Transfer of Herpes Simplex Virus Thymidine Kinase Synthesized in Bacteria by a High-Expression Plasmid to Tissue Culture Cells by Protoplast Fusion

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The introduction of a protein into living tissue culture cells may permit the *in vivo* study of functions of the protein. We have previously described a high-efficiency-expression plasmid, pHETK2, containing the herpes simplex virus type 1 thymidine kinase (TK) gene which, upon temperature induction, causes TK to be synthesized as greater than 4% of the bacterial protein. In this report we show that enzymatically active TK was transferred to mouse Ltk⁻ cells by polyethylene glycol-mediated fusion with protoplasts prepared from bacteria containing induced levels of TK. The presence of TK in the Ltk⁻ cells was detected by the incorporation of [³H]thymidine into cell nuclei as measured by autoradiography.

Methods currently used for delivery of exogenous proteins into cells include fusion with loaded erythrocyte ghosts (2-4, 6, 12), fusion with loaded liposomes (8, 9, 16), and physical microinjection (1, 13, 14). These methods usually require prior purification of the protein to be delivered. We have described high-efficiency-expression plasmids which, upon temperature induction, cause a cloned gene to synthesize 4% or more of the bacterial protein (5, 15). Cloned genes are under the control of lambda phage strong leftward and rightward promoters. The plasmids also contain the gene for the lambda phage CI857 temperature-sensitive repressor. High expression occurs when bacteria containing the plasmid are shifted from 30°C to 42°C. The procedures used to transfer plasmid DNA from bacterial protoplasts by polyethylene glycol-mediated fusion (7, 11) have been adapted for the delivery of highly expressed proteins to tissue culture cells.

Cultures of *Escherichia coli* HB101 containing plasmid pHETK2 (15 ml) were grown in L broth at 30°C to a density of 2×10^8 to 3×10^8 bacteria per ml and then shifted to 42°C for 16 h. The bacteria were sedimented and suspended in 1.25 ml of an ice-cold solution containing 20% sucrose and 50 mM Tris-hydrochloride (pH 8). A 0.25-ml volume of 0.5% lysozyme dissolved in 0.25 M Tris-hydrochloride (pH 8) was added, and the mixture was incubated 5 min on ice. A 0.5-ml volume of 0.25 M Na EDTA (pH 8) was added, and the mixture was incubated for 5 min on ice. A 0.5-ml volume of 50 mM Tris-hydrochloride (pH 8) was added, and the mixture was placed at 37°C for 5 min. A 5-ml volume of Dulbecco modified Eagle medium (DME) containing 10% sucrose and 10 mM MgCl₂ was added, and the protoplast suspension was left at room temperature for 15 min. Protoplasts of induced cultures containing pHETK2 have ca. 10^{-8} U of thymidine kinase (TK) per protoplast, where 1 U is the amount of enzyme that catalyzes the formation of 1 nmol of dTMP per min as previously described (15).

Ltk⁻ cells were grown in DME supplemented with 10% fetal calf serum on tissue culture dishes (diameter, 35 mm) to ca. 20% confluence. The medium was removed, and 1 ml of protoplast suspension containing 2×10^9 protoplasts was

added to each dish. The dishes were placed in a holder and centrifuged at $1,000 \times g$ for 15 min to sediment the protoplasts onto the cells. The medium was removed, and 2 ml of 50% polyethylene glycol 1000 (Sigma Chemical Co.) in DME was added, and the dishes were incubated for 1.5 min at room temperature. The solution was removed, and the dishes were gently washed four times each with 4 ml of DME. DME supplemented with 10% fetal calf serum, 50 µg of gentamycin per ml, and 20 µCi of [³H]thymidine per ml (60 Ci/mmol) was added to the dishes, and the cells were incubated for 24 h at 37°C. Cells were fixed by washing once in phosphate-buffered saline and twice in absolute methanol. Air-dried dishes were coated with Kodak nuclear track emulsion (NTB2) and exposed for 48 h in a refrigerator. The autoradiographs were developed for 2.5 min at 20°C with Kodak Dektol developer diluted 1:1 with water and fixed for 5 min in Kodak Rapid Fixer. Cells were viewed with a phase microscope at $\times 200$ magnification.

An autoradiograph of a representative field of Ltk⁻ cells after fusion is illustrated in Fig. 1. Cells were counted and scored positive for TK if they contained heavy localized grains over their nuclei. Over 50% of the cells expressed TK. The assay for TK depended upon the incorporation of labeled thymidine during DNA synthesis. Cells not viable after fusion or cells which did not synthesize DNA during the 24-h incubation were scored as negative. Therefore, the percentage of positive cells was a minimal estimate of those which had undergone fusion.

The following experiment suggests that autoradiographically detected TK-positive cells do not result from the uptake of [³H]thymidine which is phosphorylated by extracellular TK enzyme. Experimental cells were fused to protoplasts containing induced levels of TK, and control cells received the same treatment without the addition of protoplasts. After the cells were incubated for 24 h in medium containing [³H]thymidine, the levels of [³H]dTMP present in the medium were measured as previously described (15). No detectable [³H]dTMP was found in medium from experimental or control cells. Autoradiography demonstrated that more than 50% of the experimental cells expressed TK, whereas none of the control cells were TK positive.

The transfer of bacterially synthesized TK to Ltk⁻ cells was only detected after fusion with bacteria containing high

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FIG. 1. Autoradiograph of Ltk⁻ cells fused with protoplasts of bacteria containing plasmid pHETK2 and expressing high levels of TK.

levels of enzyme (Table 1). Furthermore, the data imply that the autoradiographically detected TK-positive cells arise from protein transfer rather than DNA transfection. Bacteria containing pHETK2, but grown at low temperature and having low levels of TK, did not yield TK-positive Ltk⁻ cells. Similarly, fusion with bacteria containing the high-expression plasmid pGM10 (5) lacking the TK gene did not produce TK-positive cells in the assay.

This report demonstrates that an active enzyme, highly expressed in bacteria, can be transferred from bacterial protoplasts to tissue culture cells. For comparison, we have employed the procedures described by Kaltoft et al. (3) to load erythrocyte ghosts with purified TK. The loaded ghosts contained ca. 10^{-8} U of TK activity per ghost or about the same as was obtained per bacterial protoplast. However, no more than 10% of the initial TK activity could be recovered in the ghosts. Similar efficiencies have been reported by others using TK (12) and other proteins (10). Fusion of the loaded erythrocyte ghosts to Ltk⁻ cells with 42% polyethylene glycol 6000 gave the same percentage of TK-positive cells as that obtained with protoplast fusion. However, the protoplast fusion method offers many advantages. The loading steps involved in procedures with erythrocyte ghosts or

liposomes are circumvented, and no purification of the protein is required. In addition, the expression in tissue culture cells of the function of a protein can be used to screen bacterial plasmids for the expression of desired genes.

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TABLE 1. Transfer of TK from protoplasts to Ltk⁻ cells^a

Plasmid	Growth temp	No. of cells screened	No. of TK-positive cells (%)
pHETK2	42°C	1,693	867 (51)
pHETK2	30°C	200	0 (0)
pGM10	42°C	200	0 (0)

^a Autoradiographs of cells were viewed under phase microscopy as described in the text. Cells with dark grains over their nuclei were counted as TK positive. Plasmids have been described previously (3, 15). Plasmid pHETK2 is a high-expression plasmid for herpes simplex virus TK, and pGM10 is the parental high-expression plasmid lacking the TK gene.

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