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High Efficiency Transformation by Direct Microinjection of DNA into Cultured Mammalian Cells

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Summary

Direct microinjection of DNA by glass micropipettes was used to introduce the Herpes simplex virus thymidine kinase gene into cultured mammalian cells. When DNA was delivered directly into the nuclei of LMTK$^-$, a mouse cell line deficient in thymidine kinase activity, 50–100% of the cells expressed TK enzymatic activity. In contrast, no TK activity could be detected when the DNA was injected into the cytoplasm. The number of injected LMTK$^-$ cells capable of indefinite growth in a TK$^+$ selective medium (that is, transformants) depended on the nature of the plasmid DNA into which the HSV-TK gene was inserted. One cell in 500–1000 cells which received nuclear injections with pBR322/TK DNA gave rise to a viable colony when grown in HAT medium (that is, a TK$^+$ selective medium). The transformation frequency increased to one in five injected cells when specific SV40 DNA sequences were also introduced into the HSV-TK plasmid. With the microinjection procedure transformation frequency was relatively insensitive to DNA concentration and did not depend on co-injecting with a carrier DNA. Most of the transformants were stable in *nonselective* medium as soon as they could be tested.

Introduction

Specific genes can be introduced into cultured mammalian cells by chromosome-mediated gene transfer (McBride and Ozer, 1973; Willecke and Ruddle, 1975) and by purified DNA-mediated gene transfer (Bacchetti and Graham, 1977; Maitland and McDougall, 1977; Wigler et al., 1977). The uptake and expression of both the metaphase chromosomes (Spandidos and Siminovitch, 1977; Miller and Ruddle, 1978) and purified DNA (Graham and van der Eb, 1973) is enhanced by the formation of a DNA-calcium phosphate precipitate. One in 10^5–10^7 treated cells becomes transformed by either chromosome- or DNA-mediated gene transfer. The rare transformant is isolated by biochemical selection.

In the initial transformation experiments using calcium phosphate precipitation to facilitate the uptake of purified DNA, the Herpes simplex viral thymidine kinase gene (HSV-TK) was transferred into LMTK$^-$, a mouse cell line deficient in thymidine kinase (Bacchetti and Graham, 1977; Maitland and McDougall, 1977; Wigler et al., 1977). This approach has been extended to the cellular genes for thymidine kinase (Wigler et al., 1978), adenine phosphoribosyl transferase (Wigler et al., 1979a) and hypoxanthine phosphoribosyl transferase (Graf, Urlaub and Chasin, 1979; Willecke et al., 1979).

For each of the above experiments a good selection procedure for isolating the transformants existed. It also became apparent that the transformation frequency was critically dependent on the particular cell line used as the recipient (Graf et al., 1979).

In an elegant set of experiments Wigler et al. (1979b) showed that nonselectable genes could be introduced into cultured mammalian cells by co-transformation with a unlinked but selectable gene. The nonselectable gene was mixed in a molar ratio of 1,000 to one with the selectable gene (HSV-TK) precipitated with calcium phosphate and layered onto LMTK$^-$ cells. More than 90% of the LTK$^+$ transformants contained multiple copies of the nonselectable gene.

In this study an alternative method of transferring purified genes into cultured mammalian cells is described. The DNA was directly injected into the nucleus using glass micropipettes (Diacumakos, 1973; Graessmann and Graessmann, 1976; Stacey and Allfrey, 1976). The transformation efficiency of the HSV-TK gene inserted into a number of different recombinant plasmids was compared. The transformants were characterized for the presence of HSV-TK enzymatic activity and for their stability in nonselective medium.

Results

Injection of pBR322/TK DNA

The microinjection experiments were initiated with two objectives in mind. The first was to determine the efficiency of DNA-mediated transformation obtained by microinjecting the DNA into cells and to compare this efficiency with that obtained with the more familiar calcium phosphate precipitation methods described by Bacchetti and Graham (1977), Maitland and McDougall (1977) and Wigler et al. (1977). The second objective was to attempt to discover the steps limiting the efficiency of the transformation process itself. DNA-mediated transformation of cultured mammalian cells can be divided into several steps including the DNA's entry into the cell, its transfer from cytoplasm to nucleus and its integration into the host genome. It seemed likely that microinjection of the DNA could be used to evaluate how much each step limits the frequency of transformation.

The experimental system chosen for these studies was the transfer of the Herpes simplex virus I thymidine kinase gene (HSV-TK) into a thymidine kinase deficient mouse fibroblast cell line, LMTK$^-$. The DNA injected was a purified preparation of E. coli plasmid pBR322 which carried the HSV-TK gene as an insert

(Enquist et al., 1979). Following injection of the pBR322/TK plasmid DNA into nuclei of LTK$^-$ cells, thymidine kinase (TK) enzymatic activity could be detected by the incorporation of ^{3}H–thymidine into DNA followed by autoradiographic analysis (Figure 1). No TK activity was detectable by this assay in cells receiving injections of buffer alone or of pBR322 DNA not containing the HSV-TK insert.

In a number of separate experiments between 50 and 100% of the LTK$^-$ cells in which a nuclear injection of pBR322/TK DNA was attempted showed TK activity. This efficiency reflects the normal rate for successful transfer of material into penetrated cells as determined either by injection of horseradish peroxidase followed by histochemical assays for peroxidase activity or by injection of radioactively labeled proteins followed by autoradiographic assays (data not shown).

In the experiment shown in Figure 1 approximately 125 molecules of plasmid DNA were injected into the nucleus of each cell. Thymidine kinase activity could be detected after injecting on the average as few as five molecules of pBR322/TK DNA per nucleus.

Nuclear vs. Cytoplasmic Injections

To assess the precision with which molecules could be delivered to selected cellular compartments, a solution of ^{125}I–IgG was injected into either the cytoplasm (Figure 2a) or the nucleus (Figure 2b). Following injection the cells were fixed with glutaraldehyde and analyzed by autoradiography. For these experiments the injections were carried out under conditions of constant flow of fluid (that is, constant pressure), the advantage of which is that by this procedure clogging the micropipette during successive injections is minimized. Under these conditions it is easy to inject fluid exclusively into the cytoplasm (Figure 2a). However, during injections intended for the nucleus the tip of the micropipette passes through the cytoplasm as it both enters and leaves the cell, depositing some fluid in the cytoplasm. Counts of exposed grains over the cytoplasm and nucleus indicate that in a nuclear injection approximately 90% of the injected material is delivered into the nucleus.

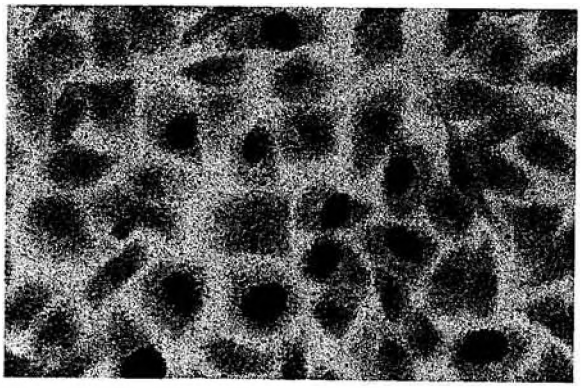

Figure 1. Autoradiographic Analysis for Thymidine Kinase Activity Present in LTK$^-$ Cells following Direct Nuclear Injection with pBR322/TK DNA

Approximately 125 molecules of pBR322/TK DNA (0.1 mg/ml) were injected per nucleus of mouse LTK$^-$ cells. Following injection the cells were incubated in MEM plus 10% fetal calf serum for 24 hr at 37°C in a 5% CO_2 incubator to allow for the expression of the injected HSV-thymidine kinase genes. The thymidine kinase (TK) activity present in each cell was determined by measuring the capacity of the cells to incorporate ^{3}H–thymidine into DNA. The cells were incubated for 12 hr at 37°C in MEM plus 10% fetal calf serum containing 25 μCi/ml of ^{3}H–thymidine. Following incubation in ^{3}H-thymidine medium, the cells were washed four times with PBS and fixed with 2% glutaraldehyde. After fixing, the cells were further washed two times with distilled water and two times with 30% ethanol. The cover slips containing the fixed cells were then mounted on microscope slides and dipped in NTB-2 autoradiographic emulsion. Following exposure for 72 hr, the slides were developed. In the above field thirteen cells received injections. Uninjected cells serve as an internal control.

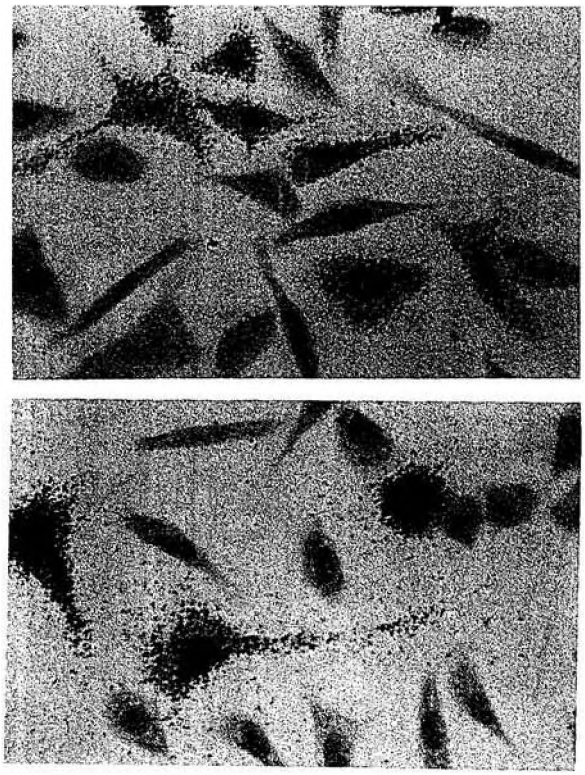

Figure 2. Autoradiographic Analysis of LTK$^-$ Cells following (a) Cytoplasmic or (b) Nuclear Injection with ^{125}I–IgG

Rabbit immunoglobulins were labeled with ^{125}I to a specific activity of 10^7 dpm/μg protein using the chloramine T procedure. A solution containing 10^6 dpm/μl of ^{125}I–IgG was selectively injected into either the (a) cytoplasm or (b) nucleus of LTK$^-$ cells. Following injection the cells were fixed with glutaraldehyde and processed for autoradiography as described in the legend to Figure 1. The slides were exposed in the dark for 10 days prior to being developed. The injections are done under conditions (constant pressure) where the micropipette is continuously flowing. As a result, during a nuclear injection some material is deposited into the cytoplasm as the tip of the micropipette passes through the cytoplasm on its way into and out of the nucleus. Counts of exposed silver grains over nuclear and cytoplasmic regions of the cell following nuclear injections with ^{125}I–IgG indicated that approximately 90% of the material is deposited in the nucleus. The ^{125}I–IgG was a gift from M. Rechsteiner.

The ability to inject pBR322/TK DNA into either the nucleus or the cytoplasm made it possible to determine whether the site of injection influences the efficiency of expression of the plasmid DNA. It will be recalled that 50–100% of the cells with pBR322/TK DNA injected into the nucleus contain detectable TK enzymatic activity. No TK activity was detected in over 1000 cells injected with pBR322/TK DNA in the cytoplasm.

Transformation of LTK$^-$ Cells to LTK$^+$

We next asked whether LTK$^-$ cells were transformed to LTK$^+$ after nuclear injection with pBR322/TK. Approximately one in 500–1000 LTK$^-$ cells injected with pBR322/TK DNA gave rise to a large colony in HAT medium (Table 1). No LTK$^+$ transformants were observed after LTK$^-$ cells received nuclear injections of pBR322 DNA. The reversion frequency of the TK$^-$ mutation in LTK$^-$ cells is extremely low. We and others have never observed a spontaneous revertant of LTK$^-$ cells to LTK$^+$ (Wigler et al., 1978).

The LTK$^-$ Transformants Contain HSV-1 Thymidine Kinase

The HSV-1 and L cell thymidine kinase activity can be distinguished by their electrophoretic separation on nondenaturing polyacrylamide gels (Figure 3). In the above experiments (Figure 3e) we show that the thymidine kinase activity in a LTK$^-$ transformant, obtained after nuclear injection with pBR322/TK DNA, was indistinguishable from the HSV enzyme with respect to this property. In a like manner five other randomly chosen LTK$^-$ transformants were shown to contain HSV-1 TK activity (data not shown).

Stability of the Transformants

Are the transformants following microinjection with pBR322/TK DNA stable in the sense that they can be

Table 1. Transformation Frequency with pBR322/TK DNA

DNA Injected	Concentration (mg/ml)	Number of Transformants per 10^3 Cells Receiving an Injection	Number of Cells Receiving an Injection
pBR322	0.33	0	10^4
pBR322/TK	0.33	2.2	5×10^3
pBR322/TK	0.33	1.2	5×10^3
pBR322/TK	0.33	2.6	5×10^3
pBR322/TK plus salmon sperm (1:5 w/w)	1.20	0.6	5×10^3

LTK$^-$ cells were grown on a 10 × 10 mm cover slip in 35 mm petri dishes. Two hundred cells per dish received nuclear injections with the respective DNA solutions. After the injections the cells were incubated for 24 hr in nonselective medium at 37°C in a 5% CO_2 incubator and then switched to HAT medium. Two weeks later the dishes were scored for the presence of a large colony.

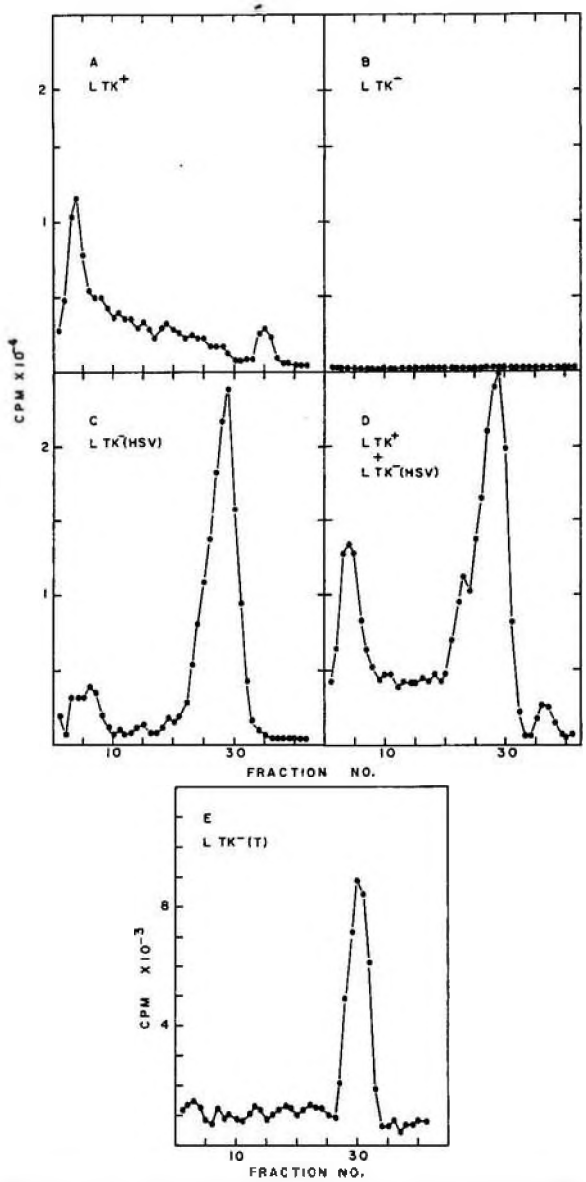

Figure 3. Electrophoretic Analysis of the Thymidine Kinase Activity in Extracts Prepared from (a) LTK$^+$ Cells, (b) LTK$^-$ Cells, (c) LTK$^-$ Cells Infected with HSV-1, (d) a Mixture of LTK$^+$ Cells and LTK$^-$ Cells Infected with HSV-1 and (e) an LTK$^-$ Transformant Obtained after Injecting pBR322/TK DNA into Nuclei of LTK$^-$ Cells

The LTK$^+$, LTK$^-$ and LTK$^-$ transformant cells were grown as monolayers in MEM plus 10% fetal calf serum. Some of the LTK$^-$ cells were infected with Herpes simplex virus I at a multiplicity of 20 and harvested 18 hr post infection. The cells were washed twice with PBS and lysed with a buffer containing 0.5% NP40, 10% glycerol, 0.1 M KCl, 0.001 M DTT, 0.001 M $MgCl_2$ and 50 μM dThd. Under this lysis condition the soluble cytoplasmic proteins are released and the remainder of the cell remains adhered to the culture dish surface. A small but detectable amount of the mitochondrial TK activity is released (A and D). The extracts were centrifuged at 30,000 × g for 30 min and applied to 5% polyacrylamide disc gels. Following electrophoresis the gels were cut into 1 mm slices and assayed for thymidine kinase as described by Lee and Chen (1976). The electrophoretic mobilities of the cellular and HSV-1 TK activities relative to the mobility of the front were 0.15 and 0.6 respectively. The R_f of the thymidine kinase activity in the LTK$^-$ transformant was 0.6, consistent with the electrophoretic mobility of the HSV enzyme.

maintained in the absence of selective medium? To answer this question each of six independent transformants was grown in nonselective medium. Every week an aliquot of each line was plated in both HAT medium (TK^+ selective medium) and nonselective medium. The number of colonies arising in both media was scored and the ratio was plotted as a function of the time the line had been maintained in nonselective medium (see Figure 4). Five of the transformants were observed to retain TK gene expression in the absence of selection. The sixth transformant did lose the ability to grow in HAT medium in the absence of selection at a rate of 3% per generation. Stable transformants could be selected from the unstable one simply by picking colonies which grew up in HAT medium after these cells had grown in nonselective medium for over 50 generations.

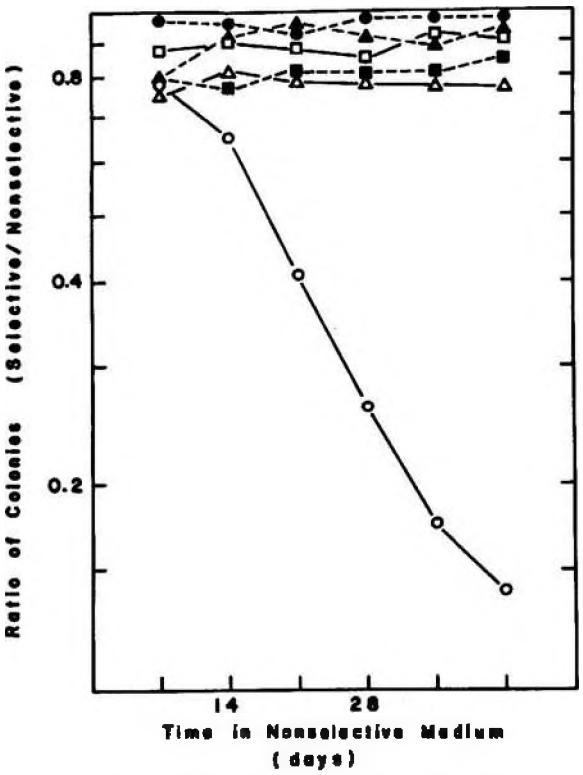

Figure 4. Stability of the TK^+ Phenotype of Six LTK^- Transformants When Grown in Nonselective Medium

Six independent TK^+ transformant cell lines obtained by nuclear injection of LTK^- cells with pBR322/TK DNA were grown in nonselective medium. Every week an aliquot of each line (approximately 3000 cells) was plated on six 100 mm petri dishes in both HAT medium (TK^+ selective medium) and nonselective medium. Ten days later the cells were fixed with methanol and stained with Giemsa. The ratio of the resultant colonies which grew up in the selective and nonselective medium was plotted as a function of the time the line had been maintained in nonselective medium (Klobutcher and Ruddle, 1979). The plating efficiency of the transformants in nonselective medium was approximately 70%.

Effect of Carrier DNA on DNA-mediated Transformation

DNA-mediated transformation of cultured mammalian cells using the calcium phosphate precipitation technique is enhanced by approximately three orders of magnitude if a carrier DNA (vertebrate DNA) is used to co-precipitate the plasmid DNA containing the desired transforming gene (Graham and van der Eb, 1973). While the role of the carrier DNA has not been fully elucidated, it appears that after the DNA is taken up by the cells as a calcium phosphate precipitate large concatemers of carrier DNA and plasmid DNA are formed. These concatemers may have the capacity to replicate independently of the host chromosome or may integrate as units into the host chromosome (Perucho, Hanahan and Wigler, manuscript submitted).

The transformation experiments in Table 1 were performed in the absence of carrier DNA. Considering the enhancement of transformation exhibited by carrier DNA with the calcium phosphate precipitation technique, it was of interest to determine whether carrier DNA would enhance transformation obtained by microinjection. As shown in Table 1 no enhancement of transformation frequency was observed when salmon sperm DNA was coinjected with pBR322/TK DNA. The same salmon sperm DNA preparation enhanced by 1000 fold pBR322/TK directed transformation of LTK^- cells to LTK^+ using the calcium phosphate precipitation technique (P. Barry, unpublished results).

Stimulation of the Transformation Frequency by Co-injecting SV40 DNA with pBR322/TK DNA

Co-injecting pBR322/TK DNA with DNA isolated from SV40 into the nuclei of LTK^- cells increases the transformation frequency to LTK^+ by a factor of approximately ten over that obtained by injection of pBR322/TK DNA alone (Table 2). The increased transformation frequency may result from SV40 providing, through recombination, DNA sequences which facilitate either replication or integration of the TK plasmid DNA. Alternatively, SV40 may provide products, in trans, which facilitate either replication or integration of the TK-plasmid DNA. To test these alternatives, recombinant HSV-TK plasmids containing different portions of the SV40 genome gene were prepared and their ability to transform LTK^- cells to LTK^+ was determined (see below).

SV40/HSV TK Recombinant Plasmids

Restriction maps of the recombinant plasmids used for the experiments to be described are illustrated in Figure 5. The first step in constructing this set of plasmids was to insert the intact SV40 genome into pBR322 through their Eco RI sites. Eco RI cleaves

Table 2. Transformation Frequency by Co-injection of pBR322–TK DNA plus SV40 DNA

DNA Injected 10^3	Concentration (mg/ml)	Number of Transformants per 10^3 Cells Receiving an Injection	Number of Cells Receiving an Injection
pBR322/TK plus SV40 (1:1 M)	0.53	15	10^3
pBR322/TK plus SV40 (1:1 M)	0.53	12	10^3
SV40	0.2	0	10^4

The experimental procedure was as described in the legend to Table 1 except that in the SV40, pBR322/TK coinjection experiments, 50 cells per dish received nuclear injections.

SV40 in the middle of the late region leaving the early region intact and functional. The orientation of the SV40 genome with respect to pBR322 was determined by cleaving the recombinant plasmid with Bam HI (Figure 6a, slot 5). The purified HSV Bam HI fragment containing the TK gene was inserted into the above pBR322/SV4O recombinant plasmid at the Bam HI sites. In the process of inserting the HSV-TK fragment a small 1 kb Bam HI fragment from pBR322/SV40 was removed. The resulting recombinant plasmid was designated pBR322/SV-O+T/TK to indicate that this plasmid contains the SV40 origin for DNA replication (Ori) as well as a functional SV40 early region (that is, capable of synthesizing T antigens). The orientation of the HSV TK gene was determined from a number of restriction enzyme digests shown in Figure 6a.

12 hr after injecting 100 molecules of pBR322/SV-O+T/TK DNA per cell into the nuclei of LTK$^-$ cells one can detect SV40 T antigen by indirect immunofluorescence assays (Figure 7). The level of T antigen per cell was comparable to that observed in SV80 cells, a human cell line which was transformed by SV40 and overproduces T antigen (Todaro, Green and Swift, 1966; Henderson and Livingston, 1974).

To generate a recombinant plasmid which still retained the SV40 origin of DNA replication but lacked sequences coding for large and small T antigen, pBR322/SV-O+T/TK DNA was partially hydrolyzed with Hind III followed by re-ligation. This DNA was used to transform E. coli. Ampicillin resistant colonies were screened on agarose gels for the presence of a recombinant plasmid missing a 1.7 kb fragment encoding for most of the early region of SV40 as well as the splicing junctions for large and small T mRNA. The resulting recombinant plasmid still retains the SV40 origin for DNA replication and was designated pBR322/SV-O/TK.

No material which cross reacts with T antigen-antiserum is observed in LTK$^-$ cells which were microinjected with pBR322/SV-O/TK DNA (data not shown).

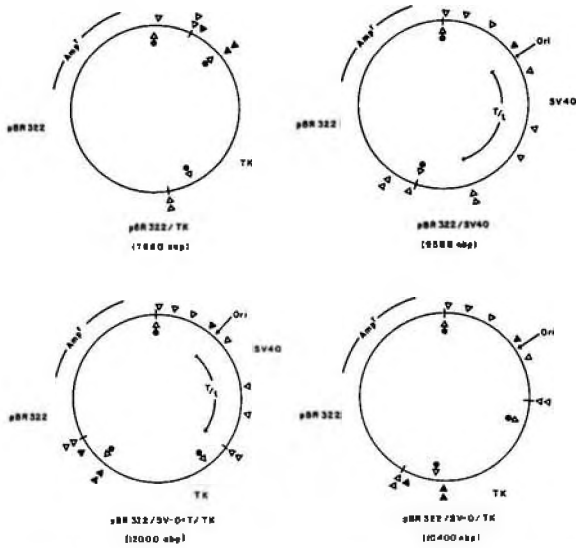

Figure 5. Restriction Maps of the Recombinant Plasmids pBR322/TK, pBR322/SV40, pBR322/SV-O+T/TK and pBR322/SV-O/TK

pBR322/TK (obtained from G. F. Vande Woude; see Enquist et al., 1979) contains the HSV-1 thymidine kinase gene as part of a 3.5 kb HSV-1 Bam HI fragment. The entire SV40 genome was ligated into pBR322 at the single Eco RI site to form pBR322/SV40. The early region of SV40 remains intact and functional in this recombinant plasmid. pBR322/SV-O+T/TK was obtained by inserting the purified 3.5 kb HSV-1 Bam HI fragment into the pBR322/SV40 recombinant plasmid. In the process of inserting the HSV-TK fragment into pBR322/SV40, a 1 kb fragment is removed which contains 0.75 kb of the coding region for SV40-VP1 and a 0.25 kb fragment of the pBR322 Tet^r gene. pBR322/SV-O+T/TK contains sequences for the SV40 origin of DNA replication (Ori), the coding sequences for large and small SV40 T antigen and the HSV-TK gene. pBR322/SV-O/TK was obtained from pBR322/SV-O+T/TK by partial hydrolysis with Hind III followed by religation. In the process two SV40 Hind III fragments (1169 and 526 nbp) were removed. These later fragments contain most of the coding sequences for SV40 large and small T antigen as well as the junctions for splicing their respective mRNAs. The symbols used to represent some of the characteristic endonuclease restriction sites of the above recombinant plasmids are: (↕) Eco RI; (▲) Bam HI; (△) Hind III; (▲) Kpn I; (▲) Bgl II.

Transformation Freguency of LTK$^-$ Cells to LTK$^+$ following Injection with Either pBR322/SV-O+T/TK or pBR322/SV-O/TK DNA

Approximately one LTK$^-$ cell in five which received nuclear injection of either pBR322/SV-O+T/TK or pBR322/SV-O/TK DNA gave rise to a cell line capable of indefinite growth in HAT medium (Table 3). For these experiments five cells per 35 mm plate were injected with one of these recombinant plasmid DNAs. The location of the five separate injected cells in each plate was recorded and the plates were monitored for colony growth in HAT medium. In some plates two or three colonies were observed growing up at the sites of the injected cells. For the data presented in Table 3 a plate was recorded as positive if it contained one or more large colonies (greater than 1000 cells per colony). This method of recording obviates problems associated with secondary colonies arising from pri-

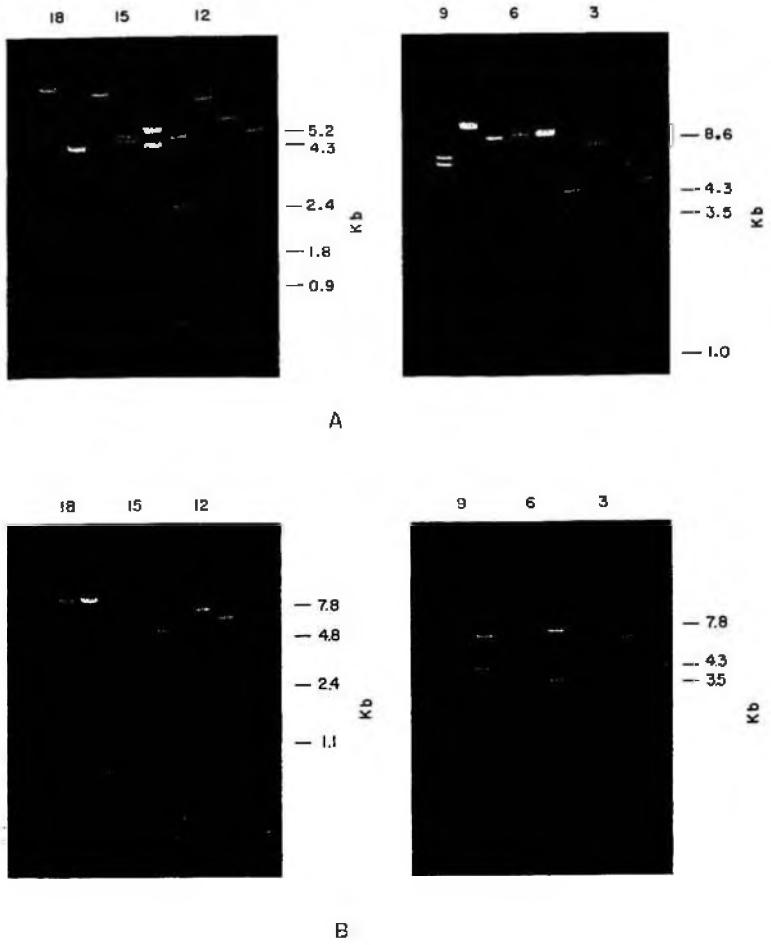

Figure 6. Restriction Fragment Analysis of the Recombinant Plasmids (a) pBR322/TK, pBR322/SV40 and pBR322/SV-O+T/TK and (b) pBR322/TK, pBR322/SV-O/TK and pBR322/SV-O+T/TK

The plasmid DNAs (0.5–1.0 μ) were digested with 2–3 units of restriction enzyme using the conditions described by Bethesda Research Lab and New England Biolabs. The DNA restriction fragments were resolved by slab gel electrophoresis in 0.8% agarose. The gels were then stained with ethidium bromide and visualized on an ultraviolet box. In (a) the uncut plasmid DNAs pBR322/TK, pBR322/SV40 and pBR322/SV-O+T/TK were applied to gel slots 1–3 and 10–12 respectively. Slots 4–6 contain the Bam HI digests of pBR322/TK, pBR322/SV40 and pBR322/SV-O+T/TK. Slots 7–9 contain Kpn I and Bgl II double digests of pBR322/TK, pBR322/SV40 and pBR322/SV-O+T/TK. Slots 13–15 contain Eco RI digests and slots 16–18 contain Hind III digests of pBR322/TK, pBR322/SV40 and pBR322/SV-O+T/TK, respectively. In (b) the uncut plasmid DNAs pBR322/TK, pBR322/SV-O/TK and pBR322/SV-O+T/TK were applied to gel slots 1–3 and 10–12, respectively. Slots 4–6 contain Bam HI digests of pBR322/TK, pBR322/SV-O/TK and pBR322/SV-O+T/TK. Slots 7–9 contain Kpn I and Bgl II double digests of pBR322/TK, pBR322/SV-O/TK and pBR322/SV-O+K. Slots 13–15 contain Eco RI digests and slots 16–18 contain Hind III digests of pBR322/TK, pBR322/SV-O/TK and pBR322/SV-O+T/TK, respectively.

mary colonies but probably underestimates the actual efficiency of transformation.

Surprisingly, the same efficiency of transformation was observed with either pBR322/SV-O+T/TK or pBR322/SV-O/TK DNA. The presence of SV40 T antigens did not influence the efficiency of transforming LTK$^-$ cells to LTK$^+$.

The efficiency of transformation was also relatively insensitive to the number of molecules of pBR322/SV-O+T/TK or pBR322/SV-O/TK DNA injected into each LTK$^-$ cell (that is, over the range from 10 to 400 molecules per cell) (Table 3).

Stability of the LTK$^-$ Transformant Resulting from Nuclear Injections with pBR322/SV-O+T/TK or pBR322/SV-O/TK DNA

The high efficiency of transformation of LTK$^-$ cells by the recombinant SV40 plasmids could result from SV40 donating to the plasmids an origin of DNA replication functional in mouse cells, thereby allowing them to be replicated efficiently without integration into the host genome. If this were the case one might anticipate that LTK$^+$ transformants arising from nuclear injections with pBR322/SV-O+T/TK or pBR322/SV-O/TK DNA would be unstable when grown in a nonselective medium. As illustrated in Figure 8 this does not appear to be the case. The stability in nonselective medium of three independent LTK$^+$ transformants arising after nuclear injections of pBR322/SV-O+T/TK and of three arising after injection of pBR322/SV-O/TK DNA was tested. All six clones were found to be stable.

The above result does not exclude efficient plasmid replication since the frequency of LTK$^-$ segregants could be very low if the cellular plasmid population were large. A segregation frequency of 0.1% per generation would have been detected. Direct evidence for the integration of the pBR322/SV-O+T/TK and pBR322/SV-O/TK plasmids into host DNA sequences by blotting analysis will be presented in a separate communication.

Extracts from the above cell lines were shown to contain TK enzymatic activities, whose electrophoretic mobility was indistinguishable from the HSV-1 enzyme (data not shown).

Discussion

When pBR322/TK DNA was injected into nuclei of mouse LTK$^-$ cells, 50–100% of the cells express

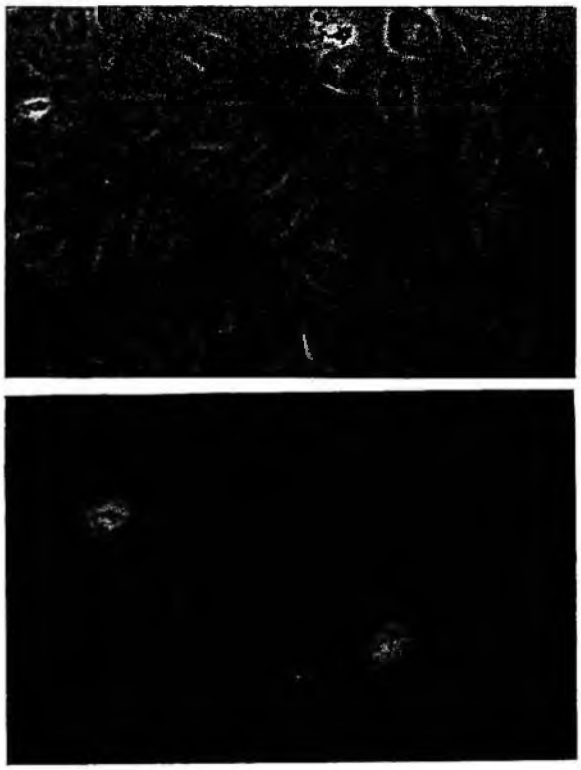

Figure 7. Immunofluorescent Assay for the Presence of SV40 T Antigen in Cells Injected with pBR322/SV-O+T/TK (a) Cells Photographed by Phase-Contrast Microscopy and (b) Cells Photographed by Fluorescent Microscopy

LTK$^-$ cells were injected with pBR322/SV-O+T/TK DNA (0.15 mg/ml) and incubated for 12 hr at 37°C in a 5% CO_2 incubator. The cells were then washed with PBS plus 1% calf serum and fixed at 0°C with acetone–ethanol (2:1). The fixed cells were then incubated with hamster anti-T serum for 30 min at 25°C, washed with PBS plus 1% calf serum and incubated with FITC-rabbit anti hamster IgG for an additional 30 min. After washing the cells with PBS plus calf serum, the coverslip containing the cells was fixed to a microscope slide upside down with 90% glycerol. For each set of experiments cover slips containing SV80 cells (human cells which were transformed with SV40 and produce high levels of T antigen) and uninjected LTK$^-$ cells were processed in parallel. Seven cells in the above field were injected with pBR322/SV-O+T/TK DNA. The amount of fluorescence observed in the injected cells was comparable to that observed in parallel reactions with SV80 cells.

thymidine kinase enzymatic activity. Of over one thousand cells injected with pBR322/TK DNA into the cytoplasm none contained detectable TK activity. Increasing the number of plasmid DNA molecules injected into the cytoplasm of each cell or increasing the time period between the injection and the assay for TK activity did not alter the results. These experiments indicate that the plasmid DNA cannot be expressed in the cytoplasm and that it does not readily gain access to the nucleus. It is not known whether the nuclear membrane itself acts as a physical barrier or whether the plasmid DNA is rapidly degraded when injected into the cytoplasm.

In light of the results described above it is interesting to speculate that the formation of the DNA-calcium

Table 3. Transformation Frequency Obtained by Injection of pBR322-SV-O+T/TK and pBR322-SV-O/TK DNA

DNA Injected	Concentration (mg/ml)	Molecules per Cell	Number of Plates Containing One or More Colonies[a]
pBR322/SV-O+T/TK	0.5	400	17/20
pBR322/SV-O+T/TK	0.125	100	19/20
pBR322/SV-O+T/TK	0.125	100	16/20
pBR322/SV-O+T/TK	0.0125	10	18/20
pBR322/SV-O+T/TK	0.0125	10	15/20
pBR322/SV-O/TK	0.4	400	13/20
pBR322/SV-O/TK	0.1	100	16/20
pBR322/SV-O/TK	0.1	100	16/20
pBR322/SV-O/TK	0.01	10	17/20
pBR322/SV-O/TK	0.01	10	18/20

[a] Five cells per plate received nuclear injections with the respective recombinant plasmid DNA. The cells were then incubated for 24 hr in nonselective medium and then switched to HAT medium. A plate was scored as positive if after two weeks of incubation in HAT medium it contained one or more large colonies (that is greater than 1000 cells per colony).

precipitate may enhance transformation by protecting the DNA as it traverses the cytoplasm. After uptake by phagocytosis a small fraction of the DNA may be shuttled into the nucleus as a precipitate where it is dissolved, releasing active genes. In such a model "competence" could reflect the cell's capacity to transport the DNA from the cytoplasm to the nucleus rather than its capacity for DNA uptake.

Although 50–100% of the cells injected with pBR322/TK DNA express TK activity, only one cell in 500–1000 injected cells can be propagated indefinitely in HAT medium. The above results can be interpreted in a number of ways. For example, it might be that only in a small proportion of the injected cells is the plasmid DNA properly integrated into the host chromosome (that is, integrated in a site that allows the HSV-TK gene to be transcribed). Alternatively, it might be that only a few of the cells that received injections provide the proper environment for independent replication of the plasmid DNA prior to integration. Such replication could result from modification of the plasmid DNA or the presence of unique factors required for replication. The above explanations are not mutually exclusive.

Most of the LTK$^+$ transformants following injection with pBR322/TK DNA were found to be stable even when grown in nonselective medium. The stability studies were initiated as soon after microinjection as possible (that is, when approximately 10^7 cells of each cell line were available). These results differ from those obtained by the calcium phosphate precipitation

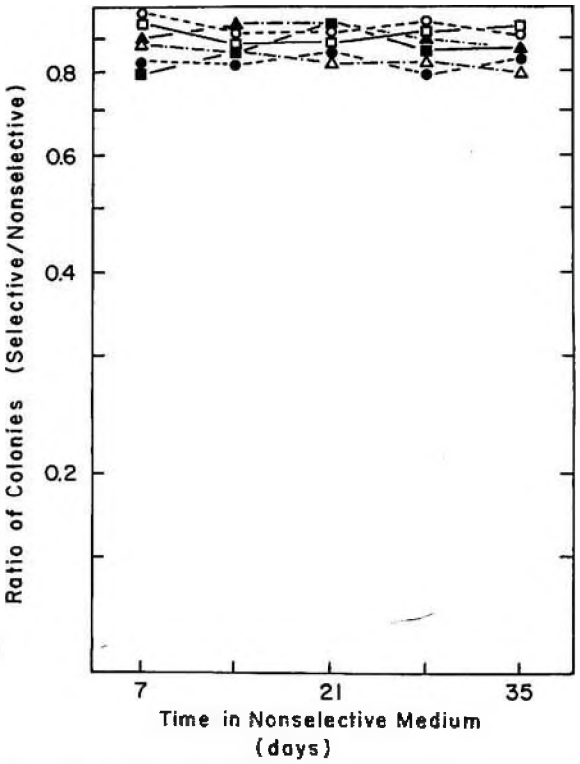

Figure 8. Stability of the TK$^+$ Phenotype of Six LTK$^-$ Transformants When Grown in Nonselective Medium

Three of the transformants were obtained by nuclear injection with pBR322/SV-O+T/TK DNA and three by nuclear injection with pBR322/SV-O-TK DNA.

The experimental protocol was as described in the legend for Figure 4. Open symbols designate transformants obtained by nuclear injections with pBR322/SV-O+T/TK DNA, closed symbols designate transformants obtained by injection with pBR322/SV-O/TK DNA.

method where stability of the transformed phenotype, in the absence of selection, is often not observed (Wigler et al., 1977; Graf et al., 1979; Wigler et al.,1979a; Willecke et al., 1979). The difference in the results obtained by the two methods could be explained if an early event in the calcium phosphate precipitation method were the incorporation of the transforming gene into the carrier DNA. The carrier-transforming gene complex may be capable of independent replication and therefore susceptible to loss due to unequal segregation during cellular division, or if incorporated into the host chromosome as a large package, the unit itself may be sensitive to loss via a recombinational mechanism. Since carrier DNA is not used with the microinjection procedure, these options are not available.

Using the microinjection procedure the number of gene copies injected into each cell can be controlled. In the range of 1–100 molecules, the number of pBR322/SV-O/TK incorporated into the transformant host DNA sequences is roughly proportional to the number of molecules injected into each cell (unpublished results). The transformation frequency to LTK$^+$ is relatively insensitive to the number of gene copies injected into each cell. The system therefore rapidly saturates and high transformation frequencies can be obtained by injecting only a few molecules per cell (that is, less than five).

Approximately 20% of the LTK$^-$ cells which received injections with either pBR322/SV-O+T/TK or pBR322/SV-O/TK DNA were transformed to LTK$^+$. The presence or absence of a functional SV40 early region did not alter the transformation frequency. The increased transformation frequency observed with these recombinant plasmid DNAs does not appear to result from independent replication. Loss of the plasmid is not observed when the transformants are grown in nonselective medium. One could argue that so many copies of the self replicating recombinant plasmid DNAs are present in each transformant that loss due to segregation could not be detected. Southern blotting analysis of these transformants indicates, however, that in most of the transformants the plasmid DNA is integrated into the host DNA sequences. Free plasmid DNA is not observed (unpublished results).

How do SV40 DNA sequences near the origin enhance the transformation frequency of the TK recombinant plasmids? A working hypothesis is that these DNA sequences facilitate the integration of the plasmid DNA into the host chromosome either because they contain palindromes and/or because they contain regions of homology to middle-repetitive host sequences. Jelinek and his colleagues (Jelinek et al., 1980) have described ubiquitous, interspersed, repeated DNA sequences in mammalian genomes which have homology to the SV40 origin of DNA replication. The SV40 sequences are not unique in their ability to stimulate the transformation efficiency of TK recombinant plasmids. Comparable transforming efficiencies have been obtained by inserting the 3′ R 5′ terminal repeats from the Schmidt-Ruppin-A strain of the avian sarcoma virus into the pBR322/TK plasmid (our unpublished results, in collaboration with P. Luciw, S. M. Bishop and H. E. Varmus). The 3′ R 5′ terminal repeat sequences of RNA tumor viruses do not have homology to the SV40 origin of DNA replication. However, they do have an intriguing DNA sequence similarity to bacterial IS sequences and are postulated to be used for integrating the provirus into the host chromosome (Hughes et al., 1978).

In this study I have emphasized the use of the microinjection technology to obtain and characterize DNA-mediated transformants. For many experiments it will be advantageous to look simply for gene expression rather than to wait for a transformant to grow up. Injection of DNA into the nuclei of cultured mammalian cells provides a convenient and sensitive bioassay for gene expression. The equivalent of 1% of a mammalian genome can be injected into a single cell. When coupled with immunofluorescent, radioimmune and autoradiographic techniques, detecting the expression of a single gene copy is possible.

In summary, microinjection of DNA using glass mi-

cropipettes provides an alternative procedure for introducing genes into cultured mammalian cells. When appropriate recombinant plasmids are used, transformation frequencies approaching unity are obtained. With these high transformation frequencies the application of the technique to embryological problems becomes feasible.

Experimental Procedures

The methods used for culturing the cells, preparing cell extract and autoradiography have been described in detail elsewhere (Sharp, Capecchi and Capecchi, 1973; Wahl, Hughes and Capecchi, 1975; Capecchi et al., 1977).

Microinjection

The recipient cells were grown on small glass slides (10 × 10 mm). The solution of macromolecules was injected into the cells via glass micropipettes having tip diameters ranging from 0.1 to 0.5 μ. The pipettes were prepared from glass capillaries (Omega Dot Tubing, 1.2 mm OD, W. P. Instruments) on a Model P77 Brown-Flaming micropipette puller (Sutter Instruments). Injections were carried out under direct visual control on a fixed stage of an inverted phase contrast microscope (Leitz Diavert, 400X). Movement of the micropipettes was controlled with micromanipulators (Narishige MO-15, modified) which have a hydrolic microdrive along the pipette axis. The fluid containing the macromolecules is forced into the cells under constant pressure supplied by a Hamilton threaded plunger syringe (Model 87000). The amount of fluid injected into each cell was controlled with moderate precision (within a factor of two) by visually monitoring changes in the cellular refractive index as the fluid enters the cell and regulating the time that the micropipette remained in the cell. The average volume (10–20 femtoliters) injected into each cell was determined by injecting ^{3}H-dTTP (10 μc/μl) into five thousand cells, washing the cells with PBS and then measuring the radioactive content by liquid scintillation counting. For L cells this volume corresponds to 1–2% of the cell volume. With practice one can attempt injections into 500–1000 cells per hour with a successful transfer of material being assured in 50–100% of them. Similar procedures for injecting macromolecules into cultured mammalian cells have been described by Graessmann and Graessmann (1976), by Diacumakos (1973) and by Stacey and Allfrey (1976).

Preparation of Recombinant Plasmids

Restriction enzymes and T4 DNA ligase were obtained from BRL and New England Biolabs and used under conditions recommended by the vendors. Plasmid DNAs were isolated from cultures of E. coli HB101, grown to saturation in NZYPD medium (per liter: 5 g NaCl, 2 g $MgCl_2$, 10 g NZ amine A, 1 g caseamino acids and 5 g yeast extract). The cells were lysed with 0.03 M NaOH, 0.003 M EDTA and 0.5% SDS. The chromosomal DNA was precipitated with 1 M NaCl and 3% PEG. Following the addition of NaCl and PEG the extracts were incubated for 2 hr at 4°C and centrifuged at 20,000 × g for 30 min. The supernatants containing the plasmid DNA were extracted twice with chloroform-phenol. The RNA was removed by gel filtration on a Biorad A50 column.

SV40 DNA was isolated from lytically infected TC7 cells and purified by the procedure of Trilling and Axelrod (1970). The HSV-1 Bam HI fragment which contains the HSV-TK gene was purified from pBR322/TK DNA by digestion to completion with Bam HI and fractionation on a 0.9% agarose gel.

SV40 was inserted into pBR322 by digesting both to completion with Eco RI. The pBR322 DNA was then treated with alkaline phosphatase [(pH 9) 68°C for 30 min] to prevent self ligation. After ligation of pBR322 and SV40 DNA with T4 ligase it was used to transform E. coli strain HB101. The resulting ampicillin resistant colonies were screened for recombinant plasmids on agarose gels. pBR322/SV-O+T/TK was prepared as described above by inserting the purified HSV-Bam HI fragment into pBR322/SV40. Following ligation the DNA was used to transform E. coli HB101. 90% of the ampicillin-resistant colonies contained the desired recombinant plasmid. pBR322/SV-O/TK was prepared from pBR322/SV-O+T/TK DNA by partial hydrolysis with Hind III followed by ligation with T4 ligase.

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