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Biochemical Transfer of Single-Copy Eucaryotic Genes Using Total Cellular DNA as Donor

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Summary

Previous studies from our laboratories have demonstrated the feasibility of transferring the thymidine kinase (tk) gene from restriction endonuclease-generated fragments of herpes simplex virus (HSV) DNA to cultured mammalian cells. In this study, high molecular weight DNA from cells containing only one copy of the HSV gene coding for tk was successfully used to transform Ltk$^-$ cells to the tk$^+$ phenotype. The acquired phenotype was demonstrated to be donor-derived by analysis of the electrophoretic mobility of the tk activity, and the presence of HSV DNA sequences in the recipient cells was demonstrated. In companion experiments, we used high molecular weight DNA derived from tissues and cultured cells of a variety of species to transfer tk activity. The tk$^+$ mouse cells transformed with human DNA were shown to express human type tk activity as determined by isoelectric focusing.

Introduction

The transfer of specific genes, free of chromosomal protein, may facilitate the analysis of the control of gene expression in complex eucaryotes. The availability of sensitive assay systems for transformation may ultimately allow the isolation of any gene for which selective growth conditions exist. To explore this possibility, we previously developed a transformation system for the thymidine kinase (tk) gene of herpes simplex virus, HSV-1. This system was chosen initially because the viral genome is orders of magnitude less complex than the cellular genome. Through a series of electrophoretic fractionations in concert with transformation assays, we isolated a unique 3.4 kb fragment of viral DNA which is capable of efficiently transferring tk activity to mutant Ltk$^-$ cells (Wigler et al., 1977). Analysis of the transformed cell DNA in molecular hybridization experiments demonstrated that a single copy of the tk gene was covalently integrated into the DNA of all transformants (Pellicer et al., 1978).

The development of a system for the transfer of the HSV tk gene to mutant mouse cells has permitted us to extend these studies to unique cellular genes. In addition, the availability of cell lines bearing a single copy of the HSV tk gene has allowed us to trace the fate of this gene when DNA from these cells is used as donor in transformation experiments. We have found that high molecular weight DNA obtained from tk$^+$ tissues and cultured cells from a variety of organisms can be used to transfer tk activity to tk$^-$ mutant mouse cells. The resulting tk activity expressed in recipient cells is donor-derived.

Results

Transformation with Viral tk Integrated in Cellular DNA

Treatment of mutant mouse cells (Ltk$^-$) deficient in thymidine kinase with the 3.4 kb Bam I restriction endonuclease fragment of HSV-1 DNA results in the appearance of numerous surviving colonies which stably express the tk phenotype (Wigler et al., 1977). By incorporating various improvements into the transformation protocol (see Experimental Procedures), we now routinely obtain efficiencies of approximately 1 colony per 10^6 cells per 40 pg of purified HSV tk gene. In the mammalian genome, a single-copy gene is present at less than one part per million. If we extrapolate from the transformation efficiency which we observe for the transfer of the viral tk gene and estimate the molecular weight of the haploid mouse genome to be 2×10^{12} daltons, we can expect to observe the transfer of a specific gene once per 10^6 cells per 30 μg of genomic DNA. Under our present transformation conditions, we can therefore expect to observe transfer of single-copy genes when total genomic DNA is used as donor.

Initial experiments designed to transfer the tk gene from cellular DNA to mutant tk$^-$ cells were performed with donor DNA purified from HSV tk$^+$-transformed Ltk$^-$ mouse cells. The choice of this donor for initial studies was dictated by several considerations. First, we have previously shown that HSV tk$^+$ cells contain only a single copy of the viral tk gene per cellular genome (Pellicer et al., 1978). Second, the properties of the viral enzyme are sufficiently different from those of the murine enzyme to allow characterization of the acquired tk activity by gel electrophoresis. Finally, the availability of purified restriction fragments containing the viral tk gene allows us to detect and analyze the physical state of the transferred gene in the DNA of the transformant.

The recipient cell chosen for these experiments

was Ltk$^-$, clone D, a clone resistant to bromodeoxyuridine and deficient in cytoplasmic thymidine kinase (Kit et al., 1963). Ltk$^-$ cells are unable to grow in medium containing HAT (hypoxanthine, aminopterin and thymidine), in which survival depends upon the presence of both salvage pathway enzymes, thymidine kinase and hypoxanthine-guanosine phosphoribosyl transferase (Littlefield, 1963). These cells have an exceedingly low rate of spontaneous reversion to the tk$^+$ phenotype, which greatly facilitates the scoring of transformants.

High molecular weight DNA (>40 kb) was purified from a number of independently derived tk$^+$ clones. This DNA was co-precipitated with calcium phosphate, and 20 μg were added to each culture dish containing 10^6 cells. After 4 hr of exposure to DNA, cells were refed growth medium, and 20 hr later, cultures were refed growth medium containing HAT. Cultures were fed HAT medium every 2–3 days, and after 2 weeks, the surviving colonies were counted. In each experiment, DNA from Ltk$^-$ was used as a control. Data from a series of transformation experiments are summarized in Tables 2 and 3.

Transformation was attempted using DNA purified from four independently derived clones of Ltk$^-$ which contain the viral tk gene (Table 1). Transformation assays with DNA purified from the four HSV tk$^+$ transformants gave rise to numerous colonies (Table 2). As expected, DNA obtained from Ltk$^-$ was unable to transfer tk activity to Ltk$^-$ cells. For clarity, we define primary transformants as the original HSV tk$^+$ mouse cells which were derived following transfer of purified viral DNA. We define secondary transformants as tk$^+$ cells obtained following transfer of cellular DNA extracted from primary transformants. It is apparent from Table 2 that the frequency of transformation varies for DNA derived from different sources. DNA derived from clones LH2b, LH7 and LHHB resulted in transformation frequencies 3–16 times greater than predicted. DNA from clone LHH5-1 generated colonies at a frequency less than that predicted above.

Origin of the tk Activity in Secondary Transformants

The transformation frequencies which we observe (Table 2) range from one colony per 1 × 10^5 cells

Table 1. Derivation of Cell Lines

Cell Line	Transforming DNA[a]
LH2b	Bam I-generated, 3.4 kb doublet from HSV DNA
LH7	Bam I-generated, 3.4 kb doublet from HSV DNA
LHHB-1	Homogeneous Bam I-generated, 3.4 kb singlet from HSV DNA
LHH5-1	Hpa 8.3 kb fragment from HSV DNA
L(LHH5-1)1	Total cellular DNA from LHH5-1
L(LHH5-1)2	Total cellular DNA from LHH5-1

[a] See Pellicer et al. (1978).

Table 2. Transformation Data: HSV tk Gene

DNA Source	Total Colonies/Total Dishes	Relative Transformation Efficiency[a]
Ltk$^-$	0/20	0.0
LH7	95/10 (9.5)	16.0
LH2b	16/9 (1.8)	3.0
LHHB	78/10 (7.8)	13.0
LHH5-1	4/20 (0.2)	0.3

[a] The calculation of transformation (colony per genome equivalents) efficiency was performed assuming that the tk gene is present only once per haploid genome. The total number of genome equivalents (in 20 μg of DNA) added per plate was directly determined from the genome size of donor. Transformation efficiency is then normalized to the transformation efficiency of the 3.4 kb Bam I HSV tk gene (one colony per 10^6 cells per 40 pg of purified DNA).

Table 3. Transformation Data: Indigenous tk Gene

DNA Source	Total Colonies/Total Dishes	Relative Transformation Efficiency[a]
Ltk$^-$ (Mouse Cells)	0/30	0.0
Drosophila Embryo Cells	0/10	0.0
Slime Mold	0/10	0.0
Salmon Sperm	0/10	0.0
LM (Mouse Cells)	63/10 (6.3)	10.5
Mouse Liver	28/4 (7.0)	12.0
CHO (Hamster Cells)	72/10 (7.2)	12.0
Chicken RBC	31/10 (3.1)	2.5
Calf Thymus	62/8 (7.8)	13.0
HeLa (Human Cells)	9/9 (1.0)	3.3

[a] See Table 2 for explanation.

to one colony per 5×10^6 cells. In our studies with the recipient cell Ltk$^-$ over the past years, we have never observed a single spontaneous revertant. Our estimate of the rate of spontaneous reversion of Ltk$^-$ to tk$^+$ is $<10^{-9}$. The appearance of even a single colony in cellular transformation experiments is therefore significant, and strongly suggests that expression of the tk$^+$ phenotype results from the introduction and expression of foreign DNA. Nevertheless, the expression of tk activity in these transformed cells conceivably could result from either reversion or reactivation of wild-type enzyme rather than the introduction and expression of a new tk gene from donor DNA. Analysis of the electrophoretic properties of the tk activities of the transformed cells allows us to distinguish among these possibilities. The size and charge of the murine and viral tk activities are sufficiently different to permit separation by nondenaturating polyacrylamide gel electrophoresis. In Figure 1A, we observe that the relative mobility (R_f) of the wild-type murine tk activity is 0.18; the R_f of HSV-1 tk, however, is 0.4. Electrophoresis of the cytosol of two secondary transformants (see Table 1) demonstrates a single peak of tk activity with an R_f of 0.40, identical to that of the donor cell. Transformation with cellular DNA therefore results in the introduction and expression of the viral tk gene from donor DNA.

Physical Presence of the tk Gene in Secondary Transformants

The use of donor DNA derived from cells originally transformed with viral tk DNA allows a direct analysis of the physical state of the tk gene in recipient cells. It is possible to determine the size, number and arrangement of the HSV tk gene in transformed cell DNA by eluting restriction endonuclease-treated DNA from agarose gels onto nitrocellulose filters. Highly radioactive tk DNA is then annealed with these filters. The distribution of tk sequences within transformed cell DNA is determined by autoradiography. This experimental design derives from the powerful hybridization technology originally introduced by Southern (1975), which was previously used to demonstrate the presence of a single integrated copy of the HSV tk gene in Ltk$^-$ cells transformed with HSV-1 DNA (Pellicer et al., 1978).

DNA fragments that contain the viral tk gene have been purified to homogeneity. One such fragment, 8.3 kb in length, is obtained following Hpa I digestion of HSV DNA. A clone of tk$^-$ cells transformed with this fragment (LHH5-1) was the source of cellular DNA in one of the transformation experiments described above. In initial experiments, it was necessary to examine the organization of the tk gene in donor DNA.

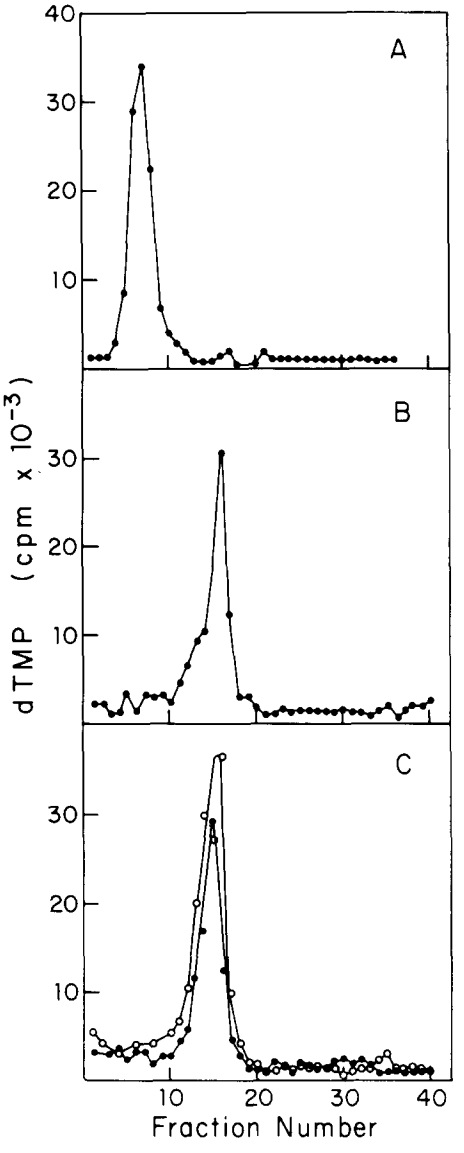

Figure 1. Electrophoretic Pattern of Thymidine Kinase Activities from Cytoplasmic Fractions of Various Cell Lines

The 30,000 × g supernatants of homogenates from four cell lines were applied to 5% polyacrylamide gels. The gels were electrophoresed and sliced into 2 mm slices. Each slice was assayed for thymidine kinase activity as described in Experimental Procedures. (A) LM (wild-type mouse extract), (B) LHH5-1 (primary transformant), (C) L(LHH5-1)1 and L(LHH5-1)2 (secondary transformants).

A restriction map of the Hpa I-generated 8.3 kb fragment of HSV-1 DNA is shown in Figure 2. This DNA fragment contains four sites of cleavage for the endonuclease Bam I. The structural gene for tk is entirely contained within the 3.4 kb Bam I fragment. If transformation resulted from the introduction of the intact 8.3 kb fragment, cleavage of cellular DNA with Bam I should generate five fragments homologous to the Hpa I fragment. Bam I-treated transformant DNA, however, contains only

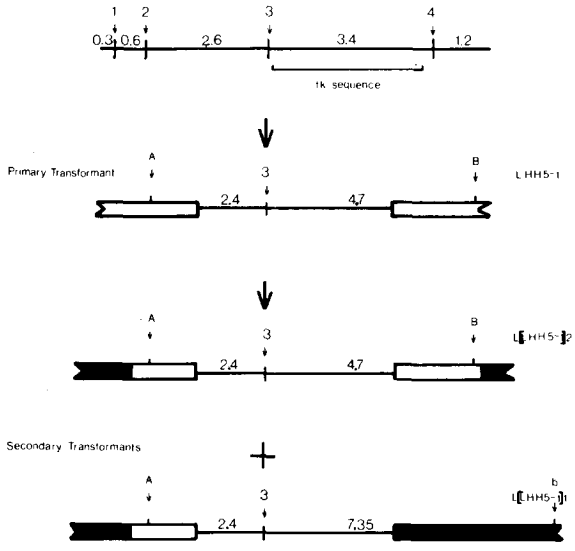

Figure 2. Restriction Map of the HSV tk Gene in Viral DNA and Cellular DNA from Primary and Secondary Transformants

The upper portion of this figure indicates the Bam I cleavage sites in the 8.3 kb fragment of viral DNA containing the tk gene. The tk structural gene sequence is noted at the leftward end of the 3.4 kb fragment. Transformation with the 8.3 kb fragment resulted in the loss of three Bam sites, with the retention of the tk structural gene sequence and BAM site number 3 generating a primary transformant. The organization of the Bam sites in DNA from the primary transformant was determined from Figure 3, slot B. Transformation with high molecular weight DNA from the primary transformant generated two secondary transformants. The organization of the tk gene and the Bam sites in these DNAs were determined from Figure 3, slots C and D. Bam sites labeled with numerals result from cleavage within viral DNA. Sites labeled with uppercase letters reflect sites in primary transformant DNA, and sites labeled with lowercase letters reflect sites in secondary transformant DNA. The size of the fragments is indicated in kb. This model is one of four logically equivalent models that fit the data from Figure 3.

two annealing fragments (Figure 3). These data suggest that nucleolytic attack of the 8.3 kb fragment occurred during the transformation process, resulting in the loss of three Bam I sites, and that the remaining fragment has integrated into host DNA (Figure 2).

High molecular weight cellular DNA from this primary transformant was used to transform fresh Ltk$^-$ cells, generating two secondary transformants L(LHH5-1)1 and L(LHH5-1)2. The DNA from these clones was then cleaved with Bam I, and the organization of tk gene sequences was analyzed. The annealing profile observed with L(LHH5-1)2 is identical to that of donor DNA. In this instance, transformation resulted from the acquisition of DNA sequences retaining the original distribution of host sequences about the tk gene (Figure 2). The pattern observed with transformant L(LHH5-1)1 is more difficult to interpret. The low molecular weight band observed in donor DNA is preserved, but the second annealing fragment is increased in size. One possible interpretation for the change in

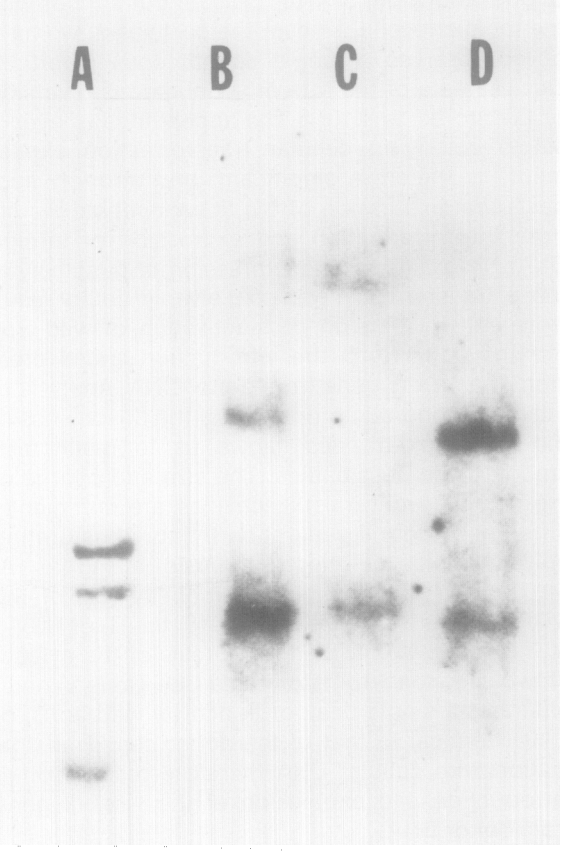

Figure 3. Identification of HSV tk-Specific Sequences in Cells Transformed by Cellular DNA Containing Only One Copy of the HSV tk Gene

High molecular weight DNAs obtained from LHH5-1, L(LHH5-1)1 and L(LHH5-1)2 were cleaved with the enzyme Bam I and electrophoresed on 0.9% agarose gels. The DNA was denatured in situ, transferred to nitrocellulose filters and then annealed with ^{32}P-tk DNA. The 8.3 kb tk gene probe was derived by cleavage of HSV-1 DNA with Hpa I. Bam I-digested DNA from LHH5-1 [a primary transformant (slot B)], L(LHH5-1)1 [a secondary transformant (slot C)] and L(LHH5-1)2 [a secondary transformant (slot D)] are shown. As a reference, 0.5 ng of HSV-1 DNA were digested with Hpa I and Bam I and run in slot A.

size of the tk fragment is described in Figure 2. These results demonstrate that secondary transformants contain at least one copy of the HSV tk gene.

Transformation with Indigenous Cellular Genes

These experiments have demonstrated the feasibility of transferring a unique gene without prior fractionation of the donor genome. We therefore attempted the transfer of indigenous cellular genes. High molecular weight DNA was isolated from LM, a line of mouse cells which expresses tk activity, and also from mouse liver. Transformation was carried out as described earlier, and after 2 weeks, colonies surviving in HAT medium were scored. With LM DNA, 65 colonies were observed in 10 culture dishes, and 28 colonies were observed

in 4 culture dishes with mouse liver DNA (Table 3). In contrast, Ltk$^-$ DNA failed to produce a single colony.

These experiments demonstrated the feasibility of intraspecific gene transfer. We next asked whether transformation could also be effected with DNA from distantly related eucaryotic organisms. High molecular weight DNA was purified from Dictyostelium, Drosophila embryo cultures, salmon sperm, chick erythrocytes, cultured hamster cells, calf thymus and HeLa cells. Chick, calf, hamster and human DNA generated numerous surviving colonies, while no transformation was observed with Dictyostelium, Drosophila or salmon DNA. We conclude that both intra- and interspecific transfer of the tk gene can be effected with high efficiency under our transformation conditions.

tk Activity of Transformants Is Donor-Derived

The appearance of surviving colonies following transformation assays with cellular DNA could result from reactivation of the murine tk, or from the introduction of a new wild-type tk gene coded for by donor DNA. As discussed earlier, the exceedingly low frequency of spontaneous reversion of the recipient cells, coupled with the inability to generate tk$^+$ transformants using Ltk$^-$ DNA as donor, argues strongly that the tk$^+$ phenotype which we observe following transformation results from the introduction of a new structural tk gene into tk$^-$ cells. The human tk enzyme displays biochemical properties distinct from those of the mouse, enabling us to determine the source of the tk expressed in transformants. The pI of human tk is 9.7, whereas the murine tk activity has a pI of 9.0 (Kit et al., 1974). Extracts of LM cells, HeLa cells and transformants generated with purified HeLa DNA were analyzed by isoelectric focusing in polyacrylamide slabs. The tk activity was localized by assaying the conversion of TdR to TMP in situ. The product of this reaction, ^{3}H-TMP, was blotted out of the gel onto PEI plates which were then analyzed by fluorography. Figure 4 demonstrates that the pI of transformed cell tk is identical to that of human tk and differs from the more acidic murine tk. Transformation must therefore result from the expression of the donor tk gene.

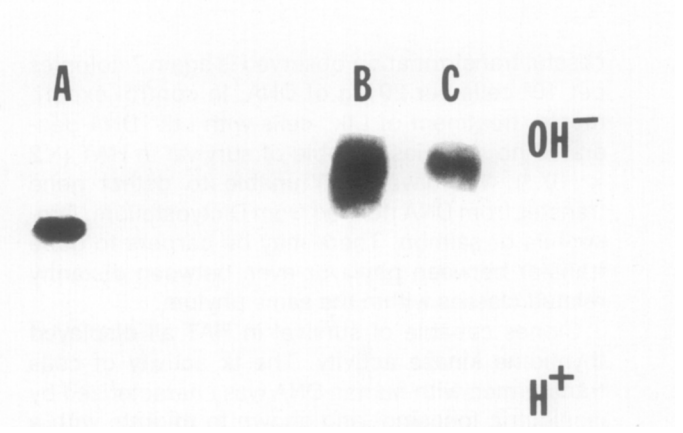

Figure 4. Isoelectric Focusing of Thymidine Kinase Activity in Gels

The 30,000 × g supernatants from homogenates of LM [a wild-type mouse cell (slot A)], HeLa [a human cell line (slot B)] and L(HeLa)-I [a tk$^-$ mouse cell transformed using DNA from tk$^+$ HeLa cells (slot C)] were focused on 4.5% acrylamide gels. Thymidine kinase activity was assayed in situ, and the product was blotted out onto PEI-cellulose and localized by fluorography as described in Experimental Procedures.

Discussion

This study demonstrates the transfer of thymidine kinase activity to mouse Ltk$^-$ cells using high molecular weight cellular DNA as donor. These experiments represent a logical extension of previous work in our laboratories, in which we demonstrated the stable transformation of tk$^-$ cells to the tk$^+$ phenotype using purified restriction endonuclease fragments of HSV-1 DNA as donor (Wigler et al., 1977).

A single copy of the viral tk gene has been found in all independently derived HSV tk$^+$ clones examined (Pellicer et al., 1978). Our initial experiments demonstrating transfer of single-copy genes with cellular DNA were therefore performed with DNA purified from HSV tk$^+$ cells. The addition of these DNA preparations to Ltk$^-$ cells, followed by selection in HAT medium, resulted in the appearance of numerous surviving colonies. The maximum frequency of transformation observed was 10 colonies per 10^6 cells per 20 μg of DNA, a frequency 40 fold higher than predicted from studies of transfection with the purified HSV tk gene. The tk activity expressed in these secondary transformants was demonstrated to be viral in origin by its electrophoretic mobility. We exploited the elegant technology of Southern (1975) to identify the number and location of the tk sequences that are liberated upon restriction endonuclease cleavage of transformed cell DNA. The data strongly suggest that the structural gene for tk is present in both primary and secondary transformants. The identification of viral tk activity and the detection of HSV tk gene sequences in the DNA of transformed Ltk$^-$ cells demonstrates that the transformation which we observe using total cellular DNA as donor results from the introduction and expression of DNA sequences coding for the viral tk.

Successful transfer of the integrated HSV tk gene immediately suggested the possibility of transfer of the indigenous cellular tk gene. We have demonstrated that DNA from various species of mammals and birds can be used to transfer tk activity to murine Ltk$^-$ cells. The maximum frequency of suc-

cessful transformation observed is again 7 colonies per 10^6 cells per 20 μg of DNA. In control experiments, treatment of Ltk$^-$ cells with Ltk$^-$ DNA generated no colonies capable of survival in HAT ($<2 \times 10^{-8}$). We have been unable to detect gene transfer from DNA derived from Dictyostelium, Drosophila or salmon. There may be barriers to gene transfer between phyla or even between distantly related classes within the same phylum.

Clones capable of survival in HAT all displayed thymidine kinase activity. The tk activity of cells transformed with human DNA was characterized by isoelectric focusing, and shown to migrate with a pI identical to human tk. Thus the conclusion that cells take up and express single-copy genes from complex eucaryotic DNA appears to be firm.

The method which we have used to transfer the thymidine kinase gene can, in principle, be applied to any gene for which conditional selection criteria are available. In preliminary experiments, we have successfully transferred the phenotypic marker, ouabain resistance, from rodent cells to primate cells. In practice, the efficiency of gene transfer can be expected to be a function of the recipient cell, the source of the gene being transferred and the stringency of the selection criteria. In order for gene transfer to be readily detectable, it must occur at a frequency higher than the spontaneous rate of mutation of the recipient to the phenotype selected. The frequencies which we observe for the transfer of the tk gene to Ltk$^-$ range from 2×10^{-7} to 1×10^{-5}. This is also the frequency range observed for spontaneous mutation at many interesting loci in cultured somatic cells. Improvements in transformation efficiency or prefractionation of donor DNA can be expected to extend the usefulness of this technique.

Transfer of single-copy genes in eucaryotes has also been achieved using metaphase chromosomes as donor (McBride and Ozer, 1973, Willecke and Ruddle, 1975). The transfer of single-copy genes using genomic DNA as donor has clear advantages: DNA can be obtained from interphase cells; genomic DNA can be cleaved with restriction enzymes and subsequently fractionated; distances between linked genes can, in theory, be precisely determined; and, most important, DNA-mediated gene transfer can be used as a bioassay allowing the purification and subsequent amplification of specific genes. Transformation with restriction endonuclease-cleaved, size-fractionated viral DNA fragments has allowed the purification of viral genes responsible for morphologic transformation (Graham et al., 1974) and the herpes simplex genes coding for thymidine kinase (Maitland and McDougall, 1977; Wigler et al., 1977). This approach, while successful with viral genomes, cannot be used to purify the single-copy genes of the vastly more complex mammalian genomes. An alternate approach involves the construction of phage libraries containing an entire eucaryote genome. Using transformation as a bioassay, clones bearing specific genes can then be identified. This approach may ultimately allow the isolation of genes for which specific hybridization probes are difficult to obtain. The availability of cloned genes and the ability to select mutants at these loci will facilitate the analysis of the nature of mutational events in higher eucaryotes.

Experimental Procedures

Cell Culture and Virus Production

Murine Ltk$^-$ cells (clone D) (Kit et al., 1963) were obtained from Dr. Patricia Spear, and maintained in DME supplemented with 10% calf serum and 30 μg/ml of BUdR without antibiotics. Prior to transformation, cells were passed in DME with serum containing antibiotics without BUdR.

The F strain of HSV-1 was grown and titrated in monolayers of Vero cells as previously described (Nishioka and Silverstein, 1977).

Isolation and Fractionation of Viral DNA and Preparation of the tk Probe

Intact HSV DNA was isolated and digested with Hpa I. The restricted DNA was fractionated by electrophoresis through 0.7% agarose gels (40 × 20 × 0.3 cm) for 40 hr at 80 V. The 8.3 kb fragment containing the tk gene was extracted from the gel as previously described (Pellicer et al., 1978). The purified fragment containing the tk gene was labeled to high specific activity by nick translation as described by Maniatis, Jeffrey and Kleid (1975). The reaction mixture contained 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 50 μg/ml BSA, 1 ng/ml DNAase I, 4.0 μM ^{32}P-deoxyribonucleotide triphosphates (200–300 Ci/mmole), 1–2 μg/ml of DNA fragment and 1 unit of DNA polymerase per 0.1 μg of DNA. The reaction was incubated at 15°C for 1 hr, and the product was isolated by phenol extraction and column chromatography on Sephadex G-50. The final product had a specific activity of $2–4 \times 10^8$ cpm/μg.

Extraction of DNA

DNA was isolated from either frozen tissue or cultured cells. If tissue was the source of DNA, it was extracted as described by Axel, Cedar and Felsenfeld (1973), using the buffers of Marshall and Burgoyne (1976). DNA was extracted from cultured cells as previously described (Pellicer et al., 1978).

Transformation

The transformation protocol used was that described by Bacchetti and Graham (1977), with modifications. Sterile, ethanol-precipitated viral or high molecular weight eucaryotic DNA was gently resuspended in 1 mM Tris (pH 7.9), 0.1 mM EDTA. DNA (at 40 μg/ml) was adjusted to 250 mM $CaCl_2$ and added slowly to an equal volume of sterile 2× HBS [280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2PHO_4 (pH 7.12)] with constant agitation. For transformation assays with the purified HSV DNA, high molecular weight salmon sperm DNA was used as carrier. The calcium phosphate-DNA precipitate was allowed to form for 30 min, and 1 ml of precipitate was added to the 10 ml of medium that covered the recipient cells. For each transformation experiment, recipient cells were plated from the same cell pool at 5×10^5 per 10 cm plate 24 hr prior to the experiment, and refed growth medium 4 hr prior to the addition of DNA. After 4 hr of exposure to DNA, the medium was replaced with fresh medium, and the cells were allowed to

incubate for an additional 20 hr, at which time the growth medium was changed to selective HAT medium (DME containing 10% calf serum, 15 μg/ml hypoxanthine, 0.2 μg/ml aminopterin, 5.0 μg/ml thymidine). The medium was changed the next day and then every third day for 2–3 weeks until tk^+ clones developed. At this time, clones were picked from individual plates. Colonies were scored following formaldehyde fixation and staining with Giemsa. As a control, in each experiment 10 plates of recipient cells were treated with Ltk^- DNA under the transformation conditions described above.

Filter Hybridization

The DNA fragments from agarose slab gels (21 × 18 × 0.6 cm) were transferred to nitrocellulose filter sheets as described by Ketner and Kelly (1976). Annealing with labeled probe was carried out as previously described (Pellicer et al., 1978).

Derivation of Viral Transformed tk^+ Cell Lines

Four independently derived biochemical transformants were used in these experiments. Two of these lines, LH2b and LH7, were derived by transformation with the Bam I-generated, 3.4 kb doublet of HSV-1 DNA. They differ only in the amount of viral DNA used to effect transformation. Each cell line contains only a single copy of the gene coding for the HSV-1-specified thymidine kinase as assayed by solution and filter hybridization (Pellicer et al., 1978). LHHB was derived by transformation with the unique 3.4 kb fragment obtained after digestion of the 8.3 kb Hpa I fragment with Bam. It too contains only a single copy of the HSV tk gene. LHH5-1 was derived following transformation with the purified 8.3 kb Hpa I fragment of HSV-1 DNA.

Electrophoresis of Thymidine Kinase Activity

Polyacrylamide gel electrophoretic analyses of the S100 fraction from tk^+ cells were performed in 5% acrylamide gels as described by Lee and Cheng (1976). The running buffer was composed of 24 mM Tris, 191 mM glycine (pH 8.6) containing 5 mM 2-mercaptoethanol and 50 μM thymidine. Gels were electrophoresed at 2 ma per gel for 30 min, and the current was then raised to 3 ma per gel for the duration of the electrophoresis. At the termination of electrophoresis, gels were cut into 2 mm slices and assayed for tk activity by immersion into 100 μl of reaction mix, which was composed of 0.1 M Tris-maleate (pH 6.5), 25 mM KCl, 20 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 10 mM ATP and 10 μl of ^{3}H-TdR (spec. act. = 50 Ci/mmole). The slices were incubated at 37°C for 2 hr, and the amount of TMP produced was measured by spotting 50 μl of reaction mix onto DE-81 paper. The paper was washed 3 times with 4 mM ammonium formate and then with methanol to remove any residual TdR. Discs were counted in 5 ml of Econofluor (NEN) in a scintillation spectrometer.

Isoelectric Focusing of Thymidine Kinase Activity

Thymidine kinase activity was localized by isoelectric focusing essentially as described by Kit et al. (1974). The activity was assayed using a modification of the technique used by Chasin and Urlaub (1976) to assay hypoxanthine-guanosine phosphoribosyl transferase. Isoelectric focusing was performed in polyacrylamide slab gels 1.5 mm thick. The slabs were composed of 4.5% acrylamide, 0.15% N,N'-methylene-bisacrylamide, 7.6% sorbitol, 6% ampholine (pH 9–11) solution (LKB), 0.34% ampholine (pH 7–9) solution (LKB), 2 mM ATP, 2 mM 2-mercaptoethanol and 0.5% ammonium persulfate. The gels were cast and focused on an LKB multiphor apparatus across the short dimension. The anode solution was 0.1% ampholine (pH 7–9) and the cathode solution was 1% ampholine (pH 9–11). A constant voltage power supply was used to deliver 400 V to the gel, whose temperature was maintained by circulating cooled (2°C) water through a glass cooling plate. Small squares of 3MM paper saturated with extract were applied to the surface of the gel toward the cathode and focused for 4 hr. At the termination of the focusing, twice-concentrated tk reaction mix was applied to the gel surface and allowed to soak into the gel at 37°C for 30 min. A sheet of plastic-backed polyethylemeimine-cellulose (PEI), cut to the size of the gel, was wetted with water, blotted to remove excess moisture and applied to the gel surface. Incubation was continued for an additional 90 min, at which time the gel and PEI sheet were inverted, and the reaction product (TMP) was blotted out onto the PEI sheet. After 30 min, the sheet was washed in running water for 20 min and dried. Tritium was visualized by fluorography. A solution of 2,5 diphenyl-oxazole in ether (100 mg/ml) was poured over the sheet and allowed to dry. The sheet was placed in contact with Cronex 2 DC X-ray film at −85°C for 4 days and then developed.

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