transfection protocols are injurious to cells. However, the PLO method appears to be better tolerated by L6 myotubes than Ca²⁺P precipitation, as reflected in cell morphology, attachment, protein content and reporter gene expression. This protocol has proven similarly effective for transient gene transfer into differentiated mouse (C2C12) myotubes. It is not, however, uniformly successful for all quiescent cells, as rat hepatoma (H4IIEC3) cells are inefficiently transfected by this method. In fact, transient transfection into hepatoma cells by this method is approximately two orders of magnitude less efficient than conventional Ca²⁺P precipitation of identical fusion gene constructs. Nonetheless, the PLO method may be useful in the transfection of cells that are not easily transfected by conventional methods.

In summary, we have employed the method of Dong et al. to accomplish efficient gene transfer into differentiated, quiescent L6 myotubes. To our knowledge, this is the first use of the PLO technique for gene transfer into a cell type that has been differentiated *in vitro*, thus allowing the study of gene regulation while minimizing or obviating any confounding effects associated with differentiation. This inexpensive transfection method is simpler to perform and considerably more efficient for gene transfer into L6 myotubes than conventional Ca²⁺P precipitation. Moreover, the use of PLO rather than Ca²⁺P may obviate problems with disrupted signal transduction in cells that depend upon Ca²⁺-dependent signaling mechanisms for endogenous or chimeric fusion gene expression.

REFERENCES

1. **Beguino, F., C.R. Kahn, A.C. Moses and R.J. Smith.** 1986. The development of insulin receptors and responsiveness is an early marker of differentiation in the muscle cell line L6. *Endocrinology* 118:446-455.
2. **Bradford, M.M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
3. **Chen, C. and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* 7:2745-2752.
4. **De Wet, J.R., K.V. Wood, M. DeLuca, D.R. Helinski and S. Subramani.** 1987. Firefly luciferase: structure and expression in mammalian cells. *Mol. Cell. Biol.* 7:725-737.

5. **Dong, Y., A.I. Skoultchi and J.W. Pollard.** 1993. Efficient DNA transfection of quiescent mammalian cells using poly-L-ornithine. *Nucleic Acids Res.* 21:771-772.
6. **Klamut, H.J., S.B. Gangopadhyay, R.G. Worton and P.N. Ray.** 1990. Molecular and functional analysis of the muscle-specific promoter region of the Duchenne muscular dystrophy gene. *Mol. Cell. Biol.* 10:193-205.
7. **Nordeen, S.K., P.P. Green and D.M. Fowlkes.** 1987. A rapid, sensitive and inexpensive assay for chloramphenicol acetyltransferase. *DNA* 6:173-178.
8. **O'Brien, R., P.C. Lucas, C.D. Forest, M.A. Magnuson and D.K. Granner.** 1990. Identification of a sequence in the PEPCK gene that mediates a negative effect of insulin on transcription. *Science* 249:533-537.
9. **Printz, R.L., S. Koch, L.R. Potter, R.M. O'Doherty, J.J. Tiesinga, S. Moritz and D.K. Granner.** 1993. Hexokinase II mRNA, gene structure, regulation by insulin, and evolution. *J. Biol. Chem.* 268:5209-5219.
10. **Rahm, M., P. Jin, J. Sümegi and T. Sejersten.** 1989. Elevated *c-fos* expression inhibits differentiation of L6 rat myoblasts. *J. Cell. Physiol.* 139:237-244.
11. **Sanes, J.R., J.L.R. Rubenstein and J.F. Nicolas.** 1986. Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. *EMBO J.* 5:3133-3142.
12. **Shainberg, A., G. Yagil and D. Yaffe.** 1971. Alterations of enzymatic activities during muscle differentiation *in vitro*. *Dev. Biol.* 25:1-29.
13. **Widmer, F. and P. Caroni.** 1993. Phosphorylation-site mutagenesis of the growth-associated protein GAP-43 modulates its effects on cell spreading and morphology. *J. Cell Biol.* 120:503-512.
14. **Zingg, J.-M., G.P. Alva and J.-P. Jost.** 1991. Characterisation of a genomic clone covering the structural mouse MyoD1 gene and its promoter region. *Nucleic Acids Res.* 19:6433-6439.

Address correspondence to Daryl K. Granner, Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN 37232-0615, USA.

Received 30 June 1995; accepted 13 September 1995.

R. Brooks Robey, Haruhiko Osawa, Richard L. Printz and Daryl K. Granner
*Vanderbilt University School of Medicine
Nashville, TN, USA*

Preparation of Electro-Competent *E. coli* Using Salt-Free Growth Medium

BioTechniques 20:42-44 (January 1996)

Bacterial electroporation is an extensively used method by which plasmid DNA can be transformed into *Escherichia coli* at high efficiency. For high efficiency electroporation, it is essential to have the resistance of the electroporation medium as high as possible. This is achieved by ensuring that all the salts from the cell suspension have been removed. Because media used to grow *E. coli* contain added salt (0.5% or 1% NaCl in LB medium) (1,3-5), the salt must be removed by extensive washing with large volumes of water or low ionic strength buffer containing glycerol (1,3) and multiple steps of centrifugation and suspension. These steps are time-consuming, salt removal is not reproducible and extensive washing and resuspension steps can result in contamination and lower electroporation efficiencies due to lower cell viability.

In our earlier studies we extensively used a rich growth medium (yeast extract and nutrient broth, YENB) that was not supplemented with NaCl and was able to support vigorous growth of most *E. coli* strains, including *recA* strains (commonly used for electroporation) (7-10). In fact, certain *E. coli* mutant strains (e.g., *D polA*) that did not grow in LB medium containing added NaCl were able to grow normally in YENB with high viability (6). Here we describe a simple method to prepare electro-competent *E. coli* cells that employs rich growth medium (YENB) without added NaCl. Our method also makes it possible to prepare electro-competent cells from bacterial strains that do not grow (or grow poorly) in growth medium with added salt. However, our method worked well for all bacterial strains used.

Preparation of Cells

We have routinely used *E. coli* strain WM1100, a *recA* derivative of MC1061 (2). This strain carries a *tet^r* marker and

Table 1. Protocol

A. Preparation of Competent Cells

1. Grow a fresh overnight culture in liquid YENB medium (Bacto yeast extract [0.75%], Bacto Nutrient Broth [0.8%]; Difco Laboratories). This is very important as it eliminates all the steps needed to remove the salts. Salts are known to reduce the efficiency of electrotransformation and cause arcing in the electroporation cuvette (1).
2. Inoculate 1 L of fresh YENB medium with 5–10 mL of fresh overnight culture and grow cells at 37°C with shaking. Cells should be harvested between an OD₆₀₀ of 0.5 to 0.9 (if cells are overgrown, dilute back to an OD₆₀₀ of ca. 0.2 and regrow to desired OD₆₀₀).
3. To harvest cells, chill the flask on ice for about 5 min and spin at 4000× *g* for 10 min at 4°C. It is important not to centrifuge at a higher *g*-value than needed to pellet the cells: more centrifugal force applied for a longer time reduces the viability of the culture, which in turn reduces the electrotransformation efficiency.
4. Remove and discard medium. As the culture medium does not contain added salt, it is not important to remove traces of medium as required in the original protocols (1,3). Wash pellet twice in 100 mL of cold water and centrifuge as in Step 3; remove and discard supernatant.
5. Resuspend in 20 mL of cold 10% glycerol (J.T. Baker, Phillipsburg, NJ, USA). Centrifuge as in Step 3; remove and discard supernatant.
6. Resuspend the cells (from 1 L of culture) in a final volume of 2–3 mL of cold 10% glycerol. The cell number in the suspension should be $2-4 \times 10^{10}$ cells/mL. These competent cells can be used fresh or be frozen (see below) for future use.
7. To freeze competent cells, aliquot into centrifuge tubes (40 µL/tube) and place tubes over dry ice in a bucket until frozen. Store at -70°C. Thaw on ice before use. These competent cells are good for 1–2 years.

B. Electrotransformation

The following protocol was used for electrotransformation using electro-competent cells made by the procedure described:

1. To one 40-µL aliquot of competent cells, add 1–5 µL of DNA in low ionic strength buffer; e.g., TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Mix well and let sit on ice for approximately 1 min.
2. Transfer the mixture of cells and DNA to a cold 0.2-cm electroporation cuvette (Bio-Rad, Hercules, CA, USA) and electroporate in a Bio-Rad Gene Pulser® according to the manufacturer's instructions. This produces a pulse with a time constant of 4–5 ms, consistent with the published value.
3. Remove the cuvette from the chamber and immediately add 1 mL of YENB medium to the cuvette. (SOC medium can also be used. SOC medium contains 2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose. Bacto tryptone was from Difco and the other chemicals were from Sigma Chemical, St. Louis, MO, USA). Resuspend the cells. Transfer the cells suspension to a polypropylene tube (17 × 100 mm) and incubate with or without shaking at 37°C for 1–3 h.
4. Plate cells on selective medium plates (YENB plates containing antibiotics) and incubate overnight at 37°C.

can be selected for ampicillin-resistance. This marker is commonly carried on a plasmid to be electroporated. Other bacterial strains used were DH5α and MC1061/p3. When MC1061/p3 were transferred from a salt medium to a no-salt growth medium, after an initial lag, MC1061/p3 grew better in growth medium without added salt. Plasmid DNAs used were pUC18, pBR322, pBSM13+, pDR2, pSP64, etc. *E. coli* strains were plated and

maintained on YENB plates (Bacto® yeast extract [0.75%], Bacto Nutrient agar [2.3%], both from Difco Laboratories, Detroit, MI, USA).

By using our method for the preparation of electro-competent cells (Table 1), we routinely obtain more than 10⁹ transformants/µg of plasmid DNA. This transformation efficiency is similar to what other more labor-intensive methods are known to give. In summary, the method described here is rapid

and less labor-intensive than methods using growth medium with added salt, it never causes arcing and gives high electrotransformation efficiencies. Furthermore, our method allows preparation of electro-competent cells from bacterial strains that either do not grow or grow poorly in growth medium with added salt.

REFERENCES

1. **Bio-Rad *E. coli* Transformation Apparatus.** 1994. Operating Instructions and Application Guide. Bio-Rad Laboratories, Richmond, CA.
2. **Dover, W.J.** 1990. Electroporation of bacteria: a general approach to genetic transformation. *Genet. Eng.* 12:275-295.
3. **Dover, W.J., J.F. Miller and C.W. Ragsdale.** 1988. High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res.* 16:6127-6145.
4. **Maniatis, T., E.F. Fritsch and J. Sambrook.** 1982. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
5. **Sambrook, J., E.F. Fritsch and T. Maniatis.** 1989. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
6. **Sharma, R.C. and K.C. Smith.** 1987. Role of DNA Polymerase I in postreplication repair: a reexamination with *Escherichia coli* *D* *polA*. *J. Bacteriol.* 169:4559-4564.
7. **Sharma, R.C. and K.C. Smith.** 1987. Comparison of the rep-38 and mmrA1 mutations of *Escherichia coli*. *Mutat. Res.* 184:23-28.
8. **Sharma, R.C. and K.C. Smith.** 1986. Repair of DNA double-strand breaks in UV-irradiated *Escherichia coli* *uvrB* *recF* cells is inhibited by rich growth medium. *Mutat. Res.* 166:23-28.
9. **Sharma, R.C. and K.C. Smith.** 1985. A mechanism of rich medium inhibition of the repair of daughter-strand gaps in the deoxyribonucleic acid of UV-irradiated *Escherichia coli* K-12 *uvrA*. *Mutat. Res.* 146:177-183.
10. **Sharma, R.C. and K.C. Smith.** 1985. A minor pathway of postreplication repair in *Escherichia coli* is independent of *recB* *recC* and *recF* genes. *Mutat. Res.* 146:169-176.

We thank Dr. Keri Tate and Ms. Theresa McRae of Stanford University for using our method and helpful discussion. Address correspondence to Rakesh C. Sharma, Department of Biological Sciences, Stanford University, Stanford, CA 94305, USA.

Received 13 July 1995; accepted 11 September 1995.

**Rakesh C. Sharma and
Robert T. Schimke**
Stanford University
Stanford, CA, USA

Rapid PCR Method for Site-Directed Mutagenesis on Double-Stranded Plasmid DNA

BioTechniques 20:44-46 (January 1996)

Site-directed mutagenesis is an important tool for precise characterization of the function of transcription factor responsive elements within promoter/enhancer regions. A number of different approaches have been reported (1,3-9,11). Among them, the polymerase chain reaction (PCR) has become a common method for introducing mutations by a procedure known as recombinant PCR (4,6). Here, a new and more efficient PCR method is described that allows construction and cloning of promoter mutations directly from genomic DNA into a luciferase vector. We have successfully used this procedure to introduce mutations into the IL-6 promoter. This technique allows mutants to be precisely placed in a promoter region, even if no unique cloning sites are available. The general procedure is illustrated in Figure 1.

The murine IL-6 enhancer region contains three "CACCC" repeats located just upstream of the classic NF- κ B binding site (2,10). [The mouse IL-6 gene sequence was obtained from GenBank®: Accession No. M20572.] To understand the functional importance of transcription factor binding to these repeats, we substituted the sequence CAAAC for each repeat (Figure 1). Two primers that flanked the IL-6 promoter were synthesized and used to amplify the relevant region. The 5' primer contained the *NheI* site (5'-TTTCGATGCTAGCCGACGTCACA-3'), which was designated EN (external *NheI* containing) and was complementary to nucleotides -179 > -158 of the murine IL-6 promoter region (10). The 3' primer containing a *HindIII* site (5'-TTGACTAAGCTTCTTGGTGGGCT-3') (called EH) was complementary to nucleotides +34 > +12 of the IL-6 promoter. The first round of PCR was performed using 100 pmol of each primer and 500 ng of mouse genomic DNA extracted from a tail biopsy. After 30

cycles of amplification [94°C for 30 s (denaturing); 56°C for 30 s (annealing); and 72°C for 30 s (extension)], a 214-bp fragment (-179 > +34) was recovered that bracketed the downstream wild-type promoter region of IL-6.

For insertion of mutations, two directly adjacent, but non-overlapping, internal primers were synthesized. A 40-nucleotide internal primer (primer 1) (In1) (-136 > -96, 5'-GGAG-TTTGGGTTTGGGGCCAGAGTG-TTTGGGGCTGATTGG-3') contained the three CAAAC substitutions (complementary sequence: GTTTG). Internal primer 2 (In2) (5'-AACAAA-GATTTTATCAAAT-3', -95 > -76) faced the opposite direction just downstream of the CACCC region. These primers, when paired with adjacent external primers, allowed generation of two abutting fragments (A and B), which were cloned directionally. To achieve correct orientation, only the 5' ends of the two internal primers but not the external ones were phosphorylated. This was accomplished using T4 kinase by the standard method: One micromole of In1 or In2 was phosphorylated at 37°C for 2 h in 50 μ L kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10 mM KCl, 5 mM dithiothreitol [DTT] and 1 mM ATP) containing 4 units of T4 polynucleotide kinase (Promega, Madison, WI, USA). Two separate PCRs were then carried out: Reaction 1 contained 100 pmol of unphosphorylated EN and phosphorylated In1, as well as 50 ng of native promoter sequence fragment obtained from the previous PCR amplification. Reaction 2 contained 100 pmol of unphosphorylated EH and phosphorylated In2, in addition to 50 ng of the native template. PCR conditions were the same as before, except the number of PCR cycles was reduced to 20. Two fragments (fragment A and fragment B) were generated, of which fragment A contained the "CAAAC" mutations (shown as a dot in the figure). Fragments A and B were separated from the primers by agarose gel electrophoresis and recovered by electroelution (Little Bluetank™ apparatus; Isco, Lincoln, NE, USA). They were ligated at 14°C overnight in 50 μ L of ligation buffer (30 mM Tris-HCl, pH 7.8, 10 mM MgCl₂, 10 mM DTT, 20 mM ATP).