay with a time constant equal to the product of the capacitance and the buffer resistance. For standard conditions (a buffer of 1X HeBS and a capacitance of 1080 μF) the time constant was approximately 7 msec.

Discharge of the large amounts of charge stored in the capacitors resulted in the conversion of ions to clearly visible amounts of gas from the



C.

MPA resistant colonies per 10^5 transfected cells	0	373	7
Transformation efficiency per viable cells	0	1.4%	>0.35%

Figure 2. Electroporation as a function of voltage.
(A) Transient expression of XGPRT activity assayed by thin layer chromatography; (B) transient expression of XGPRT and cell viability; and (C) stable expression of XGPRT: as functions of voltage. XP12RO cells were electroporated with 20 μg of pRSV-gpt plasmid and assayed for either transient or stable expression of XGPRT. For transient expression, 3×10^6 cells were plated per 85 mm dish. Cell extracts were harvested after 48 hours and assayed for XGPRT activity. For stable expression, 10^5 transfected cells were plated per 85 mm dish, grown under selective conditions after 72 hours, and stained with Giemsa after 17 days to estimate the stable transformation efficiency.

cell suspension. However, neither osmolarity nor pH of the buffer changed significantly with electroporation. There was no evidence that electroporation caused disruption of the DNA. Supercoiled DNA subjected to the pulsed electric field did not show any detectable change in mobility or conversion to nicked forms when analyzed by gel electrophoresis.

The reproducibility of the electroporation procedure was reasonably good,

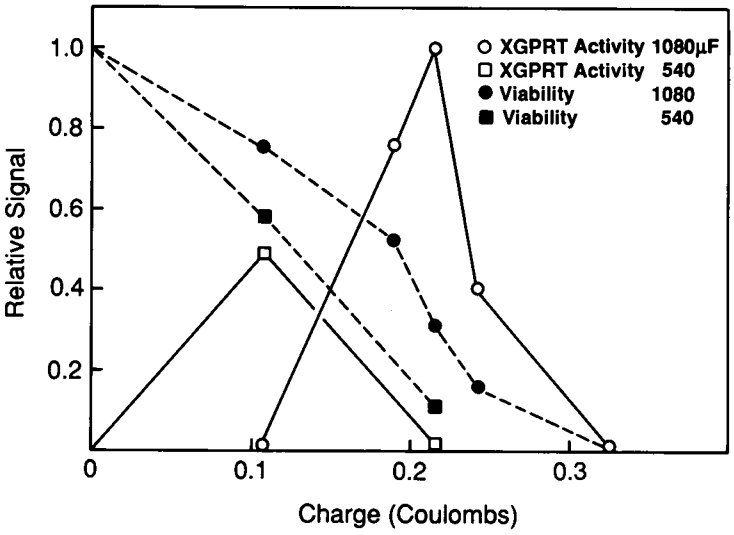


Figure 3. Electroporation as a function of capacitance.
XP12RO cells were electroporated with 20 μg of pRSV-gpt plasmid DNA at two different capacitances, 1080 μF and 540 μF. Cell extracts were harvested after 48 hours and assayed for XGPRT activity. Relative XGPRT activity and cell viability are plotted as functions of the total charge released through the cell suspension during electroporation. (The charge, in Coulombs, is equal to the product of the capacitance, in Farads, and the voltage, in volts.)

provided that growing cells were used and the electrode separation was kept constant to a tolerance of 5%. DNA transfection experiments performed either at the same time or on different days yielded results within 25% of each other. Best results were obtained when the cells were approximately 50% confluent. If fully confluent cells were used for electroporation, there was a two to tenfold decrease in signal, depending on the cell line and the state of the tissue culture.

Dependence on voltage, capacitance, salt and DNA concentration, cell number, and temperature

Voltage had an extremely important effect on the efficiency of the electroporation. Figure 2 shows the result of the electroporation of pRSV-gpt into XP12RO cells for a range of voltages. Transfection efficiency was assayed both by transient expression of XGPRT activity after 48 hours and by growth in selective medium containing mycophenolic acid after 17 days. Transient expression and colony formation showed parallel behavior as a

function of voltage, with a sharp maximum at 200 volts, which corresponds to an electric field strength of 530 volts/cm. At 200 volts the conversion of xanthine was measured to be 3.17 pmoles/min/µl, which is 20 to 50 fold higher than what was obtained by transfection using calcium phosphate coprecipitation. Above 200 volts, the drop in signal was caused by a sharp drop in cell viability. The number of mycophenolic acid resistant colonies expressed as a percentage of viable cells plated was greater than 1%, about two orders of magnitude higher than what was achieved with calcium phosphate coprecipitation. It should be noted that cell death was not detectable by trypan blue exclusion immediately after electroporation. Instead, the cells adhered to the tissue culture dish initially, only to die and float off into the medium over the next 48 hours.

The capacitance of the electroporation circuit affected transfection efficiency by determining the amount of electric charge and the duration of the voltage pulse passing through the cell suspension. The total electric charge Q is equal to the product of the capacitance C and the voltage V ($Q = CV$). The voltage pulse undergoes an exponential decay with a time constant equal to the product of the capacitance and the resistance of the buffer.

Since it was not convenient to vary capacitance, electroporation was tested at only two values, 540 µF and 1080 µF. The results shown in Figure 3 indicate that transfection was more efficient for the larger capacitance, after each was optimized for voltage. Cell viability appeared to depend primarily on the total charge passing through the cell suspension, with a weak dependence on capacitance. It is possible that a larger capacitance might produce an even stronger signal. Such a device would take longer to charge, since the time constant for charging is proportional to the capacitance in the circuit. For 1080 µF, the time required to charge to 99% capacitance was about 10 seconds.

The salt concentration of the buffer affects electroporation by multiple mechanisms. The ionic strength of the buffer at the time of the electric field pulse affects the formation of the transmembrane pores. Higher salt concentrations correspond to less electrical resistance, which leads to a shorter electric field pulse and therefore smaller pore sizes, affecting both DNA transfection and cell viability (12). The salt concentration of the buffer immediately after the electric field pulse affects cell viability by another mechanism. The transmembrane pores allow an inward sodium leak which

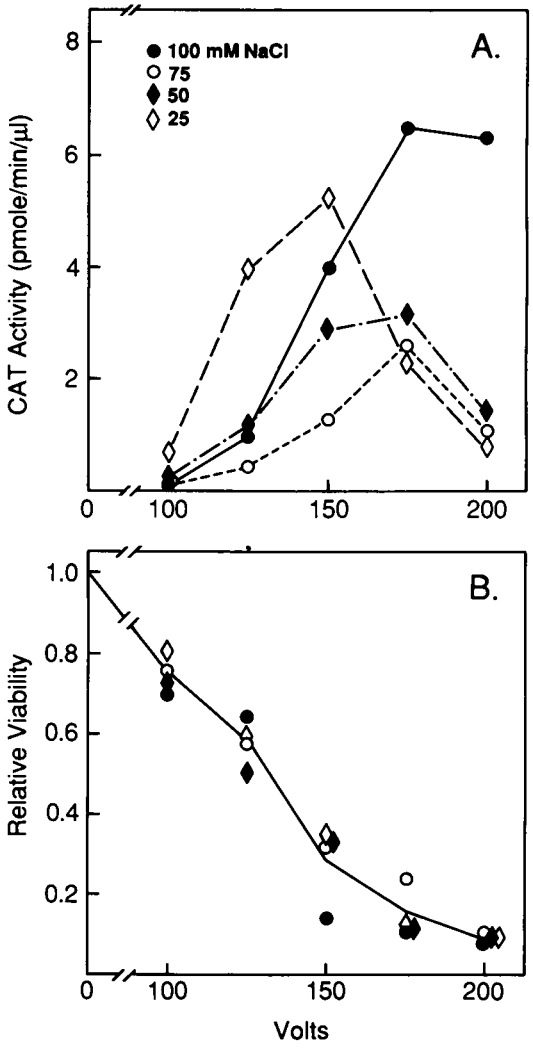


Figure 4. Electroporation as a function of salt concentration. (A) CAT activity and (B) cell viability are shown as functions of voltage. XP12RO cells were electroporated with 20 µg of pRSV-cat plasmid while suspended in buffer consisting of 20 mM Hepes pH 7.3, 5 mM KCl, and a range of salt concentrations, with sucrose added to keep the osmolarity constant: 100 mM NaCl; 75 mM NaCl with 50 mM sucrose; 50 mM NaCl with 100 mM sucrose; and 25 mM NaCl with 150 mM sucrose. For each salt concentration, a range of voltages was used to determine the voltage optimum. Cell extracts were harvested after 72 hours and assayed for CAT activity.

is driven by the transmembrane sodium concentration difference. The resulting osmotic pressure leads to cell swelling and, if severe enough, to rupture and lysis (12).

Because sucrose moves across the post-electroporation membrane much more slowly than sodium, sucrose was substituted for NaCl to maintain the osmotic pressure while decreasing the salt concentration. The voltage was varied for each salt concentration. The results are shown in Figure 4. The highest salt concentration yielded the strongest signal, with the voltage optimum at around 200 volts. As the salt concentration decreased, the voltage optimum also decreased. The height of the signal peak decreased at first and then gradually increased, probably as a result of increased cell viability. It is possible that even lower salt concentrations might yield higher levels of transfection. Cell viability at any given voltage did not appear to change over the wide range of salt concentrations tested. This was probably due to two competing effects. Lower salt concentration increases viability by decreasing the driving force for the sodium leak, but decreases viability by increasing the transmembrane pore size.

Because the strongest signal was seen with the highest salt (100 mM NaCl) under somewhat hypotonic conditions (205 mosm), subsequent experiments were done with even higher salt concentrations. The isotonic buffer 1X HeBS (137 mM NaCl, 295 mosm) was used routinely and gave somewhat higher viabilities and correspondingly higher transfection signals.

DNA concentration also affected transfection efficiency. Figure 5A shows that transfection of pRSV-cat plasmid increased linearly with DNA concentration even up to 80 µg/ml. Figure 5B shows the effect of adding carrier nucleic acid to 20 µg of pRSV-cat DNA. Sonicated salmon sperm DNA gave an increase in CAT signal up to 500 µg/ml, with a decrease at 1000 µg/ml due to a drop in cell viability. The nature of the carrier nucleic acid was important, since the addition of yeast tRNA led to a small decline in CAT activity.

Transfection efficiency was measured as a function of cell density for a fixed amount of DNA. XP12RO cells were electroporated with 20 µg of pMT-hGH-SV2 DNA, using 2.5×10^6, 5×10^6, 10×10^6, 20×10^6 and 40×10^6 cells in 1 ml. In each case, 2.5×10^6 cells were plated onto an 85 mm dish and assayed for growth hormone expression 72 hours later. The level of expression was the same for each dish, independent of the density of cells used for electroporation. Thus a relatively modest amount of DNA can be used to transfect a large number of cells.

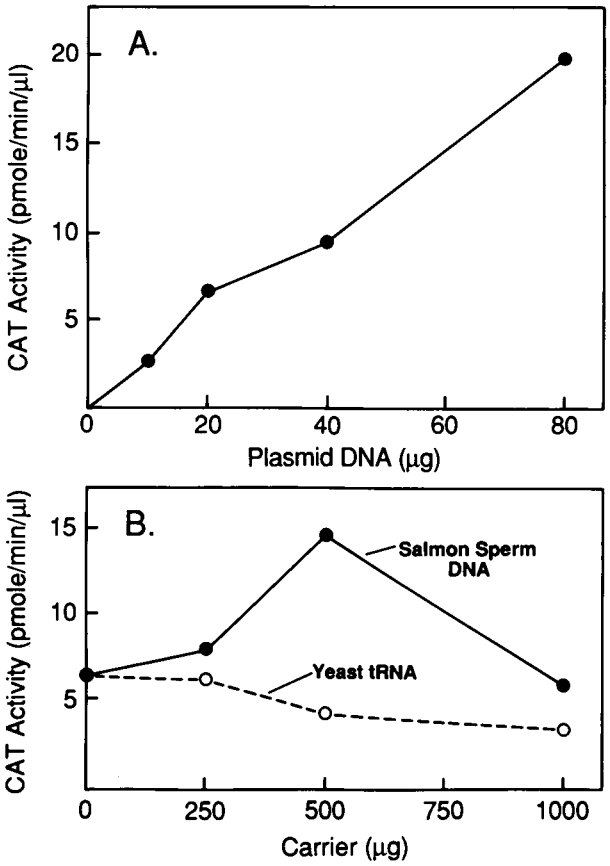


Figure 5. Electroporation as a function of DNA concentration.
The effects of (A) DNA concentration and (B) carrier nucleic acid on transfection are shown. XP12RO cells were electroporated either with different concentrations of pRSV-cat plasmid DNA or with 20 µg of pRSV-cat DNA plus different concentrations of carrier nucleic acid. Cell extracts were harvested after 72 hours and assayed for CAT activity.

Electroporation was investigated for a number of other cell lines by transfecting 20 µg of pMT-hGH-SV2 DNA and assaying for growth hormone expression 72 hours later. In addition to XP12RO cells, the experiments included other human cells derived from primary culture (IMR-90), immortalized by SV40 (VA13), and derived from a tumor (HeLa), as well as monkey (CV-1) and mouse (NIH3T3) cells. Figure 6 shows growth hormone expression and cell survival as a function of electroporation voltage for each of the six cell lines. In each case, there was a sharp voltage dependence, as already

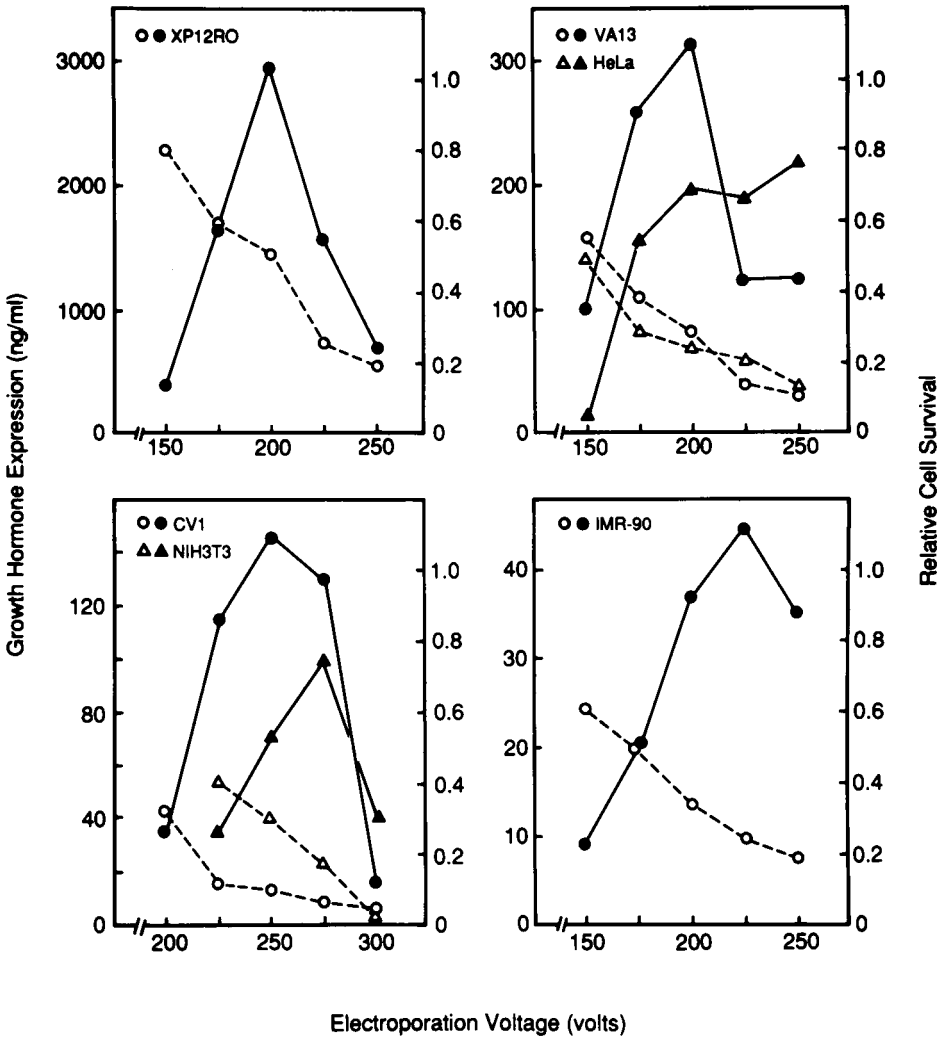


Figure 6. Electroporation for different cell lines.

Cells (3 x 10^6 cells in 1 ml) were electroporated with 20 µg of pMT-hGH-SV2 DNA and 500 µg of salmon sperm DNA for a range of voltages. The solid lines show growth hormone accumulation in the tissue culture medium assayed 72 hours after transfection. The dashed lines show relative cell survival measured 24 hours after transfection.

observed for XP12RO cells. The optimal voltage ranged from 200 to 275 volts, with the value generally varying inversely with the size of the cells in suspension. The differences in growth hormone expression between different

Table I. Comparison of transfection by electroporation and calcium phosphate coprecipitation.

Cell Line	Optimal Voltage (Volts)	Relative Cell Survival	Electroporation EP* (ng/ml hGH)	Calcium Phosphate CP (ng/ml hGH)	EP/CP
CV-1	250	0.10	145	92	1.6
HeLa	250	0.13	220	15	14.7
IMR-90	225	0.24	45	8.3	5.4
NIH3T3	275	0.18	100	13	7.7
VA13	200	0.29	312	78	4.0
XP12RO	200	0.50	2952	69	42.9

Cells were transfected with 20 µg of pMT-hGH-SV2 DNA and assayed for growth hormone expression 72 hours later. Electroporation was done at the optimal voltage with 3 x 10^6 cells and included 500 µg of carrier salmon sperm DNA (see Figure 6). Relative cell survival is shown for reference. Calcium phosphate coprecipitation was done in at least two independent experiments on 85 mm dishes which had been seeded on the previous day with 10^6 cells. Thus, on the day of transfection, approximately equivalent numbers of cells were used for both methods.

*If the results for electroporation are expressed as signal per viable cell, they would be 2 to 10 fold higher than indicated in the table, depending on the cell line.

cell lines do not necessarily reflect differences in transfection efficiency. For example, we found that pRSV-cat DNA produced almost equal CAT activity in XP12RO and VA13 cells, while pMT-hGH-SV2 DNA produced ten fold more growth hormone expression in XP12RO cells than VA13 cells. Comparison of electroporation with the calcium phosphate method is shown in Table I. Electroporation was more efficient for each cell line, with increases in transfection efficiency from 1.6 to 40 fold depending on the cell line. The results in Table I are expressed for a given number of cells transfected and not in terms of viable cells following transfection. If transfection is normalized for cell viability, the increases in efficiency with electroporation would be somewhat greater. For example, in the case of CV-1 cells the improvement would then be approximately 16 fold.

Other electroporation systems have been reported to be more effective when the cells were transfected at 0°C rather than 20°C (18). We therefore transfected CV-1, HeLa, VA13, and XP12RO cells with 20 µg of pMT-hGH-SV2 DNA at 0°C and at 20°C, using 200 volts and 1080 µF, and assayed for growth hormone expression after 72 hours. In each case, efficiency was greater at 20°C, with the improvement ranging from 1.6 to 50 fold, depending on the cell

Table II. Stable transformation efficiency by electroporation.

Cell Type	DNA	Viability	Colonies	Colonies per viable cell
VA13	mock	25%	0	0
	supercoiled	25%	572	2.3%
	linear	25%	552	2.2%
XP12RO	mock	50%	0	0
	supercoiled	50%	152	0.3%
	linear	50%	592	1.2%

XP12RO and VA13 cells were transfected with 20 µg of pRSV-neo DNA and 500 µg of sonicated salmon sperm DNA, plated at a density of 10^5 cells per 85mm dish, grown in G418 starting on day 3, and assessed for transformed colonies on day 20. The plasmid was either supercoiled or linearized at the Bgl I restriction site located outside the neo transcription unit.

line. Our results may differ from earlier results because of the use of different cell lines, or because we use a voltage pulse of lower magnitude and much longer duration.

Stable transformation by electroporation

Stable transformation frequency was assayed by transfecting XP12RO and VA13 cells with pRSV-neo and measuring the number of colonies resistant to G418. The conditions used were: 3 x 10^6 cells in 1 ml 1X HeBS, with 20 µg pRSV-neo and 500 µg salmon sperm DNA, electroporated at 200 volts and 1080 µF. The DNA was either supercoiled or linear (Table II). For supercoiled plasmid, an extremely large number of small colonies appeared by day 10 at a time when the background from untransfected cells was negligible. However, by day 20 many of these colonies had disappeared, leaving a far smaller number of actively growing colonies. The colonies which disappeared probably survived initially by expression of unintegrated plasmid DNA and then died after the DNA was degraded or diluted by cell division, since the plasmid does not replicate autonomously. Almost all of the colonies still present on day 20 continued to grow in G418, presumably because they had integrated the neo marker into the host chromosome.

For linearized plasmid, there were somewhat fewer colonies on day 10. Transient expression of linearized plasmid was 50%-60% that of supercoiled plasmid (data not shown). However, by day 20, the results were markedly different. For VA13 cells, the number of transformants was now equal to that obtained from supercoiled plasmid. For XP12RO cells, the number of

transformants was four fold higher than that from supercoiled plasmid. Therefore, linearization of the plasmid appeared to facilitate stable transformation in XP12RO cells.

DISCUSSION

The transfection of DNA by electroporation was studied for a number of cell lines. Different parameters were studied using both transient expression and stable colony formation to measure the efficiency of transfection.

The electroporation procedure described in this paper is simple, reproducible, and highly efficient. In every cell line studied, electroporation was more efficient than the calcium phosphate method, with increases in transient expression ranging up to 50 fold. A capacitor was charged by a conventional power supply and then discharged through a cell suspension containing DNA, exposing the cells to a pulsed electric field. Transfection efficiency increased with increased plasmid DNA concentration even up to 80 μg/ml of DNA. Sonicated salmon sperm DNA could be used to boost efficiency with lower plasmid DNA concentrations. Linearization of the plasmid resulted in a moderate decrease in transient expression but a large increase in the stable transformation efficiency in XP cells. With pRSV-neo or pRSV-gpt DNA, stable transformation to G418 or mycophenolic acid resistance was obtained in more than 1% of the viable cells with viabilities in the range of 25% to 50%.

Voltage was a critical parameter for electroporation. For a given capacitance and buffer, there was a sharply defined voltage for optimal transfection efficiency. The signal rose with increasing voltage until it was offset by a marked decrease in cell viability. Application of this procedure to other cell lines should include a careful measurement of transfection efficiency over a range of voltages. The local potential difference across the cell is the driving force for pore formation and is proportional to the product of the capacitor voltage and the cell diameter (16). Therefore, the voltage optimum for transfection should depend inversely on cell size.

The stable transformation frequencies reported here for mammalian cells are one to two orders of magnitude higher than previously reported for electroporation applied to a number of different cell lines (16-18,28). The most likely explanation for this large difference is that other methods have generally employed an electric field pulse with strengths in the range of 2 to 10 kilovolts/cm and durations of 0.5 to 5 μsec. By contrast, our best results were obtained with a pulse of about 0.53 kilovolts/cm and durations of 7000

μsec, a somewhat weaker electric field of much longer duration. Indeed, it is possible that pulses of even longer duration or of some other shape could yield even higher transformation frequencies.

Electroporation probably involves a physical interaction between the cell membrane and the applied electric field. Therefore, efficient transfection might be relatively independent of cell type. Others have reported successful electroporation of number of lymphoblastoid cell lines (18,28,29), which have been otherwise resistant to transfection by the calcium phosphate method. Optimal conditions, especially for voltage and DNA topology, should be adjusted for each cell line. If this is done, electroporation should allow highly efficient DNA transfection of a broad range of mammalian cells.

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