Study of mechanisms of electric field-induced DNA transfection IV

Effects of DNA topology on cell uptake and transfection efficiency

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ABSTRACT Electric parameters and solvent conditions are known to influence the efficiency of DNA transfection of cells by a pulsed electric field (PEF). A previous study (Neumann, E., M. Schaefer-Ridder, Y. Wang, and P. H. Hofschneider. 1982. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:841–845) has indicated that DNA topology is also an important determinant. We report an investigation of the PEF induced uptake, stability, and expression of three different topological isomers, circular supercoiled (scDNA), circular relaxed (crDNA), and linearized (lnDNA) forms of the plasmid pBR322, by *Escherichia coli* strain JM105. Monomeric pBR322 prepared by the electroelution from an agarose gel was in the supercoiled form. Treatment of the scDNA with wheat germ topoisomerase I removed the superhelicity and the DNA assumed the relaxed circular form. Treatment of scDNA by a restriction endonuclease, EcoRI or Hind III, linearized the DNA. The $MgCl_2$-dependent bindings of all three forms of DNA to the cell surface were indistinguishable. So was the PEF induced cell uptake. In contrast, the transfection efficiency (TE) for the scDNA and the crDNA were high (approximately 2×10^8 μg^{-1} DNA at neutral pH), whereas that for the lnDNA was approximately five orders of magnitude lower (less than 1×10^3 μg^{-1} DNA). Analysis by agarose gel electrophoresis indicated that the PEF loaded lnDNA was degraded by the host cell within 3 h. However, the loaded scDNA and the crDNA were stable and expressed in the cytoplasm. We conclude that first, the PEF induced DNA entry into *E. coli* did not depend on the topology of the DNA. As cellular uptake of DNA also correlated with the surface binding, these data support electrophoresis of surface bound DNA as the dominating mechanism for the DNA entry. Second, the variations of TE for different topological forms of DNA reflected their relative stability in the host cells. Third, since the loaded DNA could be either rapidly degraded by the host enzyme or expressed, they were unlikely coated with a layer of protective lipid membrane. Thus, PEF induced cellular uptake of DNA is unlikely by the endocytotic mechanisms as was reported previously for the liposomes (Chernomordik, L. V., A. V. Sokolov, and V. G. Budker. 1990. *Biochim. Biophys. Acta.* 1024:179–183).

INTRODUCTION

Electroporation is a convenient method for introducing foreign genes or plasmid DNA into living cells (1–3). The transfection efficiency by electroporation is generally much higher than those by chemical methods and it may soon approach the level useful for gene therapy (4–6). With its versatility and ease of use, there are widespread applications of the electrotransfection method in molecular biology, genetic engineering and biotechnology (1–8). Despite all these developments, it remains unclear how a pulsed electric field (PEF) can facilitate DNA uptake by cells without severely impairing the normal function of the cell membranes. Likewise, little is known about the factors which govern the efficiency of the electrotransfection and the cell recovery after electric shock. Because of this lack of knowledge of the basic chemistry of electrotransfection, consistent results have been difficult to obtain and the success or failure of an experiment relies heavily on luck rather than on ability and experience. Therefore, further improvement in transfection efficiency (TE) to attain the level required for gene therapy is not assured.

Several mechanisms have been discussed. Neumann and co-workers have suggested that cell uptake of DNA is by the electrophoresis of DNA in solution toward the cell membrane and then across the electropores (9–10). This thesis has been tested by Chizmadzhev and co-workers (11) who have shown that the TE is much higher if an electric pulse drives the negatively charged DNA towards the cell on the anode side. TE is reduced by one order of magnitude if the polarity of the field is reversed. They have also shown that increasing the viscosity of the solution reduced TE, consistent with electrophoresis of DNA through the bulk solution before its entrance into the cells. Another interesting observation made by these authors is that the DNA taken up by liposomes using the electroporation method may be enclosed in a shell of lipid and is inaccessible for binding by ethidium bromide (12).

DNA uptake by electroosmosis is also considered a plausible mechanism. Sowers et al. (13, 14) have shown that a hydrodynamic flow towards the cathode is associated with the electroporation event. DNA near the cell membrane which faces the anode may be carried into the cell by this hydrodynamic flow. Klenchin et al. (11) have observed an elevated TE above the control sample when the polarity of a PEF counters the electrophoresis of DNA towards the cells. Electroosmosis is known to be opposite to the direction of the electrophoretic movement of DNA (11).

Our previous studies (15, 16) have shown that at low DNA to cell ratios (<0.5), binding of DNA to the cell surface was a prerequisite for the electrotransfection of *Escherichia coli*. DNA binding to *E. coli* was facilitated by cations, with effectiveness in the order of $Ca^{++} > Mg^{++} \gg Na^+$. It was also found that, DNA added to the

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cells pre-treated with a PEF could also transfect (i.e., transfection in the absence of an electric field), although the TE in this case was low compared to that if DNA was added before the electroporation (11, 15). These results suggest that surface binding followed by the diffusion of DNA through the electropores is a likely mechanism for the electrotransfection of *E. coli*. The molecular weight dependence of TE was also reminiscent of the diffusion of flexible molecules on highly structured solid surface for DNA uptake by the cells (16).

Despite these studies, investigators are confounded with several crucial questions. First, does the TE reflect the efficiency of DNA entry into the cells? Second, how does the TE and DNA uptake depend on the topology of the DNA? Third, how is the TE related to the stability of DNA in the cytoplasm after the cellular uptake? And, fourth, is the PEF loaded DNA enclosed in lipid or membrane vesicles, which protect them from degradation by the host enzyme? The PEF induced transfection of *E. coli* JM105 by the plasmid DNA pBR322 has been used to answer these questions.

MATERIALS AND METHODS

Preparation of ^{3}H-labeled supercoiled plasmid pBR322 DNA

The plasmid (carrying a gene encoding ampicillin-resistance) was loaded into *E. coli* JM105 by electroporation (see below). The cell suspension was then plated on LB-agar solid selective culture medium (LBASSCM) which contained 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 5 g NaCl, 15 g agar, 25 mg streptomycin, and 30 mg ampicillin per liter. After overnight incubation at 37°C, a single colony was used to inoculate 25 ml of LB selective liquid culture medium (LBSLCM) which had the same composition as LBASSCM except the agar was omitted. The sample was incubated at 37°C overnight in a shaker. One ml of the culture was then used to inoculate 500 ml of a special culture medium which contained 7 g K_2HPO_4, 3 g KH_2PO_4, 0.05 g Na-citrate, 0.1 g $MgSO_4$ and 1 g $(NH_4)_2SO_4$ per liter. Immediately, 25 mg streptomycin, 30 mg ampicillin, 10 ml 20% glucose, and 1 ml of a 4 mg ml^{-1} vitamin B1 solution, per liter were added to the culture. The culture was incubated at 37°C in a shaker. At the mid-log phase (specific optical density OD_{600nm} of 0.8–1.0), a 2.5 ml of solution containing 34 mg ml^{-1} chloramphenicol, with or without 1 ml of ^{3}H-thymidine (1 mCi), was added. The culture was further incubated at 37°C for 12–16 h. Cells were harvested and lysed with NaOH and SDS. Plasmid DNA pBR322 (both radioactively labeled and unlabeled) was precipitated by isopropanol and ethanol; purified by ultracentrifugation in a cesium chloride/ethidium bromide density gradient. The monomeric scDNA was isolated by the electroelution; purified by phenol, chloroform and ethanol extraction; and redissolved to appropriate concentrations in a TE buffer (10 mM Tris buffer containing 1 mM EDTA, at pH 7.4).

Preparation of crDNA and lnDNA of plasmid pBR322

lnDNA (^{3}H-labeled or unlabeled) was prepared by the restriction endonuclease treatment of the scDNA. The mixture (400 μl) contained 100 μg scDNA (in 120 μl), 245 μl double-distilled water, 200 units EcoR I (10 μl), 200 units Hind III (10 μl), 15 μl 10× buffer (enzymes and buffer were supplied by Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). The mixture was thoroughly mixed and reacted overnight at 37°C. The sample was then heated at 75°C for 10 min to inactivate the enzymes. The lnDNA was then purified by phenol, chloroform and ethanol extraction and redissolved in a TE buffer to appropriate concentrations.

crDNA was prepared by the treatment to completion of scDNA with wheat germ topoisomerase I. The reaction mixture (400 μl) containing 100 μg scDNA (in 120 μl), 40 μl 10 mM dithiothreitol, 30 units Topo I (3 μl), 40 μl 10× buffer, 80 μl glycerol and 117 μl double distilled water was thoroughly mixed and kept at 37°C overnight. crDNA was then purified by phenol, chloroform and ethanol extraction and redissolved to appropriate concentrations in a TE buffer.

Cell culture

E. coli JM105 was grown in LB liquid culture medium (LBLCM) containing 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 5 g NaCl and 25 mg streptomycin per liter with vigorous shaking at 37°C. The overnight culture grown to stationary phase was diluted 100× with the LBLCM medium and incubation was continued until it reached the mid-log phase (OD_{600nm} of 0.7 to 0.9). Cells were harvested by centrifugation at 4,000 *g* for 10 min at 4°C, and washed twice with the binding and electroporation medium (BEM), which contained 1 mM (or 0.1, 0.3, 3 and 10 mM) $MgCl_2$, 30 mM sucrose, 1 mM Tris buffer at pH 7.4, and resuspended in BEM at a desired cell concentration (usually 1 × 10^{10} ml^{-1}). Cell suspensions were kept on ice before use.

DNA binding experiment

0.1 μg of ^{3}H-labeled pBR322 (scDNA, lnDNA, or crDNA) was added to 500 μl of cell suspension in BEM. After mixing thoroughly, the sample was incubated on ice for 5 min and then centrifuged at 5,000 *g* for 2 min. The radioactivity in the supernatant and the pellet were counted with a Packard Model 1600CA liquid scintillation analyzer (Packard Instrument Co., Inc., Downers Grove, IL). The DNA binding ratio (BR) was defined as the ratio of cpm of the pellet and the total cpm of the sample.

Electroporation and assay for DNA uptake and transfection

The apparatus for electroporation has been described (15). Basically, it consists of a Cober 605P high voltage generator, an electroporation chamber with two platinized platinum electrodes in contact with cell suspension. The PEF (square waves of different field strengths and pulse durations) was directly monitored with a Tektronix Model 5103 storage oscilloscope. The distance between the two electrodes was 0.15 cm. The sample volume was 50 μl. The chamber was maintained at 2°C by circulating water and an PEF did not heat the sample by more than 2°C, as was monitored by a thermistor probe with a time constant of 0.1 s. When a sample was treated with multiple pulses, the time interval between pulses ranged from 3 s to 10 min. After PEF treatment, the sample was immediately transferred into 500 μl of LBSLCM in an eppendorf tube and incubated at the room temperature (23–25°C) for a specified time and then assayed.

For transfection efficiency, the control and the PEF treated samples were incubated at 37°C for 1 h before they were diluted 10× or more with the LBASSCM. Aliquots, each of 100 μl, were plated on LBASSCM. After overnight incubation (>12 h) at 37°C, the number of colonies on each plate were counted. For the assay of cell survival, ampicillin was omitted from the culture. Transfection efficiency (TE) is defined as colonies μg^{-1} DNA.

For the assay of ^{3}H-labeled DNA uptake by cells, surface-bound DNA was first removed by washing the PEF treated cells twice with the LBSLCM, followed by treatment with DNAse I. The radioactivity in the supernatant and pellet (by centrifugation at 5,000 *g* for 2 min) was counted. Transfer ratio (TR) is defined as the ratio of CPM in the cell pellet divided by the total CPM of the sample.

To assay for the plasmid DNA inside the PEF treated *E. coli*, a sample and its control were incubated at 37°C for varying lengths of time (0 min, 10 min, 30 min, 1 h, 3 h and 5 h). Residual surface bound DNA was then removed by washing with buffer followed by the DNAse

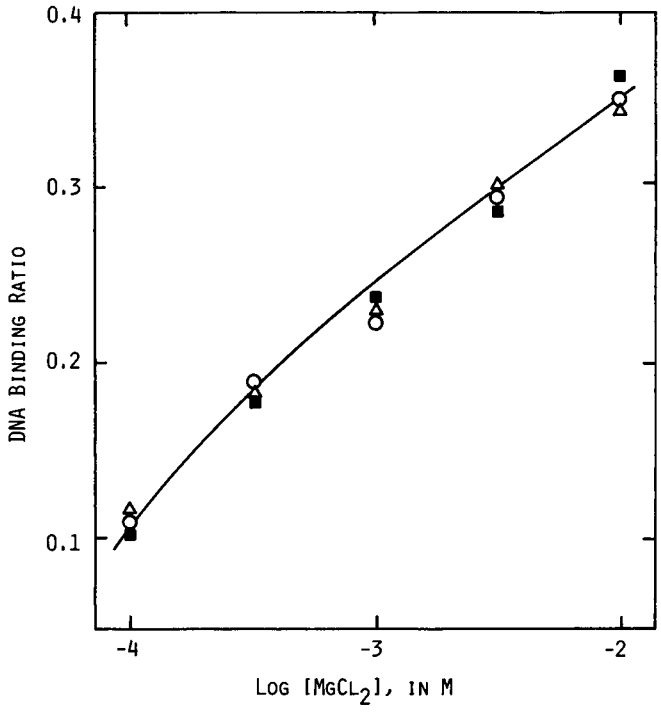

FIGURE 1 $MgCl_2$ facilitated binding of the three topoismers of pBR322 DNA to *E. coli* JM105. Each sample (500 μl), contained 5×10^9 cells in BEM buffer (1 mM Tris buffer at pH 7.4, 30 mM sucrose, and a given concentration of $MgCl_2$), kept on ice, was added 0.1 μg of ^{3}H-labeled pBR322 DNA and thoroughly mixed. After 5 min of incubation, the mixture was centrifuged at 5,000 *g* for 2 min. Radioactivities in the supernatant and the cells were determined separately. DNA transfer ratio is defined as CPMs in the cells divided by the total CPMs of sample. Data in ○ are for scDNA, in ■ for crDNA, and in ▲ for lnDNA.

I treatment, as described above. After centrifugation, the cells were lysed with NaOH and SDS. DNA was isolated and purified by ethanol, phenol and chloroform extraction, followed by a treatment with RNAse to remove RNA. The plasmid DNA was identified by agarose gel (0.8%) electrophoresis with TAE buffer (40 mM Tris-acetate, 1 mM EDTA, at pH 8.0).

RESULTS

Similar surface binding and PEF-induced cell uptake for the three topological isomers of DNA

As has been shown previously, binding of plasmid DNA to the cell surface is essential for PEF induced transfection of *E. coli* and this binding is facilitated by the millimolar concentration of Ca^{++} or Mg^{++}. Fig. 1 presents the DNA binding ratio (DNA bound/total DNA) as a function of the added Mg^{++} for the three topological isomers of the ^{3}H-labeled pBR322 DNA. Each sample contained 0.2 μg ml^{-1} of DNA and 1×10^{10} ml^{-1} of cells. All three forms of DNA (scDNA, crDNA and lnDNA) showed identical binding and similar dependence of binding on the Mg^{++} concentration.

The PEF induced DNA uptake of the three forms of DNA is compared in Fig. 2. A broad range of experimental conditions was tested to see if subtle difference existed in the PEF induced uptake for the three forms of DNA. When the duration of PEF was fixed at 1 ms and only a single pulse was applied to each sample, the logarithm of the DNA transfer ratio (TR) increased monotonically with increasing field strength (Fig. 2 *A*). No difference was observed for the three forms of DNA. Similarly, when the field strength was fixed at 6 kV cm^{-1}, log [TR] increased with increasing pulse width (Fig. 2 *B*). Again, no difference was discernible for the three forms of DNA. In Fig. 2 *C*, log [TR] is plotted against number of pulses applied to each sample, with two conditions (*curve 1* for data using 6 kV cm^{-1}–1 ms pulses and *curve 2* for data using 4 kV cm^{-1}–2 ms pulses). No difference in TR was detected for the three forms of DNA. Varied durations between pulses also did not demonstrate any differences in TR for the three forms of DNA. Fig. 2 *D* shows the results of such an experiment using three pulses of 8 kV cm^{-1}–1 ms PEF.

Reduced transfection efficiency and stability for lnDNA in E. coli

In contrast to the DNA transfer ratio, the TE was found to be several orders of magnitude lower for the lnDNA than those for the crDNA and the scDNA. For an experiment using a single PEF of 1 ms duration, plots of log [TE] versus field strength for the three topoisomers of pBR322 are shown in Fig. 3. Percent cell survival is also shown. At the optimal field strength (~10 kV cm^{-1}), the TE for the scDNA and crDNA were approximately five orders of magnitude higher than the TE for the lnDNA. Since PEF induced DNA uptake by *E. coli* was identical for the topoisomers of DNA, this difference in TE could mean either that lnDNA was unstable in the cytoplasm or that it could not be expressed in the host cells.

The stability of the loaded plasmid was examined by agarose electrophoresis, as shown in Fig. 4. After electrotransfection with a single PEF of 8 kV cm^{-1}–1 ms, samples and control (untreated samples) were incubated for different periods of time and analyzed for the presence of plasmid DNA in the cells by agarose gel electrophoresis. Lane 1 shows the marker genes. Lane 2 is a control sample in which no plasmid DNA was present. Lanes 3, 4, 5, and 6 show the lnDNA in the cytoplasm after 5, 3, 1, and 0 h of incubation, respectively. lnDNA was completely degraded in the cytoplasm 3 h after loading by electroporation. Lanes 7 and 8 are the crDNA, 3 and 0 h after electric loading, respectively. crDNA was found to be stable in the cytoplasm. Lanes 9 and 10 are the scDNA, 3 and 0 h after electric loading, respectively. scDNA was also stable in the cytoplasm.

Effect of molecular size on PEF induced cell uptake

In a previous study (16) we have reported that the efficiency of transfection depends on the molecular weight of the plasmid DNA. An experiment with four scDNAs,

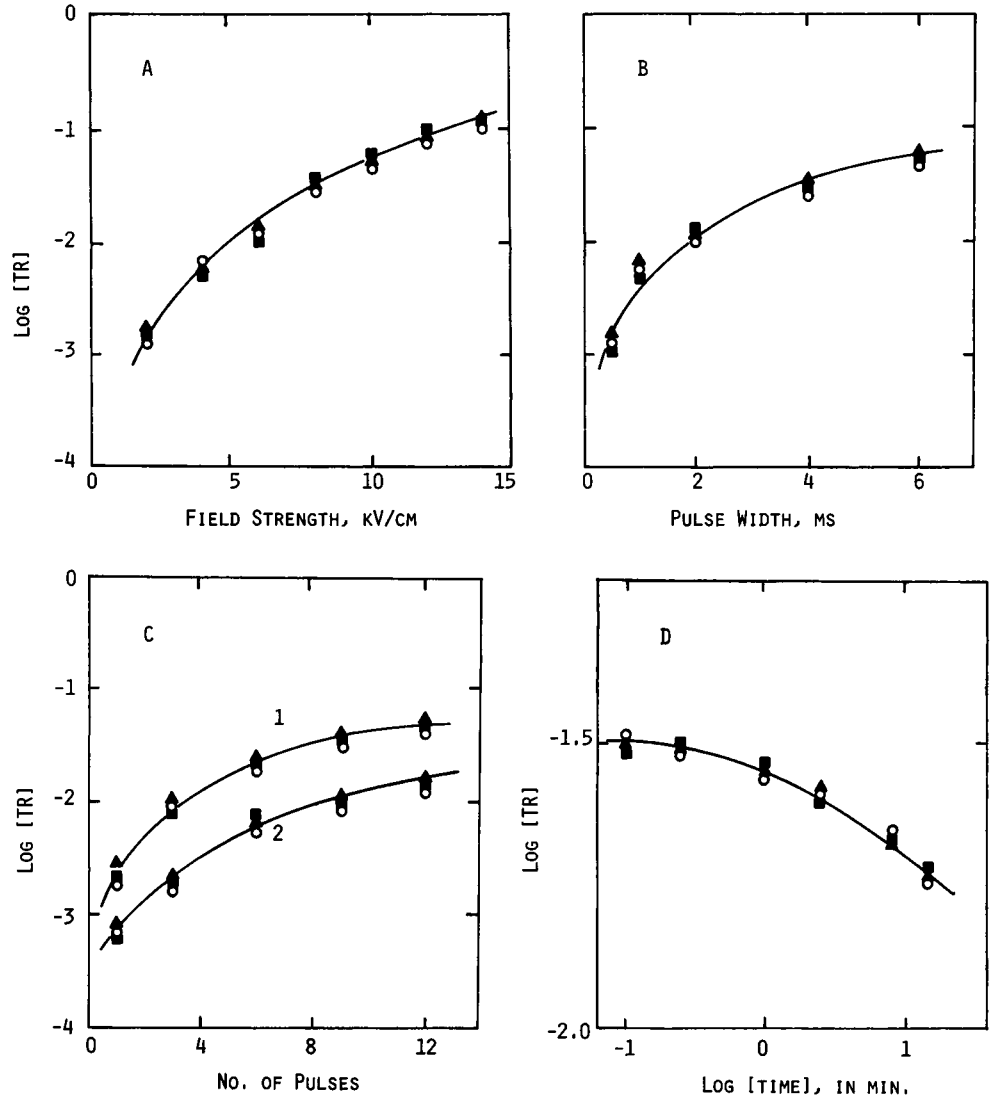

FIGURE 2 PEF induced uptake of pBR322 DNA by *E. coli* JM105. (*A*) Dependence on field intensity. 0.5 μg of ^{3}H-labeled pBR322 was added to 50 μl of cell suspension (1×10^{10} ml^{-1}) in BEM buffer. After incubation on ice for 5 min, a single 1 ms-PEF of varying field strengths was applied. The DNA transfer ratio (TR) was determined as described in Materials and Methods section. Data symbols are the same as those of Fig. 1. (*B*) Effects of electric field duration. A single PEF (8 kV cm^{-1}) of varying durations was applied to 50 μl of cell suspension and the DNA transfer ratio was determined, as in A. (*C*) Effects of multiple electric pulses. Multiple pulses of 6 kV cm^{-1}–1 ms PEF (*curve 1*) or 4 kV cm^{-1}–2 ms PEF (*curve 2*) were applied to each sample and DNA transfer ratio was assayed. Other experimental conditions were the same as those in *A* and *B*. (*D*) Effects of duration between electric pulses. Three pulses of 8 kV cm^{-1}–1 ms PEF were applied to each sample, with the duration between pulses (time) ranging from 0.1 to 10 min. Other experimental conditions were the same as those of *A* and *B*.

pUC18 (2686 base pair, mol wt 1.6×10^6), pUC19 (2686 base pair, mol wt 1.6×10^6), pBR322 (4362 base pair, mol wt 2.2×10^6) and PMSG (7626 base pair, mol wt 4.7×10^6), showed that TE was the highest for the pUC18 and the pUC19, next for the pBR322 and the lowest for the PMSG. PEF dependent cell uptake of these plasmid DNAs was not measured. We have performed a series of experiments similar to those shown in Fig. 2 to compare the Mg^{++} facilitated surface binding and the PEF induced uptake by *E. coli* under various sets of conditions. The results were similar to the data of Fig. 2, namely that, there were no difference in the DNA binding ratio and the transfer ratio for the three different sizes of the plasmid DNAs under a broad range of experimental conditions.

DISCUSSION

PEF induced DNA uptake and transfection of cells

These results demonstrate unequivocally that the PEF induced DNA uptake by *E. coli* was not dependent on DNA topology. Neither was it dependent on the molecular weight in the range 1.6×10^6 to 4.7×10^6. The much lower TE of lnDNA compared to crDNA and scDNA was due to the instability of the lnDNA in the host cell.

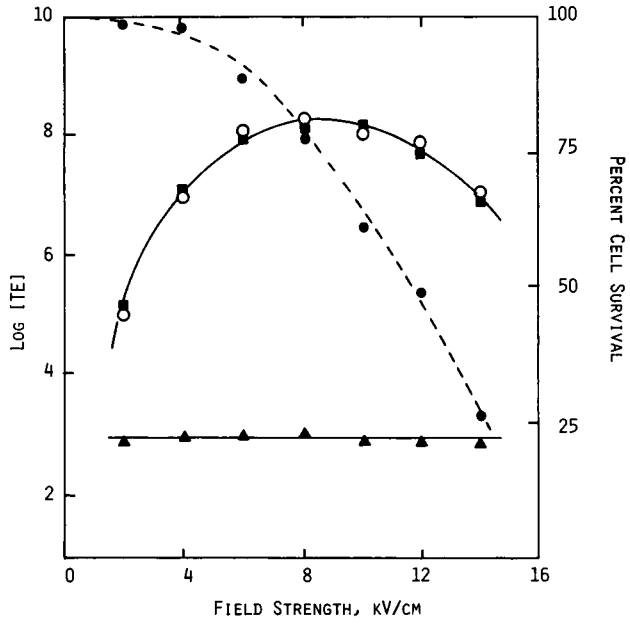

FIGURE 3 Efficiency of PEF induced DNA transfection. Cells (1×10^{10} ml^{-1}) and DNA (0.01 μg ml^{-1}) in BEM were treated with a single PEF of 1 ms duration, with varying field strengths. Data in ○ are for scDNA, in ■ are for crDNA, and in ▲ are for lnDNA. Percent cell survival are also shown in the dashed curve.

lnDNA was rapidly degraded by the host enzyme. In contrast, the crDNA and scDNA were stable and were expressed.

The TE observed for the lnDNA which was low but still higher than the control level (Fig. 3) could have come from the contamination of scDNA and crDNA in the lnDNA preparations. lnDNA prepared by using two restriction enzymes, EcoR I and Hind III was purer than the lnDNA prepared by using EcoR I only. The former retained only 0.001% of the residual scDNA while the latter retained 0.1% of the residual scDNA. The TE for the latter was higher than that for the former (data not shown). These observations indicate that lnDNA has little resistance to the host enzyme and can not express in the host cells. This is contrary to the observation of Neumann and co-workers for the electrotransfection of the mouse L-cell (10). In this case, linearized DNA was more effective for electrotransfection than the circular DNA. However, in this case, loaded DNA must be integrated into the chromosome of the host cell, and the TE could depend both on the stability of the loaded DNA in the cytoplasm and on the efficiency of its integration into the chromosome. It remains unclear why the higher molecular weight scDNA (PMSG) had a lower TE than the TE of the lower molecular weight scDNA (pUC18 and pUC19) (16) despite their equal efficiency in cell uptake.

Different mechanisms for DNA uptake

Our results indicate that DNA transfer ratio rather than the TE should be used for formulating mechanisms of PEF induced DNA uptake by cells. The TE can depend on many factors which are not related to the electric loading of DNA. Several of these factors which are relevant to the PEF induced DNA uptake by cells are considered. (*a*) Electrophoresis of DNA across the cell membrane. The results presented here support the electrophoresis mechanism as the main contribution to the PEF induced DNA uptake by cells (~90%). However, electrophoresis of DNA from the bulk solution would be ineffective for an electric pulse shorter than 1 ms because the distance of DNA electrophoresis would be small. Our previous data show that DNA binding to the cell surface is essential. Thus, electrophoresis of surface bound DNA would be the most plausible mechanism for the electrotransfection of cells. The reduced TE on a viscous medium does not necessarily mean that DNA must diffuse through the bulk solution. The viscosity additives could equally interfere with surface diffusion or DNA entrance across the cell membrane. (*b*) Diffusion of surface bound DNA across the electropores. This mechanism is supported by the observation that DNA added after the electroporation can also transfect cells (11, 15). The low TE in this case could mean either that the electropores rapidly shrank in size or that the contribution via this pathway is minor. The contribution to the overall TE through this pathway is small, in the range of a few per-

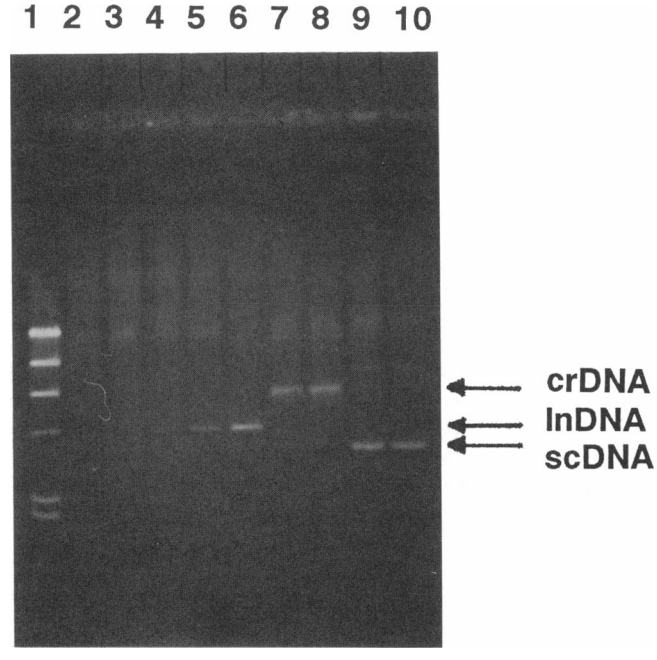

FIGURE 4 Identification of PEF loaded pBR322 DNA in *E. coli* by the agarose gel electrophoresis. Cells and DNA in BEM were treated with a single 8 kV cm^{-1}–1 ms PEF at 2°C. The sample was then incubated at 37°C for a given period of time. Cells were washed, collected, lysed, and DNA extracted. DNA was run in 0.8% agarose gel, as described in the Methods. Lane 1 shows marker genes; lane 2, a control sample (without PEF loading of pBR322 DNA), lanes 3–6, lnDNA incubated in cells for 5, 3, 1, and 0 h, respectively; lanes 7 and 8, crDNA incubated in cells for 3 and 0 h, respectively; lanes 9 and 10, scDNA incubated in cells for 3 and 0 h, respectively. lnDNA was unstable in the cytoplasm but crDNA and scDNA were stable.

cent (11, 15). (*c*) DNA entry by electroosmosis. Klenchin et al. (11) have shown that even if the polarity of an electric field was to drive DNA away from the cells, the efficiency of the field induced transfection was still much higher than that of the control sample. Since hydrodynamic flow induced by the electroosmosis is opposite to electrophoretic movement of DNA, the transfection in this case could have come from both the electroosmosis and the surface diffusion of DNA across the cell membrane. Electroosmosis should become more important when PEF's of longer duration are used (14). Contribution via electroosmosis of DNA is estimated to be 10% from the data of Klenchin et al. (11). (*d*) DNA entry by endocytotic mechanisms. An PEF can induce many morphological changes in cell membranes, one of which appears to be the endocytosis of the plasma membrane. Chernomordik et al. (12) have shown that in liposomes, DNA taken up after electroporation was inaccessible to binding by ethidium bromide. They have suggested that endocytosis of lipid membrane could be involved in DNA uptake. In such a case, DNA uptake would not depend on the topology of the DNA. Neither would the endocytotic mechanism be sensitive to the size of DNA unless the difference in size were great. While, our results can not unequivocally rule out endocytotic mechanisms for DNA uptake, we do not favor such a mechanism. The lnDNA taken up by the cells was accessible to the host enzyme for rapid degradation. Thus, the loaded DNA was not protected by lipid or membrane enclosure. It remains possible that a small fraction of DNA may enter the cells by endocytosis. Quantitative analysis of contributions due to these four different effects of a PEF remain to be done.

One should mention that in experiments involving bipolar oscillating field of high frequencies, diffusion of DNA through the bulk solution can be ruled out because DNA can not travel far before the polarity of the field is reversed. However, bipolar PEF has been shown to be more efficient for electrotransfection than unipolar PEF (17, 18). If DNA is surface bound, an oscillating field should greatly enhance the efficiency of transfection. Low amplitude bipolar electric fields have also been shown to induce DNA transfection (19). Since, the field strength was insufficient to cause electroporation of cell membranes in this case, electroconformational changes of membrane proteins or lipids have been invoked to interpret the results (19–21).

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