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High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment

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ABSTRACT

Using a plasmid containing the bacterial chloramphenicol acetyl transferase gene, we have assayed for transient expression of DNA introduced into mouse L cells by a variety of transfection conditions. High efficiency uptake and expression of this foreign DNA have been achieved by modifying the DEAE dextran mediated transfection procedure of McCutchan and Pagano (1) to include a shock with either dimethyl sulfoxide or glycerol. Inclusion of the shock step can increase expression of the transfected gene a surprising ~50 fold. With plasmid constructs that do not replicate after transfection, we can readily detect CAT activity in an overnight autoradiographic exposure from less than 0.1% of an extract from a 60 mm dish of transfected cells. We have determined the amounts of DNA, the amount and time course of DEAE-dextran and dimethyl sulfoxide treatments, the effects of additional DNA, and the time after transfection which yield maximal expression. Overall, this transfection protocol using DEAE-dextran coupled to a shock treatment is simple, straightforward, and gives consistently high levels of expression of the input DNA.

INTRODUCTION

Introduction of exogenous purified DNA into eukaryotic cells by transfection allows the study of gene expression in molecular detail. This analysis may be accomplished through transient expression of transfected DNA in the absence of selection or alternatively by long term expression, following selection of cell colonies in which the transfected DNA is either integrated into the host chromosomes (2) or contained on a replicating, non-integrating bovine papilloma virus vector (3,4). Transient expression offers several substantial advantages over either long term expression protocol. In particular, it obviates the time consuming procedure of isolating cloned cell lines containing the transfected DNA. It also obviates the clonal variability introduced by integration of transfected DNA, where the level of expression in each resultant cloned line is highly dependent on the location and the number of the integrations.

Currently, two basic protocols for transiently transfecting cells are

widely used, the DEAE-dextran method of McCutchan and Pagano (1) and the calcium phosphate precipitation method of Graham and van der Eb (5). More recent modifications of the calcium phosphate method have allowed increased levels of expression by the introduction of a dimethyl sulfoxide (6,7) or glycerol (8) shock step to induce DNA uptake. On the other hand, the potential difficulty of obtaining reproducible calcium phosphate precipitates (see 7,9) has persuaded many investigators to turn instead to the technically simpler DEAE-dextran protocol. In the present report, we have found that surprisingly high levels of expression of transfected DNA can be obtained by combining these methods to include a dimethyl sulfoxide (DMSO) or glycerol shock step in a DEAE-dextran mediated transfection.

MATERIALS AND METHODS

The transfection protocol

The transient transfection protocol utilizes the DEAE-dextran method of McCutchan and Pagano (1) as modified by Danna and Sompayrac (10) in combination with a DMSO (6,7) or glycerol (8) shock treatment. This approach was inspired by Sussman and Milman (personal communication; 13) who have successfully obtained a high fraction of transfected cells using a qualitatively similar protocol which includes an additional CO_2 shock step for optimal results. However, the specific transfection conditions that provide us with maximal levels of transient expression differ from those of Sussman and Milman and we further find that higher levels of expression are not obtained by inclusion of the somewhat inconvenient CO_2 shock step.

For our transfections, mouse L cells (kindly provided by G. Khoury) were seeded into 60 mm dishes at 6 x 10^5 cells/dish and grown overnight in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. After washing in 2.5 mls of DMEM without serum, the medium was replaced with 1.5 mls of DMEM without serum but containing supercoiled pSV2-CAT plasmid DNA (11, generously provided by B. Howard) complexed to DEAE-dextran (M_r = ~500,000 from Pharmacia). Maximal levels of expression were obtained with 8-16 µg/ml pSV2-CAT DNA and 200µg/ml DEAE-dextran. After 4 to 20 hours in a 5% CO_2 incubator, the transfection solution was removed by aspiration and replaced with 1 ml of HBS (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose, and 21 mM Hepes, pH 7.1) containing 10% DMSO. Following this DMSO shock (generally 2 minutes duration) at room temperature, the cells were washed with 2 mls of phosphate buffered saline (PBS) and then incubated for times up to 72 hours in a 5% CO_2 37°C incubator

in 5 mls of DMEM containing 10% fetal calf serum.

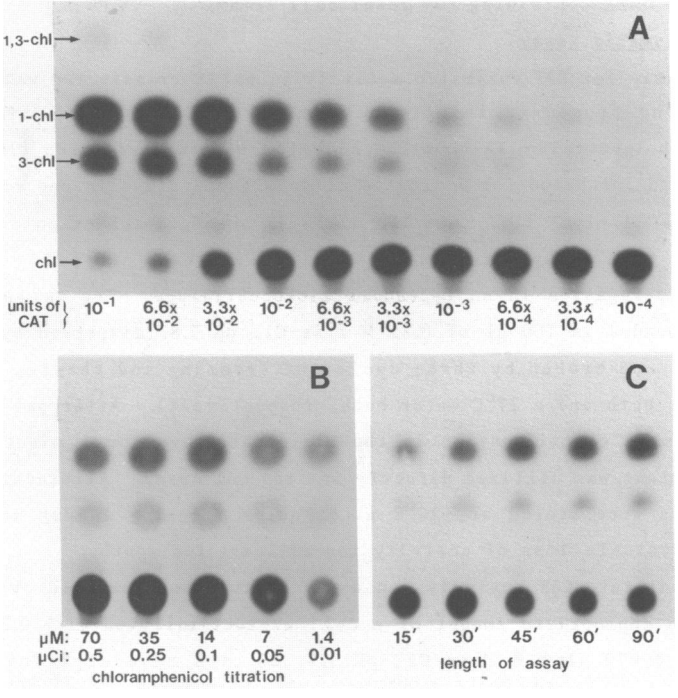
The CAT enzymatic assay

The assay for CAT enzymatic activity in cells transfected with pSV2-CAT followed the procedure in Gorman et al (11). At various times post-transfection (generally 72 hours), the cells were washed three times with 2 ml of PBS and incubated for 5 minutes at room temperature in one ml of 40 mM Tris-Cl, pH 7.4, 1 mM EDTA, and 150 mM NaCl. Using a rubber policeman, the cells were then scraped into a 1.5 ml microfuge tube and pelleted by a 2 minute centrifugation in an Eppendorf microcentrifuge. The pelleted cells were resuspended in 100 µl of 0.25 M Tris-Cl, pH 7.8, dispersed by vigorous vortexing, and broken by three cycles of freezing and thawing (in a dry ice/ethanol bath and a 37°C water bath, respectively). After pelleting the cell debris by centrifugation in the microcentrifuge for 5 minutes at 4°C the supernatant was utilized directly for the CAT assay. Alternatively, the supernatants were stored at -20°C after quick freezing in dry ice/ethanol without detectable loss of activity for at least two months.

To quantitate CAT activity, an assay mixture was prepared by combining 55 µl of an appropriate amount of a cell extract (diluted with 0.25 M Tris-Cl, pH 7.8), 70 µl 1 M Tris-Cl, pH 7.8, 20 µl 4 mM acetyl CoA (freshly prepared), and 1 µl ^{14}C-chloramphenicol (NEN: 46.9 mCi/mmol, 0.1 mCi/ml). This mixture was incubated at 37°C for various times (usually 60 minutes). The ^{14}C-chloramphenicol and its acetylated derivatives were then extracted into 1 ml of ethyl acetate and the organic phase was collected and evaporated to dryness in a Savant Speed-vac. The residue was resuspended in 30 µl of ethyl acetate and spotted in 5 µl aliquots on a silica gel thin layer chromatography plate (EM reagents). The plate was developed in $CHCl_3$:methanol (9:1) until the solvent front had ascended approximately 12 cm and the radioactive chloramphenicol was visualized by autoradiography using Kodak XAR film.

RESULTS

To study the efficiency of transient transfection, we have utilized the chloramphenicol acetyl transferase (CAT) gene contained on the plasmid pSV2-CAT. This hybrid plasmid has been engineered by Gorman et al (11) to place the coding portion of the bacterial CAT gene under the eukaryotic transcriptional control of the SV40 early region promoter/enhancer sequences and also to contain the SV40 small t splice and polyadenylation signals. Since CAT has no eukaryotic counterpart, expression of this gene can be



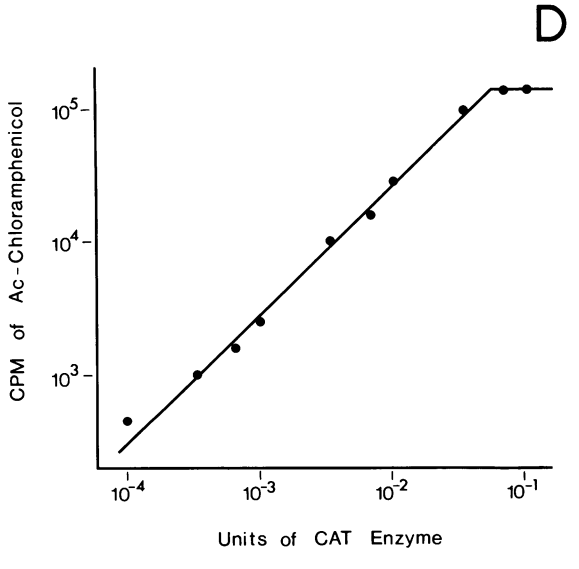


Figure 1. Establishment of the parameters of the enzymatic assay for CAT activity. A and D) The range of CAT activity which can be quantitated in a standard assay. (A) Dilutions of commercial CAT enzyme (PL Biochemicals)

were added to a standard CAT assay mixture containing 14 μM ^{14}C-chloroamphenicol (see Materials and Methods) and incubated for 60 minutes at 37°C. The reaction products were analyzed by thin layer chromatography and visualized by autoradiography. Exposure was for 21 hours. The positions of the unreacted chloramphenicol (chl), chloramphenicol acetylated on the 1 position (1-chl) or the 3 position (3-chl) and the 1,3-diacetylated chloramphenicol (1,3-chl) are shown. (D) The results of part A were quantitated by scintillation counting of the product spots excised from the chromatogram. B) Determination of minimum ^{14}C-chloramphenicol concentration which confers maximal sensitivity. Identical aliquots of extracts from pSV2-CAT transfected cells were assayed for CAT activity in the presence of decreasing amounts of ^{14}C-chloramphenicol. C) The time course of the CAT assay. Aliquots of a standard assay mix containing extract from pSV2-CAT transfected cells were removed at the specified times of incubation and analyzed for acetylated chloramphenicol.

readily detected in the absence of endogenous background after transfection.

In order to analyze transfections of the CAT gene quantitatively, it is essential to verify that the CAT assay faithfully reflects the amount of enzyme present. The CAT assay involves the enzymatic conversion of ^{14}C-labelled chloramphenicol to mono- and di- acetylated forms using acetyl CoA as the acetyl donor. The acetylated products are readily separable from the unreacted species by chromatography on silica gel thin layer chromatography plates (11). As shown in Figure 1A and confirmed in Figure 1D by scintillation counting of the excised product spots, our standard CAT assay is indeed linear over one hundred fold range in enzyme activity (from $3x10^{-2}$ to $3x10^{-4}$ units of commercial [PL Biochemicals] enzyme in a 150 μl reaction volume). Moreover, since ^{14}C-chloramphenicol, the substrate of the CAT assay, is quite expensive, we have found it advantageous to minimize the chloramphenicol content of each assay. As shown in Figure 1B, the chloramphenicol can be reduced by as much as five fold from the standard 70 μM (i.e., 0.5 μCi/reaction; 11) without detectable loss of sensitivity. Further, as shown in Figure 1C, the sensitivity of the CAT assay can be significantly increased by simply extending the reaction time from the usual 30 minutes (11).

With the parameters of the CAT assay thus defined, we have attampted to maximize expression of transfecting DNA following DEAE-dextran mediated DNA transfer into mouse L cells. In Figure 2, the parameters that we have varied are: 1- the presence or absence of a 2 minute DMSO shock, 2- the concentration of DEAE-dextran employed, 3- the concentration of the transfecting DNA, and 4- the length of time of exposure of the cells to the DNA/DEAE-dextran. Transfections on the left hand side of Figure 2 (parts A,B,E,F) were subjected to a DMSO shock while those on the right hand side

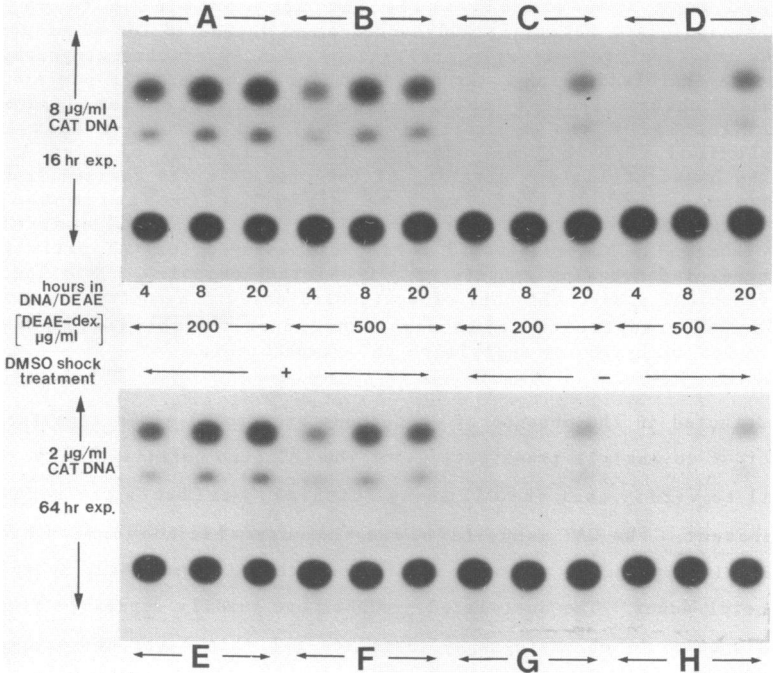


Figure 2. Assay of CAT activity following transfection of pSV2-CAT into L cells in the presence or absence of a DMSO shock, at varying concentrations of transfecting DNA and DEAE-dextran, and at varying times of transfection. The three lanes of each part represent the activity obtained from cells transfected by incubation for 4, 8 or 20 hours with DNA/DEAE-dextran. Parts A-D represent transfections with 8 µg/ml DNA; parts E-H represent 2 µg/ml DNA. Parts A, C, E and G show assays of extracts from cells transfected with 200 µg/ml DEAE-dextran; parts B, D, F and H are from extracts of cells transfected with 500 µg/ml DEAE-dextran. Parts A and C, B and D, E and G, and F and H represent duplicate transfections except that the first of each pair included a 10% DMSO shock step for 2 minutes. Each assay represents CAT activity in 1/100th of a 60 mm dish of cells at 72 hours post-addition of DNA. Autoradiograms of parts A-D (8 µg/ml of DNA) have been exposed for 16 hours; autoradiograms of parts E-H (2 µg/ml of DNA) have been exposed for 64 hours. Consistant with previous reports (12), cell damage becomes visible after 12 hours exposure to 500 µg/ml DEAE-dextran.

(parts C,D,G,H) were not. In agreement with previous reports (10,12) for cells transfected in the absence of a shock, overall levels of expression increase monotonically between 4 and 20 hours of exposure of the cells to the DNA and DEAE-dextran. This is true both at 200 µg/ml (Fig. 2C,G) and 500 µg/ml (Fig. 2D,H) DEAE-dextran concentrations and at 8 µg/ml (Fig. 2C,D) and 2 µg/ml (Fig. 2G,H) of transfecting DNA. Approximately four fold lower absolute activity levels are obtained with 2 µg/ml than with 8 µg/ml of

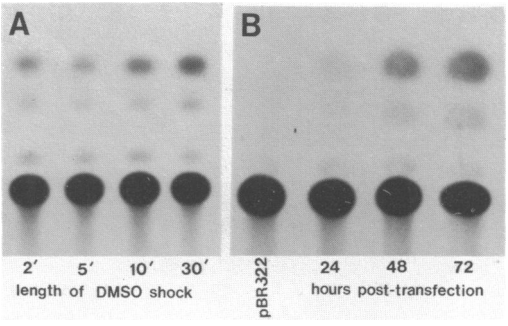


Figure 3. Dependence of CAT expression on length of dimethyl sulfoxide shock and on time of cell harvesting after transfection. A) Aliquots of extract from 1/100th of a 60 mm dish of cells were assayed following transfections with pSV2-CAT/DEAE-dextran. These transfections utilized a 10% dimethyl sulfoxide shock varying in length from 2 minutes to 30 minutes. B) Using our standard transfection conditions and a 2 minute dimethyl sulfoxide shock, total CAT activity was determined at the indicated times post-transfection.

transfecting DNA; hence the autoradiograph in Fig. 2E-H has been exposed four times longer than that of Fig. 2A-D. Remarkably, although the level of CAT expression varies greatly according to the transfection conditions, in all cases addition of a DMSO shock markedly increases the expression level relative to the parallel unshocked cells. Specifically, stimulation of expression by a shock treatment occurs upon transfection at low (Fig. 2A,E) and high (Fig. 2B,F) concentrations of DEAE-dextran, at low (Fig. 2E,F) and high (Fig. 2A,B) concentrations of transfecting DNA and at all times of DEAE-dextran treatment. [Compare the left and the right halves of Fig. 2.] The maximal expression is obtained with 8-20 hours exposure to 200 μg/ml DEAE-dextran and 8 μg/ml DNA, followed by DMSO shock treatment.

We also examined the dependence of the level of expression on the length of time that the DEAE-dextran transfected cells are exposed to the DMSO shock. There is a broad optimum, with comparable high levels of expression obtained with between 2 and 30 minutes in DMSO (Figure 3A). This represents a somewhat shorter time of shock than has previously been determined to be optimal for DMSO shock using the calcium phosphate transfection protocol (7). Not surprisingly, the level of CAT expression also depends on the time interval between the DNA/DEAE-dextran treatment and cell harvesting, increasing over a three day period post-transfection (Figure 3B).

To further optimize the overall level of transient expression of the

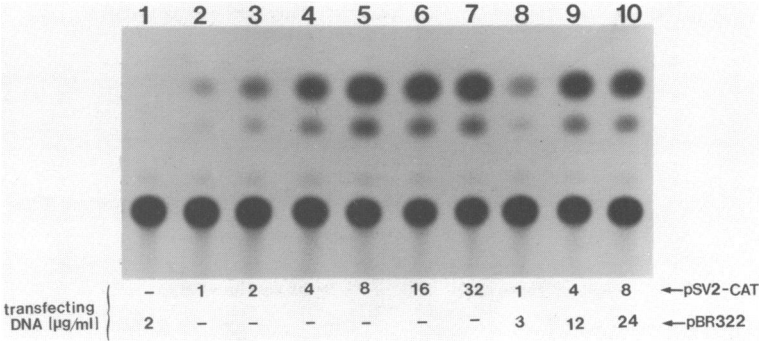


Figure 4. Dependence of CAT activity on the concentration of transfecting DNA. The concentration of pSV2-CAT DNA (transfected with 500 μg/ml DEAE-dextran and a 2 minute DMSO shock) was varied between 1 and 32 μg/ml in the presence or absence of competitor pBR322 plasmid DNA, as indicated. In each case extract derived from 1/100th of a 60 mm dish of cells was assayed and a 16 hour exposure is shown. (Lane 1)- transfection with 2 μg/ml of pBR322 DNA alone; (lanes 2 to 7)- 1, 2, 4, 8, 16, and 32 μg/ml pSV2-CAT; (lane 8)- 1 μg/ml pSV2-CAT plus 3 μg/ml pBR322; (lane 9)- 4 μg/ml pSV2-CAT plus 12 μg/ml pBR322; (lane 10)- 8 μg/ml pSV2-CAT plus 24 μg/ml pBR322.

CAT gene introduced into cells by DEAE-dextran and DMSO treatment, we have varied the concentration of the transfecting DNA (Figure 4). Between 1 and 8 μg/ml, CAT expression increases in proportion with the concentration of input plasmid. Above 8 μg/ml the signal plateaus and then slowly decreases. That the important parameter in increasing CAT expression is the specific gene concentration and not the total DNA concentration is shown in Figure 4 lanes 8-9, where the CAT enzymic activity directed by suboptimal amounts of the transfecting CAT plasmid is not increased by raising the total DNA concentration three fold by addition of carrier DNA (compare lanes 2 and 8, 4 and 9).

Finally, a comparison of the overall level of expression of DNA transfected by the DEAE-dextran and the calcium phosphate methods in the presence and absence of a shock treatment is shown in Figure 5. Using DEAE-dextran, expression of the transfecting gene is enhanced to the same extent - about 50 fold - by the use of either a 10% DMSO or a 15% glycerol shock. In our hands, these shocked, DEAE-dextran transfected cells (Fig. 5, lanes 1, 3 and 4) reproducibly exhibit a 5 to 10 fold higher level of expression than do cells that are transfected in the presence of calcium phosphate and subjected to a glycerol shock treatment (Fig. 5, lanes 6 and 7). Indeed, using pSV0-CAT, a CAT gene construct containing no eukaryotic gene promoter sequences (11), we can easily detect CAT activity in extracts from 1/100th

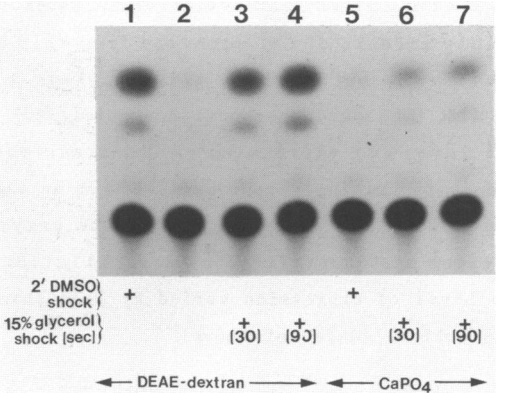


Figure 5. Comparison of CAT activity following transfection using DEAE-dextran or calcium phosphate mediated DNA uptake in the presence or absence of DMSO or glycerol shock treatments. Identical aliquots of cells were transfected with pSV2-CAT DNA. Lanes 1-4 were transfected using the DEAE-dextran mediated protocol (2 μg/ml final DNA concentration): including a two minute, 10% dimethyl sulfoxide shock (lane 1), in the absence of any shock treatments (lane 2), or in the presence of a 30 second (lane 3) or a 90 second (lane 4) shock with buffer containing 15% glycerol. Lanes 5-7 were transfected using the calcium phosphate protocol (8) including our standard shock step of 10% DMSO for 2 minutes (lane 5) or substituting a 15% glycerol shock step for 30 seconds (lane 6) or for 90 seconds (lane 7). All cell aliquots were harvested and assayed for CAT activity 48 hours post-transfection.

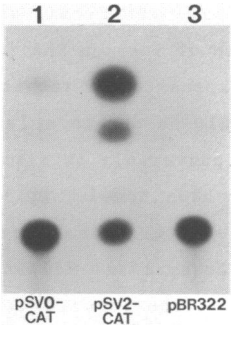


Figure 6. Detection of activity from the promoterless plasmid, pSV0-CAT. Plasmids containing the CAT gene constructs lacking (pSV0-CAT) or containing (pSV2-CAT) the SV40 promoter/enhancer region or pBR322 were transfected into L cells and the resultant CAT activity was assayed. (Lane 1)- pSV0-CAT; (lane 2)- pSV2-CAT; (lane 3)- pBR322. Note the product spot from pSV0-CAT.

of a 60 mm dish of L cells after DEAE-dextran/DMSO shock transfection (Fig. 6, lane 1). Detectable amounts of CAT activity from this pSV0-CAT plasmid have not been previously obtained by transfections with the calcium phosphate protocol (11; G. Khoury, personal communication).

Moreover, the levels of expression of DNA transfected by the DEAE-dextran/DMSO method is surprisingly reproducible. In an experiment designed to address this question, the chromatogram of the enzymic assay of ten separate transfections was quantitated by scintillation counting of the product spots. The level of expression varied by less than 20% between all the different transfections (data not shown).

DISCUSSION

The protocol we have identified to yield maximal levels of expression of transfected (non-replicating) plasmid DNA utilizes 8 µg/ml plasmid DNA in 200 µg/ml DEAE-dextran incubated with the cells for 8-20 hours, followed by a 2-30 minute shock treatment with 10% DMSO or a 0.5-2 minute shock treatment with 15% glycerol, and then a 3 day recovery period in serum-containing medium. The level of expression is increased up to 50 fold simply by inclusion of either of these shock steps. This final level of expression is 5-10 fold higher than we have been able to obtain with L cells using the calcium phosphate/glycerol (8) or calcium phosphate/DMSO (7) transfection protocols. Overall, we feel that the DEAE-dextran/DMSO protocol provides a convenient, efficient, and reproducible method for effecting short-term expression of foreign DNA in eukaryotic cells.

The increased level of expression of the transfected DNA in response to the DMSO shock shown above could be due to a large percentage of the cells transcribing the input DNA or conversely to a small percentage of the cells expressing this DNA at a very high level. By monitoring the conversion of Ltk^- cells to a tk^+ phenotype after transfection with the Herpes simplex virus thymidine kinase gene, Sussman and Milman (13) have shown that up to 80% of recipient cells express the transfecting DNA following the DEAE-dextran/DMSO shock protocol. Under their conditions this represents an approximate 15 fold increase in efficiency of transfection over that obtained without the shock treatment. Further, using a line of mouse B lymphocytes, M. Hauser and P. Gearhart (personal communication) obtain up to 97% transfection efficiency with this shock protocol.

Finally, although we would caution that the optima for transfection (DNA concentration, DEAE-dextran concentration, etc.) may be somewhat

dependent upon the recipient cell type and upon the specific transfecting DNA (e.g., its size, etc.), in our hands the addition of a DMSO or glycerol shock step to the basic DEAE-dextran protocol has resulted in a marked increase in expression of the transfected gene under all conditions thus far tested.

ACKNOWLEDGEMENTS

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REFERENCES

1. McCutchan, J.H. and Pagano J.S. (1968) J. Nat. Can. Inst. 41:351-356.
2. Wigler, M., Pellicer, A. Silverstein, S. and Axel, R. (1978) Cell 14:725-731.
3. Sarver, N., Gruss,P. Law, M.F., Khoury, G. and Howley, P.M. (1981) Mol. Cell Biol. 1:486-496.
4. DiMaio, D., Treisman, R. and Maniatis, T. (1982) Proc. Natl. Acad. Sci., U.S.A. 79:4030-4034.
5. Graham, F.L. and van der Eb, A.J. (1973) J. Virology 52:456-467.
6. Stow, N.D. and Wilkie, N.M. (1976) J. Gen. Virol. 33:447-458.
7. Lewis, W.H., Srinivasan, P.R., Stokoe, N. and Siminovitch, L. (1980) Somatic Cell Genetics 6:333-347.
8. Parker, B. and Stark, G. (1979) J. Virol. 31:360-369.
9. Corsaro, C.M. and Pearson, M.L. (1981) Somatic Cell Genetics 7:603-616.
10. Danna, K.J. and Sompayrac, L.M. (1982) J. Virol. Meth. 5:335-341.
11. Gorman, C., Moffat L. and Howard, B. (1982) Mol. Cell Biol. 2:1044-1054.
12. Sompayrac, L.M. and Danna, K.J. (1981) Proc. Natl. Acad. Sci., U.S.A. 78:7575-7578.
13. Sussman, D. and Milman, G. (1984) Mol. Cell. Biol., in press.