High-Frequency Transfer of Cloned Herpes Simplex Virus Type 1 Sequences to Mammalian Cells by Protoplast Fusion

ROZANNE M. SANDRI-GOLDIN,¹ ALAN L. GOLDIN,¹ MYRON LEVINE,^{1*} AND
JOSEPH C. GLORIOSO²

*Department of Human Genetics¹ and Unit for Laboratory Animal Medicine,² University of Michigan
Medical School, Ann Arbor, Michigan 48109*

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The protoplast fusion technique of Schaffner (W. Schaffner, Proc. Natl. Acad. Sci. U.S.A. 77:2163-2167, 1980) has been adapted to introduce cloned herpes simplex virus genes into cultured mammalian cells. The technique involves digesting bacterial cell walls with lysozyme to produce protoplasts and then fusing the protoplasts to mammalian cells by treatment with polyethylene glycol. For monitoring transfer, protoplasts were labeled with the fluorescent dye fluorescein isothiocyanate before fusion. After fusion, greater than 50% of the mammalian cells were fluorescent, demonstrating that bacterial material was transferred with high frequency. Transfer of plasmid pBR325 occurred at frequencies of 1 to 2%, as measured by *in situ* hybridization. Fusion transfer of a chimeric plasmid consisting of the herpes simplex virus type 1 (strain KOS) *Eco*RI fragment F in pBR325 resulted in expression of some viral genomic sequences in about 5% of the mammalian cells, as detected by indirect immunofluorescence. One Ltk⁻ cell in 300 to 500 was transformed to the TK⁺ phenotype after fusion with protoplasts carrying the chimeric plasmid pX1, which consists of pBR322 and the *Bam*HI fragment coding for the herpes simplex virus type 1 thymidine kinase gene.

The transfer of purified genetic sequences to mammalian cells in tissue culture has proven to be a useful approach for the analysis of gene expression. The most extensively applied technique for the introduction of exogenous deoxyribonucleic acid (DNA) into cells has been the DNA-calcium phosphate precipitation method developed by Graham and van der Eb (15). With this method, viral and cellular genes coding for the enzymes thymidine kinase (TK) (2, 21, 29, 35-37, 47, 48), adenine phosphoribosyltransferase (50), hypoxanthine phosphoribosyltransferase (14, 42, 52), and a methotrexate-resistant dihydrofolate reductase (51) have been incorporated into mammalian cells not expressing those functions, and transformants were isolated in selective medium. The frequency of transformation of Ltk⁻ cells by the herpes simplex virus type 1 (HSV-1) TK gene or by a cellular TK gene ranged from one transformant in 10⁶ cells to one transformant in 10⁵ cells (39, 47). This method of transformation may be difficult to apply to many other somatic cell loci, however, because the frequency of transformation is in the range of spontaneous mutation at these loci (39), making it difficult to identify transformants from revertants even when a selective system is available. Cells transformed with nonselectable ge-

netic markers cannot be isolated directly by the calcium phosphate precipitation technique because of the low frequency of transformation. Transformants carrying and expressing nonselectable genes have been isolated by transfecting with the HSV-1 TK gene linked to DNA coding for rabbit β -globin (31), chicken ovalbumin (25), and the simian virus 40 (SV40) genome (17, 27) and by cotransfecting with the HSV-1 TK gene and unlinked DNA coding for ϕ X174 (49), plasmid pBR322 (19, 49), adenovirus type 2 (16), rabbit β -globin (53), and human β -globin (7, 33, 40).

Various investigators have attempted to improve the frequency of gene transfer to mammalian cells. Microinjection of DNA or ribonucleic acid directly into the cell is a highly efficient, although technically difficult, means of delivery (10, 13, 28). Capecchi (5) demonstrated expression of the HSV-1 TK gene in nearly all cells that were injected, with up to 1 cell in 500 becoming stably transformed. Up to one cell in five was stably transformed, however, when an SV40 origin of replication was covalently linked to the HSV-1 TK gene. Milman and Herzberg (34a) used the diethylaminoethyl-dextran method of McCutchan and Pagano (32) to transfer viral genes to mammalian cells and found

transient gene expression in 0.1 to 1% of the transfected cells. However, no stable transformants were detected with the HSV-1 TK gene. Schaffner (45) has reported a method for the direct transfer of cloned SV40 DNA from bacterial to mammalian cells by converting bacteria containing a chimeric SV40 plasmid to protoplasts and then fusing the protoplasts to mammalian cells with polyethylene glycol. Up to 6% of the recipient cells expressed the SV40 genome, as detected by the release of infectious virus. We have used a modification of this protoplast fusion method to determine if cloned HSV-1 sequences can be transferred to mammalian cells and expressed at high frequency. Evidence which shows transfer of material from bacteria by protoplast fusion to as many as half of the recipient mammalian cells is presented. In addition, stable TK⁺ transformation occurred at frequencies of 1 cell in 300 to 500.

MATERIALS AND METHODS

Mammalian cells, virus, and bacteria. African green monkey kidney (Vero) cells were used as the recipients for all protoplast fusion experiments except those involving transfer of the plasmid containing the TK gene, for which Ltk⁻ cells (24) were used. Cells were grown in Eagle minimal essential medium supplemented with nonessential amino acids, 100 μ g of streptomycin per ml, 100 U of penicillin per ml, and 5% fetal calf serum (GIBCO Laboratories). Ltk⁻ cells were grown in the same medium containing 30 μ g of bromodeoxyuridine per ml. Cells transformed to a TK⁺ phenotype were selected in medium containing HAT (15 μ g of hypoxanthine, 1 μ g of aminopterin, 5 μ g of thymidine, and 15 μ g of glycine per ml [47]). HSV-1 (strain KOS) stocks were grown in Vero cells as described previously (1). *Escherichia coli* K-12 strain 1100 derivative DH-1 (*recA1 hsdR hsdM⁺ NalA96^r thi-1 endA1 supE44*) (12) was used as the donor for all protoplast fusion experiments. All plasmids were transferred to strain DH-1 by transformation to keep the donor strain consistent in the fusion experiments. Plasmid pBR322 (3) DNA was isolated from *E. coli* K-12 strain ED8654 (*met hsdR supE tyrT trpR*) (6), and plasmid pBR325 (4) DNA was isolated from *E. coli* K-12 strain GM31 (*dcm-6 thr-1 leu-6 thi-1 his lacY galK2 galT22 ara-14 tonA31 tsx-78 supE44*) (46). Strains DH-1, ED8654, and GM31 were all obtained from Rex Chisholm (Massachusetts Institute of Technology). Plasmid pX1 consists of the *Bam*HI 3.5-kilobase fragment containing the HSV-1 TK gene inserted in pBR322 (11) and was obtained from Kate Denniston-Thompson (National Institutes of Health). Plasmid pSG18 contains the HSV-1 strain KOS *Eco*RI fragment F inserted in pBR325 and is described in detail elsewhere (12). All experiments with recombinant DNA were performed in accordance with the National Institutes of Health guidelines.

Formation of protoplasts. DH-1 bacteria containing the appropriate plasmids were grown in 50 ml

of M9 salts (34) containing 0.5% Casamino Acids, 0.4% glucose, 0.012% MgSO₄, 5 μ g of thiamine per ml, and 50 μ g of ampicillin per ml to an absorbance at 600 nm of 0.7 to 0.8. Chloramphenicol or spectinomycin was added to 250 μ g/ml, and the culture was incubated at 37°C for an additional 12 to 16 h to amplify the plasmid copy number (18). The cells were then centrifuged at 3,000 $\times g$ for 10 min at 4°C and suspended in 2.5 ml of chilled 20% sucrose in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0). Lysozyme was added [0.5 ml of a freshly prepared solution of 5 mg of lysozyme per ml in 0.25 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 8.0], and the mixture was held on ice for 5 min. Ethylenediaminetetraacetic acid was added (1.0 ml of 0.25 M ethylenediaminetetraacetic acid [pH 8.0]) for an additional 5 min on ice, and then 1.0 ml of 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0) was added slowly. The suspension was incubated at 37°C until all the bacteria were converted to protoplasts, as monitored by phase-contrast microscopy. For strain DH-1 this required about 10 to 15 min. The protoplast solution was then carefully and slowly diluted with 20 ml of prewarmed medium containing 10% sucrose and 10 mM MgCl₂ and held at room temperature for 15 min. This solution contained about 10⁹ to 1.5 $\times 10^9$ bacteria per ml and was added directly to the cell monolayers. For labeling protoplasts with fluorescein isothiocyanate (FITC), 1 mg of FITC was added to the suspension immediately after the addition of 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride.

Nick-translation of plasmid DNA. pBR325 DNA was labeled with ³²P to 10⁸ to 2 $\times 10^8$ cpm/ μ g by a modification of the procedure of Maniatis et al. (30) as described previously (12).

In situ hybridization. Vero cells used for in situ hybridization were grown for 24 h on acid-cleaned, 22-mm, square glass cover slips in six-well dishes. Protoplasts containing pBR325 were fused to the cells, followed by incubation in medium containing 5% fetal calf serum and 100 μ g of kanamycin per ml for 3 h. The medium was removed, fresh medium was added, and the cells were incubated for another 18 h, after which they were washed three times in phosphate-buffered saline (PBS) and fixed for 10 min in ethanol-acetic acid (3:1). The cells on the cover slips were then dehydrated by immersion for 5 min each in PBS, 70% ethanol, 100% ethanol, and 100% ethanol again. They were dried completely and stored in darkness at 4°C until use. DNA in the cells was denatured by immersing the cover slips in boiling 0.1 $\times$ SSC (SSC equals 0.15 M NaCl plus 0.015 M sodium citrate [pH 6.8]) for 10 min, followed immediately by immersion in ice-cold 2 $\times$ SSC. The cells were then dehydrated and air dried as before. Hybridization was carried out in 6 $\times$ SSC-0.01 M ethylenediaminetetraacetic acid-Denhardt solution (9)-30% formamide-1 mg of sonicated denatured salmon sperm DNA per ml. Labeled pBR325 DNA was denatured by heating at 117°C for 7 min and then diluted in hybridization mix so that 10 μ l contained 10⁵ cpm. A 10- μ l amount was placed on a siliconized microscope slide which was then covered with the cover slip containing the cells face down. The slides were placed on three sheets of Whatman 3 MM

paper saturated with $6\times$ SSC in a baking pan which was then tightly covered with aluminum foil. The pan was floated in a 65°C water bath for 16 h, after which the cover slips were removed from the slides by immersion in $2\times$ SSC. They were then washed for 96 h at 4°C in 4 liters of $2\times$ SSC, changed twice daily for a total of eight buffer changes. The cells on the cover slips were dehydrated as before, air dried, and coated with Kodak NTB-3 emulsion. The emulsion was exposed for 2 weeks at 4°C and developed in Kodak D-19 developer for 2 min. The cells were stained with Giemsa stain in 0.1 M citric acid (pH 5.8) for 30 min, rinsed with water, air dried, and mounted with Permount.

HSV-1 antiserum and immunofluorescence procedures. Antiserum against HSV-1 (strain KOS) virions was prepared by hyperimmunization of New Zealand white rabbits. The virions were purified from the extracellular virus released into the medium from 20 roller bottles of Vero cells infected with strain KOS at a multiplicity of infection of 0.1. The virus was pelleted at $7,500\times g$ for 6 h and suspended in PBS, and the residual cellular debris was removed by centrifuging at $100\times g$ for 10 min. The virus was again pelleted by centrifuging in an SW27 rotor at 20,000 rpm for 1 h and resuspended in 1 ml of PBS. The virus suspension was layered onto 10 to 50% sucrose step gradients in PBS and then centrifuged in an SW27.1 rotor at 24,000 rpm for 2 h at 4°C . The viral band between the 25 and 40% layers was collected, diluted with PBS, and pelleted by spinning in an SW27.1 rotor at 20,000 rpm for 2 h. The pellet was resuspended in PBS and clarified by centrifuging at $100\times g$ for 10 min, and the virus was pelleted as before. The final pellet containing 10^{10} plaque-forming units was resuspended in 5 ml of PBS and ultraviolet light irradiated at $12\text{ ergs/mm}^2\text{ per s}$ for 20 min. Aliquots (0.25 ml each) were diluted 1:1 in complete Freund adjuvant and injected intramuscularly into the rabbits at 2-week intervals, for a total of eight injections, after which serum was collected. The serum was heated at 56°C for 30 min to inactivate complement. This serum was used as the primary antibody for indirect immunofluorescence followed by treatment with FITC-conjugated swine immunoglobulins against rabbit immunoglobulins (DAKO Immunoglobulins), as described previously (26).

RESULTS

Protoplast formation and fusion to mammalian cells. To study the direct transfer of cloned sequences to mammalian cells, we first modified Schaffner's procedures (45) for the formation of protoplasts and their fusion to mammalian cells. This was necessary because our early attempts with these techniques were unsuccessful. Protoplasts were formed from *E. coli* strain DH-1 bacteria containing different plasmids, as described in each experiment. The plasmid copy number was amplified by chloramphenicol or spectinomycin (18) before protoplast formation. The bacterial cell wall was digested

with lysozyme in the presence of ethylenediaminetetraacetic acid in 10% sucrose, as described in Materials and Methods, and the formation of protoplasts was monitored by phase-contrast microscopy. No significant lysis of the bacteria was observed with this procedure.

Protoplasts were then diluted in medium containing 10% sucrose and 10 mM MgCl_2 to a concentration of 10^9 to 2×10^9 protoplasts per ml. They were added to mammalian cells in 24- or 6-well dishes at a ratio of 10^4 to 2×10^4 protoplasts per cell, and the protoplasts were pelleted onto the cells by centrifuging at $1,500\times g$ for 8 min in a swinging microtiter dish rotor of a Beckman TJ-6 centrifuge. The protoplasts were fused to the cells by a modification of the procedure described by Davidson and Gerald (8) for somatic cell fusion. After centrifugation, the supernatant was removed by aspiration, and the dish was drained by tilting for 30 s. A 2-ml amount of polyethylene glycol solution (50 g of PEG-1000 [Sigma Chemical Co.] in 50 ml of medium) was added to each well of a 6-well dish or 1 ml was added to each well of a 24-well dish. This solution was left on the cells for the times indicated in each experiment. The polyethylene glycol solution was removed, and the plates were rinsed rapidly with 4 ml of medium (1.5 ml for 24-well dishes) three successive times. Fresh medium containing 5% fetal calf serum and $100\text{ }\mu\text{g}$ of kanamycin per ml was added to each well. The kanamycin was included to prevent the growth of any bacteria which had escaped conversion to protoplasts.

Transfer of FITC from labeled protoplasts. The formation of protoplasts or spheroplasts can be readily monitored by phase-contrast microscopy. A rapid and easy procedure was sought to monitor the transfer of material from protoplasts to animal cells after fusion. Huang et al. (20) reported transfer of the fluorescent dye FITC from labeled liposomes to animal cells by fusion. We labeled protoplasts with FITC to monitor transfer of material from protoplasts to cells after fusion for various times.

In the experiment shown in Fig. 1, protoplasts labeled with 1 mg of FITC per 5 ml of protoplast suspension were fused for 1, 2, 3, 4, or 5 min to Vero cells grown on cover slips. The cells were then incubated in the presence of kanamycin at 37°C for 60 min, at which time they were fixed in acetone and observed under a microscope with epifluorescent illumination. Figure 1 shows the bright fluorescence observed when cells were fused with protoplasts for 2 min (Fig. 1D) or 4 min (Fig. 1F), indicating transfer of the dye from the protoplasts to the Vero cells. As controls, protoplasts were pelleted onto the monolayers

but not fused to the cells. No fluorescence was observed in this case (Fig. 1B). In early experiments, ca. 1% (after a 1-min fusion) to ca. 5% (after a 4- or 5-min fusion) of the cells fluoresced. In more recent experiments, greater than 50% of the cells fluoresced after a 2-min fusion (Fig. 1D).

Transfer of plasmid DNA by protoplast fusion. To be sure that plasmid DNA was being transferred after fusion with bacterial proto-

plasts, we carried out *in situ* hybridization experiments with radiolabeled plasmid DNA as the hybridization probe. Protoplasts containing pBR325 were fused for 90 s to Vero cells grown on cover slips. The cells were then incubated in medium containing 5% fetal calf serum and 100 μ g of kanamycin per ml for 21 h after the fusion, at which time the cells were fixed, and the DNA was denatured. 32 P-labeled pBR325 DNA was hybridized to the cells, and the slides were

FIG. 1. Transfer of FITC from labeled protoplasts to Vero cells after polyethylene glycol fusion. Bacterial protoplasts were labeled with 1 mg of FITC per 5 ml of suspension for 15 min before use. Protoplasts (5×10^8 to 1×10^9) were pelleted onto Vero cells on cover slips (5×10^4 cells per cover slip) and then fused for 0 min (A and B), 2 min (C and D), or 4 min (E and F). Cells were incubated for 60 min after the fusion and then fixed in acetone and observed under epifluorescent illumination. (A, C, and E) Photomicrographs of cells with phase-contrast illumination. (B, D, and F) Photomicrographs of cells with fluorescent illumination.

coated with autoradiographic emulsion. As controls, Vero cells underwent fusion and hybridization without the addition of any protoplasts. A random and somewhat heavy background scatter of grains can be seen (Fig. 2A). We have observed this level of background grains in a number of *in situ* hybridization experiments in which ^{32}P -labeled DNA was used as the hybridization probe. It is clear, however, that the grains are not clustered over any of the cells. Autoradiographs of cells to which protoplasts containing pBR325 were fused are shown in Fig. 2B and C. In contrast to Fig. 2A, the grains are clustered over some of the cells in each field. Lightly stained negative cells can also be seen. Because the efficiency of hybridization was not uniform on all parts of the slide, it was difficult to quantitate the percentage of positive cells, but it was estimated that about 1 to 2% were positive for plasmid DNA.

Expression of HSV-1 sequences after transfer by protoplast fusion. Having demonstrated that plasmid DNA could be transferred efficiently from bacterial protoplasts to Vero cells, we sought to determine whether HSV-1 DNA inserted into a bacterial plasmid could be expressed after transfer by protoplast fusion. The chimeric plasmid pSG18 (12), which consists of the HSV-1 (strain KOS) *Eco*RI fragment F inserted in pBR325, was selected for transfer. Fragment F has a mass of 10.4 megadaltons and maps between coordinates 0.31 and 0.42 on the HSV-1 map, a region that contains sequences coding for the HSV-1 DNA polymerase (23, 43), a major glycoprotein (glycoprotein B [44]), and at least three other polypeptides (36).

Protoplasts containing pSG18 were fused to Vero cells for 90 s. At 24 h after fusion, cells were fixed in acetone and treated with rabbit antibodies against HSV-1 virions and then with fluorescein-conjugated anti-rabbit antibodies. Figure 3D shows that some of the cells observed in the phase-contrast micrograph (Fig. 3C) were definitely fluorescent, indicating that at least some HSV-1 sequences were expressed. As controls, Vero cells were fused with protoplasts containing pBR325 but not HSV-1 DNA (Fig. 3A and B). Little background fluorescence could be seen, indicating that the fluorescence observed after transfer of the HSV-1 chimeric plasmid was specific for HSV-1 antigens (Fig. 3D). In this experiment, about 5% of the cells fused with protoplasts containing pSG18 showed positive fluorescence, indicating that both the transfer and expression of HSV-1 sequences were quite efficient.

Transformation of Ltk⁻ cells with the HSV-1 TK gene. To determine whether stable

FIG. 2. *In situ* hybridization of ^{32}P -labeled pBR325 DNA to Vero cells fused with protoplasts carrying pBR325. Protoplasts containing pBR325 were fused to Vero cells on cover slips for 90 s. The Vero cells were then incubated for 21 h in medium containing 5% fetal calf serum and 100 μg of kanamycin per ml and fixed in ethanol-acetic acid (3:1). DNA in the cells was denatured by immersion of the cover slips in boiling $0.1\times$ SSC. 10^5 cpm of ^{32}P -labeled pBR325 (specific activity, 10^8 cpm/ μg) was added and hybridized to cells for 16 h at 65°C . After hybridization, cells were coated with autoradiographic emulsion and exposed for 2 weeks. (A) Photomicrograph of cells to which no protoplasts were added but which underwent the fusion and hybridization procedures ($\times 400$). (B) Photomicrograph of cells fused with protoplasts carrying pBR325 ($\times 400$). (C) Photomicrograph of cells fused with protoplasts carrying pBR325 ($\times 1,000$).

FIG. 3. Immunofluorescence assay for the expression of HSV-1 antigens in Vero cells after fusion with protoplasts. Bacterial protoplasts carrying pBR322 (A and B) or the chimeric pBR322/HSV-1 plasmid pSG18 (C and D) were fused to Vero cells on cover slips for 90 s. At 24 h after the fusion, the cells were washed extensively with PBS and then fixed in acetone. Cover slips were incubated with rabbit antiserum against HSV-1 virions, followed by treatment with FITC-conjugated swine immunoglobulins against rabbit immunoglobulins. (A) Phase-contrast photomicrograph of cells fused with protoplasts carrying pBR325. (B) Fluorescence photomicrograph of cells fused with protoplasts carrying pBR325. (C) Phase-contrast photomicrograph of cells fused with protoplasts carrying pSG18. (D) Fluorescence photomicrograph of cells fused with protoplasts carrying pSG18.

transformants could result from the transfer of DNA by protoplast fusion, we transferred the HSV-1 TK gene to Ltk⁻ cells and selected for TK⁺ transformants in HAT medium, as described by Wigler et al. (47). Bacterial protoplasts containing pX1 (11), which consists of pBR322 and the 3.5-kilobase *Bam*HI fragment of HSV-1, including the entire TK gene, were fused to Ltk⁻ cells for 1 to 3 min. In control experiments, protoplasts containing only pBR322 were fused to Ltk⁻ cells. The fusions were performed with monolayers of cells in six-well dishes, with 10⁵ cells per well. After a 24-h incubation in medium containing 5% fetal calf serum and 100 μ g of kanamycin per ml, the cells in each well were trypsinized, serially diluted, and reseeded in 25-cm² flasks at various concentrations. HAT medium was added to half the flasks to select for TK⁺ cells, whereas medium without HAT was added to the remainder to determine the total number of surviving cells. Flasks were refed every 3 days. Nonselected

colonies were counted after 1 week, and TK⁺ colonies were scored after 3 weeks. Table 1 shows that 50 to 80% of the cells survived the fusion and reseeded procedures. Of the surviving cells, 0.2 to 0.3% were TK⁺, indicating that transformants were formed at a relatively high frequency. No TK⁺ colonies were found in cells to which pBR322 alone was transferred.

DISCUSSION

We have modified the protoplast formation and fusion technique described by Schaffner (45) for the direct transfer of cloned genes from bacteria to mammalian cells, and we have confirmed that this method is very efficient in delivering exogenous DNA to cells. Schaffner (45) reported the expression of SV40 T antigen and the release of infectious virus in up to 6% of cells fused to protoplasts carrying cloned trimeric SV40 DNA. The efficiency of transfer in our early experiments as measured by FITC uptake or by in situ

TABLE 1. *TK⁺ transformation of Ltk⁻ cells by protoplast fusion^a*

Fusion time (min)	No. of surviving colonies ($\times 10^4$)	No. of TK ⁺ colonies ($\times 10^2$)	Frequency of transformation (%) ^b
1.0	7.7	1.6	0.21
1.5	7.8	2.2	0.28
2.0	4.7	1.5	0.32

^a All values represent the average from four dishes used for each transformation. 10^5 cells per dish were used in each fusion experiment.

^b Frequency of transformation = number of TK⁺ colonies/number of surviving colonies.

hybridization was from 1 to 5%, and expression of transferred HSV-1 sequences detected by indirect immunofluorescence occurred in about 5% of the recipient cells. These values are in agreement with Schaffner's results. In addition to high levels of transfer, we found that 0.2 to 0.3% of Ltk⁻ cells which had been exposed to the HSV-1 TK gene were transformed to the TK⁺ phenotype. More recent FITC uptake experiments showed that greater than 50% of the cells were positive, indicating that the level of transfer can be greatly increased. We are currently investigating whether the frequencies of expression and transformation increase concomitantly with the increase in transfer efficiency.

The higher frequency of TK⁺ transformation by protoplast fusion highlights some interesting questions concerning the limiting event in transformation. Wigler et al. (49) suggested the existence in a culture of a subpopulation of competent cells that were likely to integrate exogenous DNA sequences at frequencies higher than the general population. Our results suggest that the number of cells competent for transformation may in fact be relatively large. The factors limiting the number of cells that are transformed probably depend on the technique used and may be a function of at least three nonexclusive steps in the transformation process: the uptake of exogenous DNA by the cell, the transport of DNA from the cytoplasm to the nucleus, and the stabilization of the newly acquired DNA.

That competence is not solely dependent on the capacity of the cell to take up exogenous DNA is suggested by our results and by those of Milman and Herzberg (34a) and Capecchi (5). Milman and Herzberg (34a) observed transient gene expression in 0.1 to 1% of cells transfected by the diethylaminoethyl-dextran method but were unable to detect any stable TK⁺ transformants. Capecchi (5) found expression of HSV-1 TK activity in 50 to 100% of cells injected into the nucleus with DNA from a pBR322/TK chimeric plasmid, but only 1 cell in 500 to 1,000 was

stably transformed. We have demonstrated HSV-1 gene expression in up to 5% of the recipient cells after protoplast fusion but TK⁺ transformation of only 0.2 to 0.3% of cells. These findings indicate that transformation occurs at lower levels than exogenous gene expression (from 10- to 20-fold lower levels in our experiments to no transformation at all in the experiments of Milman and Herzberg [34a]), suggesting that transformation is not just limited by the percentage of cells receiving, or even initially expressing, exogenous DNA.

It also seems unlikely that transformation is limited simply by the transport of acquired DNA from the cytoplasm to the nucleus. Capecchi (5) found that although 50 to 100% of the cells injected in the nucleus with pBR322/TK DNA expressed TK activity, no cells injected in the cytoplasm with the same plasmid DNA expressed any TK activity. This indicates that transport of the DNA from the cytoplasm to the nucleus is necessary for gene transcription and, hence, expression. Because Milman and Herzberg (34a) demonstrated gene expression in 0.1 to 1% of transfected cells, transport to the nucleus did occur, even though transformation did not. Our results suggest a similar conclusion, because 5% of recipient cells expressed HSV-1 sequences, indicative of transport to the nucleus, but only 0.2 to 0.3% were transformed. The fact that the presence of DNA in the nucleus is not sufficient for transformation was confirmed by Capecchi (5), who bypassed the transport step by injecting pBR322/TK DNA directly into the nucleus, but still found that only 1 cell in every 500 to 1,000 cells was transformed.

The limiting factor in transformation may in fact be the stabilization of the transforming DNA in the recipient cell. Perucho et al. (41), using the DNA-calcium phosphate precipitation technique, found that exogenously acquired sequences were integrated in high-molecular-weight nuclear DNA of the host but that the flanking sequences were derived from the carrier DNA used in the transfection. Because Milman and Herzberg (34a) used no carrier DNA in their transfection experiments, the exogenous DNA may have had no opportunity to form similar stabilizing structures, resulting in the complete absence of stable transformants. However, Capecchi (5) found that TK plasmid DNA was integrated into the host DNA sequences in transformants resulting from microinjection, although, again, no carrier DNA was used. In fact, the injection of carrier DNA along with the TK plasmid did not increase the frequency of transformation in his experiments. Protoplast fusion presents large quantities of plasmid DNA to the

recipient cells and, in addition, the entire *E. coli* chromosome is available as a carrier. It is possible that this excess of plasmid or bacterial DNA or both enhances the frequency of occurrence of a stabilizing DNA structure and, hence, of the transformation event. We are currently analyzing the state of the transforming DNA in TK⁺ cells after protoplast fusion to determine what associations occur.

The frequency of stabilization of the transforming DNA is also dependent on factors other than carrier DNA. Capecchi (5) found that the frequency of TK⁺ transformation by microinjection was increased from 1 cell in 500 to 1 cell in 5 if the TK plasmid also contained DNA sequences from near the origin of replication of SV40. He suggested that these SV40 DNA sequences facilitate integration into the host chromosome because of homology to middle-repetitive host sequences, homology which has been reported by Jelinek et al. (22). Similarly, Mulligan and Berg (37) found a high transformation frequency, approximately 10⁻⁴, when a recombinant plasmid containing the SV40 origin of replication and early promoter linked to the *E. coli* xanthine-guanine phosphoribosyltransferase gene was used to transform Lesch-Nyhan cells lacking hypoxanthine phosphoribosyltransferase. This frequency of 10⁻⁴ is considerably higher than the frequency of one TK⁺ transformant per 10⁵ or 10⁶ cells that has been reported by others using transfection (39, 47). The mechanism of this enhanced transformation is unknown.

We are currently determining the percentage of cells that continue to express HSV-1 antigens when no selective pressure is applied. In this respect, it will be important to see if stable transformants can be isolated in the absence of selection. The high efficiency of delivery of DNA to cells by protoplast fusion could prove to be advantageous in the construction of cells transformed for nonselectable markers.

In summary, protoplast fusion is a highly efficient method for effecting gene transfer to cells in culture. The technique is rapid and requires considerably less skill than is needed for the microinjection procedure. There is no need to isolate and purify DNA, as is required for transfection and microinjection. Expression of transferred sequences can easily be detected in at least several percent of recipient cells by immunofluorescence or radioautography. Finally, long-term expression of markers can occur under selective conditions in up to 0.3% of the cells, suggesting that transformation after DNA delivery by fusion occurs at high frequency.

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