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Long duration electroporation for achieving high level expression of glucocorticoid receptors in mammalian cell lines

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Abstract

A method is presented that utilizes long duration electroporation (LDE) to more efficiently introduce DNA into mammalian cell lines than standard electroporation techniques. With SV40-based vectors, more than 550,000 glucocorticoid receptors (GRs) per cell could be obtained in COS-7 cells with good cell survival. In experiments with a CMV-driven vector expressing an enhanced Green Fluorescent Protein (EGFP), 54% of the cells were transfected, and 77% of EGFP positive cells expressed EGFP at moderate to high levels. In cell lines not containing the large T antigen, a CMV-driven vector for the GR was superior to the SV40-based vector. In EDR3, DG44, and CV-1 cell lines approximately 220,000, 190,000 and 150,000 GRs/cell were obtained, respectively. Transfection efficiency of the EGFP vector ranged from 44 to 55% for the three cell lines. Cortisol treatment of COS-7 and DG44 cultures cotransfected with vectors expressing the GR and a GRE driven luciferase gene produced 4 to 12 times more enzyme activity per plate with LDE than conventional electroporation protocols. LDE allows transient overexpression of proteins in COS-7 cells at the high levels generally achieved by mammalian overexpression systems only in stable cell lines.

1. Introduction

Electroporation is a popular, powerful, and efficient method to introduce genes into mammalian cells. It involves the application of a brief electrical field to a suspension of cells in the presence of DNA. The mechanisms at work during electroporation are not thoroughly understood but appear to involve some aspect of membrane streaming that momentarily forms holes or ''pores'' that allows the DNA to enter the cell [1]. The electrical field is usually applied by the discharge of a capacitor through the cell suspension. This results in a waveform with a rapid rise time and exponentially decaying tail. Typical field strengths for mammalian cells range from 550 to 750 V/cm (equivalent to 220–300 V in a 0.4 cm cuvette). The duration of the electrical field is measured by the time constant given by the peak width of the waveform at a value of 1/e ($\sim$1/3) of the maximum voltage. Typical values for a 0.4 cm cuvette are 25–40 ms, depending on the volume and density of the cell suspension and the resistance of the medium.

LDE uses lower field strengths (440–500 V/cm) but much higher time constants ($\sim$140 ms). The extended duration of the electrical field in LDE may allow the pores to remain open longer than in conventional methods, presumably letting more DNA into the cell and resulting in higher expression levels.

To maximize DNA uptake, the electrical field must be optimized for voltage and duration, in conjunction with DNA concentration, cell number, temperature, and buffer composition. Recent attempts to reduce the number of experiments needed for parameter optimization have resulted in universal protocols [2]. These protocols are very convenient but in our hands give less than optimal reporter gene expression. The method presented here requires two optimization experiments to obtain high expression: one to determine the optimal voltage, and a second to determine the optimal DNA concentration.

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2. Materials and methods

2.1. Cell lines and culture

EDR3, a generous gift from Dr. Gary Firestone, is a minimal hepatoma cell line derived from the Fu5 cell line [3]. It contains no detectable GR. CV-1 and COS-7 cells are African green monkey kidney fibroblast lines containing ~10,000 GRs that are transcriptionaly inactive. They were obtained from the American Type Culture Collection (Rockville, MD). COS-7 cells are derived from CV-1 and contain the large T antigen which allows replication of vectors based on the SV40 origins of replication. DG44 cells, a generous gift from Dr. Larry Chasin, is a Chinese hamster ovary epithelial cell line that has no endogenous dihydrofolate reductase [4] and contain ~10,000 GRs/cell that appear to be functional. All cell lines were grown at 37°C with 5% CO_2. COS-7 and CV-1 cells were grown in DMEM (4.5 g glucose/l) supplemented with 39.5 mg proline/l. EDR-3 cells were grown in DMEM/Hams F12 (1/1) and DG-44 cells in alpha MEM plus nucleosides (Life Technologies, Grand Island, NY). Iron-fortified calf serum (Sigma, St. Louis, MO, 10%) was used for all lines and was charcoal stripped twice [5] for electroporation experiments

2.2. Plasmids

The plasmids pSV2 WT2X and pCMV5mGR express the mouse GR. PSV2 WT2X was constructed from pSV2WREC [6] by excising a ~200 base pair fragment from the 3′ untranslated region with *Xba*I followed by religation of the vector. PCMV5mGR was a generous gift from Dr. Chris Jewel and Dr. John Cidlowski. It was constructed by cloning the mouse GR from pSV2WREC into the pCMV5 vector (obtained from Dr. David Russell) utilizing the *Bgl*II and *Xba*I restriction sites. PXP2 G2T-JCT was made by excising the region containing the tandem GREs and TATA region from GRE2-CAT [7] with *Xho*I and *Bam*HI and inserting into the *Sal*I and *Bgl*II sites in PXP2 [8].The vector pEGFP-N1 (Clontech, Palo Alto, CA) expresses an enhanced green fluorescent protein (EGFP) better suited than wild type GFP for excitation at 488 nm.

2.3. Long duration electroporation (LDE) protocol

One of the most critical parameters in the procedure is that cells should be in log phase growth and less than 70% confluent in culture. The day before an experiment, cells growing in 175 cm^2 flasks, are split 1 : 2. Prior to the start of an experiment, medium containing 10% 2× charcoal stripped serum (10 ml/10 cm plate) is dispensed to 100 ml serum bottles and gassed with 5% CO_2. Cells are harvested with 2 ml of a 2× trypsin-EDTA solution (Sigma, St. Louis, MO, 10× stock diluted 5-fold) for 10 min at 37°C, then collected in medium by centrifugation (3 min, 700×*g*, ~23°C). Cells are resuspended with 9 ml of PERM buffer (10 mM PIPES, pH 7.4; 137 mM NaCl; 5.6 mM glucose; 2.7 mM KCl; 2.7 mM EGTA, 1 mM Na-ATP; [9]). The cell volume is the total volume read from the pipet minus 9 ml. An additional 25 ml of buffer is added and the suspension repipeted five times. Cell numbers were determined on a model ZF Coulter Counter (Hialeah, FI). After centrifugation the supernatant is aspirated and the cells resuspended in PERM to a final concentration of 3.33 to 10×10^7 cells per ml of suspension. The cell suspension is allowed to cool on ice for 15 min. Cuvettes (0.4 cm gap, BTX or BioRad) are placed on ice at the start of (or before) the cell cooling period. During the cell cooling period the appropriate amount (Fig. 1A) of DNA (at 1 μg/μl in 1 mM EDTA; 10 mM TRIS, pH 8, buffer) is brought up to 50 μl with HBS (20 mM HEPES, pH 7.05; 142 mM NaCl; 5.4 mM KCl; 1.3 mM Na_2HPO_4; 6 mM Glucose [1]) and kept on ice. Cells (300 μl) are mixed thoroughly but gently and transferred to a cold cuvette. Accurate transfer of the cells is important for reproducible results, as the method is sensitive to small changes in volume. After all of the cells are distributed, DNA is added to the cuvette and gently repipeted 10 times. The sides of the cuvette are carefully wiped to remove ice and moisture. Cells are then electroporated and the cuvette is placed back on ice. After the voltage and time constant are recorded, the cells are gently resuspended with 1 ml of medium and transferred using a sterile plastic transfer pipet to bottles containing aliquoted medium. After all of the cells are electroporated they are distributed to 10 cm tissue culture petri dishes. After 18–24 h cells are analyzed for GR or EGFP.

2.4. LDE electroporation settings

A BTX ECM 600 electroporation system (BTX, a division of Genetronics Inc., San Diego, CA) was used for these studies. It is important that the electroporator is able to deliver high capacitance (3150 μF) with small increments of adjustment (25 μF) in order to maintain time constants in the 140 ms range. The number 10 timing resistor (720 Ω) was used for all cell types tested. Optimal voltage depended on the cell type and was determined from pilot experiments (not shown). Briefly, cells were electroporated with 15 μg of pSV2WREC following the above protocol. Capacitance was adjusted to produce a time constant of 135–140 ms at 170 V. The voltage was varied between 150 and 210 and GR levels determined. Optimal voltage was 175 V for COS-7, 185 V for CV-1

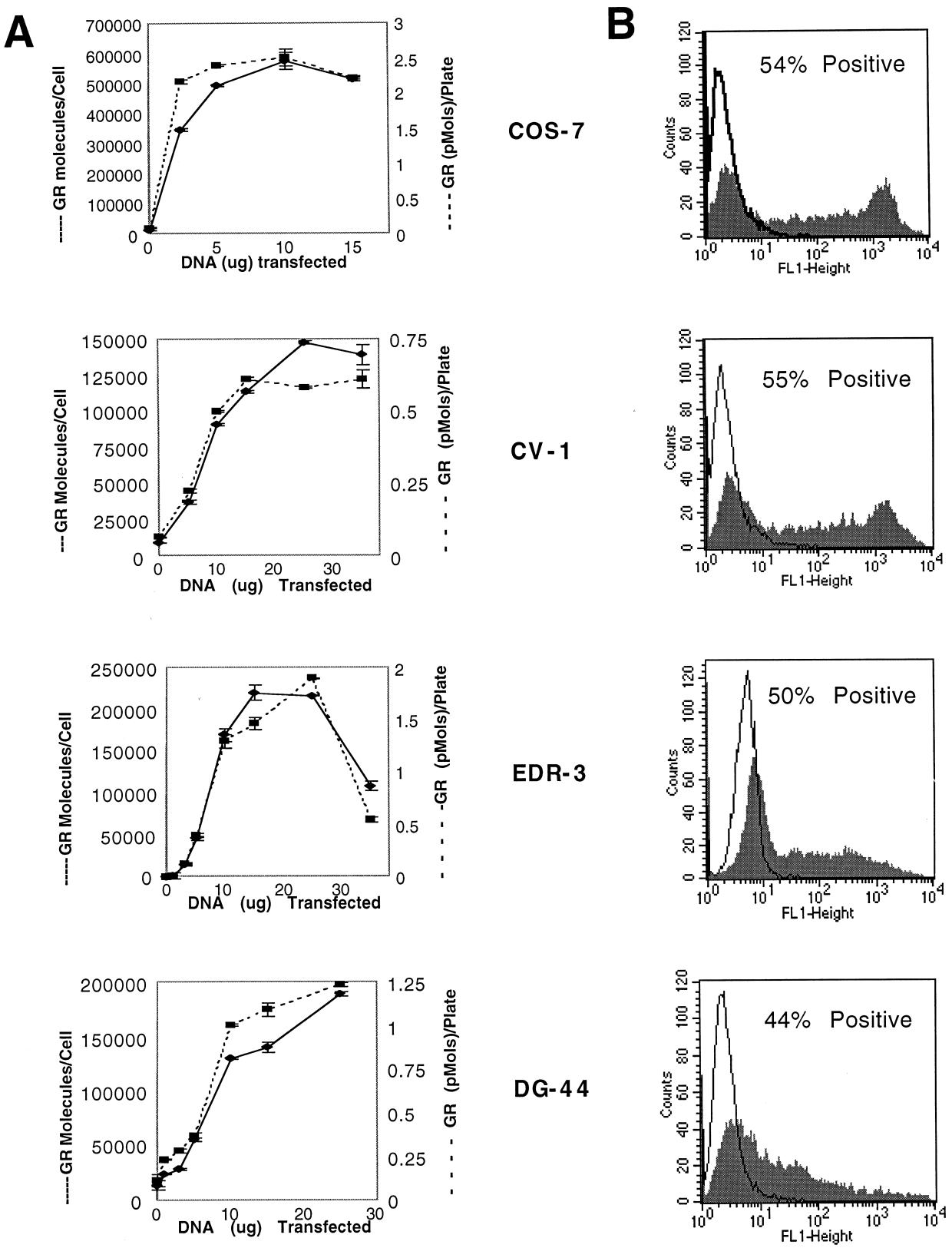

Fig. 1. DNA response curves and EGFP analysis of transfected cell lines. (A) Cell lines were transfected with increasing concentrations of pSV2WREC (COS-7) or pCMV5mGR DNA (CV-1, EDR-3, and DG-44). After 18–24 h cells were assayed for GR content as described in the methods section. Solid lines represent molecules of GR per cell and dashed lines represent the total number of pmol of GR per plate. A representative experiment is shown for each cell line. Data points represent the mean of duplicates; error bars are one half of the range. (B) Cells were transfected with an optimal amount (determined from curves in (A)) of pEGFP-N1 and fluorescence analysis determine by flow cytometry 18–24 h later. Black tracing represents control cells and filled tracing represents EGFP-transfected cells. The insert in each graph represents the percent of EGFP-transfected cells with fluorescence greater than the second log decade. See text for details.

and 200 V for EDR3 and DG44. Actual capacitance values depend on the cell type and concentration. Typical values for 2×10^7 cells were: 2500 μF for COS-7, 2850 μF for CV-1, and 3150 μF for EDR3 and DG44. Cos 7 and CV-1 cells are very large and changes in cell number greatly alters the resistance of the cells suspension. For these cells, a plot of cell number versus the resistance, *R*, proved useful for predetermining capacitance settings. From a trial electroporation *R* can be calculated from the equation (BTX electroporator manual): time constant (ms) = capacitance (F)$\times R$. Once *R* is determined for a particular cell concentration it can then be used with the equation to determine the proper capacitance.

2.5. Alternate electroporation protocols

LDE was compared to two published methods (Fig. 2). Parameters for the HBS method [1] were: 1.1×10^7 COS-7 or 2.1×10^7 DG44 cells (all three methods electroporated the same number of cells) in 500 μl of HBS, DNA in 50 μl of HBS (all reagents on ice), 260 V (280 V for DG44 cells), 975 μF capacitance, and a timing resistance of 129 Ω. Parameters for the optimized method [2] were: cells in 400 μl of DMEM medium with 10% charcoal stripped calf serum, DNA in 50 μl of HBS (all reagents at ~23°C), 260 V (280 V for DG44 cells), 1050 μF capacitance, and a timing resistance of 129 Ω. Optimal amounts of DNA were used

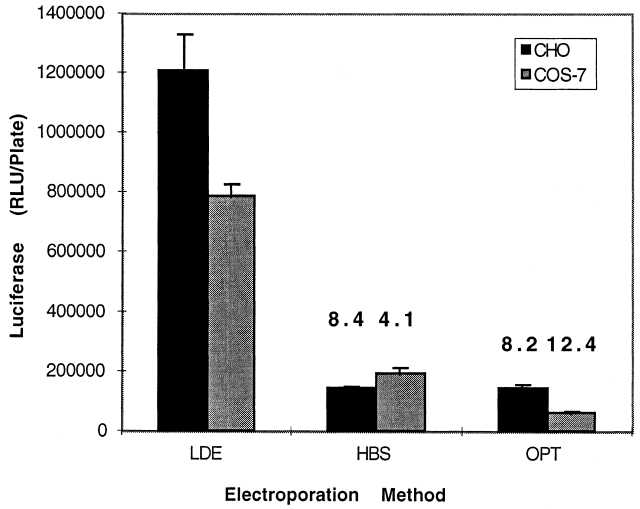

Fig. 2. Comparison of LDE to other electroporation protocols. COS-7 cells (stippled bars) or DG44 cells (black bars) were cotransfected with a GR expression vector and a luciferase reporter gene driven by tandem GREs. Cells were treated at ~24 h post transfection with 100 nM cortisol for 20 h and the luciferase activity per culture plate was determined. See material and methods section for experimental details. Data points represent the mean of duplicates; error bars are one half of the range. Numbers (in bold type) represent the ratio of LDE luciferase activity to that of the compared method.

for each method. The DNA composition for the COS-7 cell comparison was 7.5 μg of the pSV2-WT2X vector and 7.5 μg of PXP2 G2T-JCT for LDE, 10 μg of each vector was used for the two other methods. For the DG44 comparison, 8 μg of pCMV5mGR and 12 μg of PXP2 G2T-JCT were used for each method.

2.6. Luciferase and protein assays

Transfected cells in cell culture dishes were rinsed with 2×10 ml phosphate buffered saline with 5 mM glucose (PBS-G) briefly at room temperature. Plates containing cells were chilled on ice for 5–10 min, then cells were scraped from plates in 1.5 ml PBS-G. After pelleting (3 min, $700 \times g$), cells were lysed in 220 μl freeze–thaw buffer, (0.025 M TES, 0.02 M $NaMO_4$, 0.05 M NaF, 10% glycerol, 0.002 M EDTA, 0.002 M EGTA) containing 5 mM chaps((3-([(3-chloamidopropyl)dimethyammonio]-1-propanesulfonate) Sigma) for 5 min at 4°C. Cell lysates were centrifuged for 10 min, $12{,}000 \times g$. 200 μl of the cell lysate was transferred to a new tube and samples were assayed for luciferase activity on a Berthold microplate luminometer (model LB96V). Luciferase buffer (150 μl; 30 mM Hepes pH 7.8, 30 mM $MgSO_4$, 5 mM ATP pH 7.0, 2 μM pyrophosphate) was injected into 50 μl of cell lysate and the reaction started 7 s later by injection of D-luciferin potassium salt (50 μl, 1 mM in water) into the cell lysate-buffer mixture. Light emission (Relative light units-RLU) was measured in integration mode for 10 s/sample after a 2 s delay. Luciferase standards (Analytical Luminescence Laboratory) were included (2.5–20 ng) for each experiment to ensure the assay was linear. A_{260} and A_{280} measurements were obtained with a Beckman DU-64 spectrophotometer. Cell lysate protein concentrations were determined using the following formula: $[((A_{280} \times 1399) + (A_{260} \times 699)) \times \text{dilution factor}]/1000 = \text{mg/ml}$. Duplicate samples were measured for all luciferase and protein assays. RLU per plate was determined by multiplying the RLU/MG times the total mg of protein recovered from the plate.

2.7. GR binding assay

Cellular GR content was determined by a whole cell binding assay as previously described [10]. Total GRs/Plate was determined by multiplying the GRs/cell by the total number of cells recovered from the plate.

2.8. EGFP fluorescence analysis

Cells were collected by trypsinization as described above, resuspended in PBS at ~10^6 cells/ml, and kept on ice until FACS analysis. EGFP was analyzed on a Becton Dickinson FACScan flow cytometer, using 488

nm excitation from an argon ion laser and measuring fluorescence at 530 nm (30 nm band width) with standard fluorescein filters.

3. Results and discussion

Prior to analyzing the effectiveness of LDE, the voltage optimum was determined for each of the four cell lines as described in the methods section. A DNA response curve was then determined for each cell type at the optimal voltage. COS-7 cells were transfected with the vector pSV2 WREC. This vector contains the SV40 early promoter and origins of replication and in the context of the large T antigen present in COS-7 cells allows plasmid replication. As a result, average GR expression level in COS-7 cells is very high, ~550,000 molecules/cell (Fig. 1, Panel A, top graph). Other experiments yielded levels up to 750,000 GRs/cell (data not shown). Both the maximum number of GRs/cell and of GRs /plate occurred at 10 μg of DNA per transfection. The cell lines CV-1, EDR-3 and DG-44 do not contain the large T antigen. In these lines the pCMV5mGR vector expressed 2–5× as many GRs/cell (data not shown) as the pSV2 WREC vector. Their DNA response curves are displayed in the bottom three graphs of Fig. 1, Panel A. Maximal values of 150,000, 220,000 and 190,000 GRs/cells were obtained for CV-1, EDR-3, and DG-44 respectively. Interestingly, the maximal values occurred at a DNA concentration of around 25 μg, which is more than twice that needed for maximal expression in COS-7 cells (subsequent experiments showed that 25 μg is optimal for DG-44).

For all of the cell lines, the concentration of DNA that gave the most GRs/cell also gave optimal or near optimal GRs/plate. Note that the total GRs/plate are not comparable between the different cell lines as cells were plated at different densities depending on the needs of the experiment.

The number of GRs per cell obtained for the four cell lines are average values that include cells that did not take up DNA. In order to determine the distribution of positively transfected cells, we used the CMV driven vector pEGFP-N1, which expresses an engineered green fluorescent protein (EGFP).

Cells were transfected with pEGFP-N1 at the optimal voltage and DNA concentration (10 μg for COS-7 cells and 25 μg for the other cell lines). After 18–24 h cells were analyzed for EGFP. The flow cytometer was adjusted so that ~1% of control cells (transfected with no DNA) had fluorescence into the second log decade (i.e. 10^1). Fig. 1B displays the control cells with a black tracing and the EGFP transfected cells with a filled tracing, fluorescence is on the *x*-axis (FL1-height), and the number of cells at a particular fluorescence on the *y*-axis (counts). Clearly, all of the cell lines transfected with the EGFP vector shown substantial shift to the right. If we consider anything above the weak control cell fluorescence to be EGFP positive, then 54% of COS-7, 55% of CV-1, 50% of EDR-3 and 44% of DG-44 cells were positive for EGFP. There are also differences in the distribution of the EGFP in the different cell types. COS-7 and CV-1 have a much higher proportion of brighter cells (77 and 72% of positive cells were in the 3rd and 4th log decade, respectively) than EDR-3 and DG-44 (46 and 43% respectively). The DG-44 line seems to have a significant number of cells that, while not measuring above the second log decade, are clearly brighter than control cells, suggesting a low level of expression. This may account for the apparent lower transfection efficiency relative to the other lines.

Assuming that the transfection efficiencies are the same for GR and EGFP vectors, then approximately half the cells express transfected GRs. That means that the actual average number of GRs per cell in GR-positive cells is about twice the values shown in Fig. 1A which for COS-7 cells would be close to a million GRs/cell.

LDE was compared to two published electroporation protocols. One method electroporated cells in HBS at 0°C [1] while the other method utilized used complete culture medium at 23°C [2]. The three methods were used to cotransfect COS-7 or DG44 cells with a GR expression vector and a luciferase reporter gene (PXP2 G2T-JCT) driven by tandem GREs. As shown in Fig. 2, cortisol (100 nM) treatment of these cultures resulted in significantly more luciferase activity per plate (4.1 to 12.4 fold) with LDE. Likewise, the luciferase activity per mg of cellular protein was also enhanced (3.6 to 18.6 fold) with LDE (data not shown).

In cotransfection studies where the relative amount of GR DNA is diluted by multiple DNA partners, the greater efficiency of LDE makes it possible to work with a larger range of GR. LDE should also be advantageous in situations where it is desirable to work with greater expression of partner DNAs or where multiple partners are needed to produce an effect. At the very high levels of GR attainable in COS-7 cells when only GR DNA is transfected, squelching effects might be observed. However in cotransfection experiments where the reporter gene must be kept in excess to avoid being limiting, the amount of GR DNA that can be used is restricted. Maximum GR levels in this situation is $2–4\times10^5$ molecules/cell for COS-7 cells. Although we have not done experiments to study squelching per say, at these GR levels luciferase activity is still increasing (data not shown).

LDE has consistently given significantly enhanced

expression of GR over other electroporation protocols in all cells tested to date.

In summary, when this transient transfection method is used with an appropriate vector and COS-7 cells, it is capable of producing high level protein expression similar to that seen with stably transfected mammalian overexpression systems.

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