DNA-mediated Cotransfer of Unlinked Mammalian Cell Markers into Mouse L Cells

HANS WARRICK, NANCY HSIUNG, THOMAS B. SHOWS, and RAJU KUCHERLAPATI
Department of Biochemical Sciences, Princeton University, Princeton, New Jersey 08544, and Biochemical Genetics Section, Roswell Park Memorial Institute, Buffalo, New York 14263

ABSTRACT Purified DNA from three different types of mammalian cells was precipitated with calcium phosphate and added to mouse L cells deficient in thymidine kinase (TK). Donor DNA was prepared from three cell lines: (*a*) mouse cells transfected with UV-inactivated herpes simplex virus (HSV) type 1, or a purified fragment of HSV carrying the TK gene (*b*) human HeLa cells, and (*c*) CHO, a cell line derived from Chinese hamster ovaries. Several hypoxanthine-aminopterin-thymidine resistant colonies were isolated from each experiment. The origin of the TK that is expressed in these cells was studied by polyacrylamide gel electrophoresis, isoelectric focusing, or heat stability. The TK in all instances was of the donor origin. To determine the extent of gene transfer we have assayed the CHO and HeLa DNA transfectants for galactokinase (GALK), a marker closely linked to TK, and 25 other isozymes representing a large number of different chromosomes. No cotransfer of GALK was observed, indicating that the size of the transferred DNA segment is limited. We observed that, in one instance, esterase-D, an unlinked marker of Chinese hamster origin, was transferred along with TK. These experiments indicate that nonselected markers can be transferred by this method, although at a low efficiency.

Studies on mammalian gene expression and gene interaction are facilitated by various gene transfer methods. There are four methods now available to transfer genetic information from one mammalian cell to another (for a review see reference 24). The first and most widely used of these systems is somatic cell hybridization. In this method the total genome of one cell is introduced to another. Certain classes of these hybrids segregate chromosomes, resulting in variable portions of the genome of a cell to be retained (see reference 10 for a review of the properties of such hybrids). The second of these methods to introduce partial genomes is to produce and isolate microcells or microkaryoplasts and to fuse them with intact cells (5). Single chromosomes can be transferred by this method. The third method involves isolation of metaphase chromosomes and incubating them with whole cells. Under appropriate conditions the chromosomes are taken up by cells, and genes borne by them are expressed (14). Miller and Ruddle (17) have been able to enhance the extent of such chromosome-mediated gene transfer and estimate that the size of the genomic portion retained in the recipient cells (transgenote) is 40 kilobase pairs or larger. The development of methods to transfer genes using purified DNA constitutes the fourth and most recent technique (1, 16, 26). In this type of experiment either purified DNA fragments (16, 26) or total cellular DNA (25) were used as donors. In instances where total cellular DNA served as the donor, the extent of genetic information that is transferred is not known. Since this kind of transfer could be used to study gene structure and function, we have begun a series of studies to determine the parameters of such DNA-mediated transfer. We have been able to transfer the gene for thymidine kinase (TK) from herpes simplex virus (HSV) type 1, human HeLa cells, and Chinese hamster ovary (CHO) cells into mouse L cells. We observed that a gene closely linked to TK, galactokinase (GALK), is not transferred. However, an unlinked gene esterase-D was transferred at a very low frequency.

MATERIALS AND METHODS

Cells

Mouse L cells deficient in thymidine kinase (LTK^-) were obtained from Dr. Richard Axel of Columbia University. These cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (Flow Laboratories, Inc., Rockville, Md.), 100 U/ml of penicillin, 50 U/ml of streptomycin, and 1 mM glutamine.

HeLa-S3 cells were obtained from American Type culture collection and maintained in DMEM. CHO cells were obtained from Dr. E. Postel of Princeton University.

UV Inactivation of HSV

Virus was irradiated with UV light at a dose of 23 erg/s/mm^2 for 10 min. This irradiated virus was used for transfection at an equivalent dose of 5 plaque-forming units/cell.

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DNA Isolation and Transfer

DNA was isolated from cultured cells by a method described earlier (21). Briefly, cell nuclei were isolated and lysed by addition of SDS. After digestion of nuclei with pronase the DNA was extracted with phenol and precipitated with absolute ethanol. The DNA was treated with RNase, and re-extracted and precipitated with ethanol. Dried DNA was solubilized in Tris-EDTA (10 mM Tris-2 mM EDTA) and stored at 4°C. The DNA:protein ratio as determined by absorbance at 260/280 nm exceeded 1.7.

Mouse LTK$^-$ cells were plated at a density of ~10^6 cells/T75 flask. The medium was replaced after 20 h. A mixture of high molecular weight DNA, calcium, and phosphate was prepared as described by Wigler et al. (26). The final concentration of the various components is 20 μg/ml DNA, 125 mM $CaCl_2$, 25 mM HEPES buffer, pH 7.10, 0.75 mM phosphate. The precipitate was kept at room temperature for 30 min, and 1 ml of the precipitate was added to each flask. The cells were incubated at 37°C for 4 h, excess DNA precipitate was removed and replaced with fresh medium. Selective medium was added 24 h later. The selective medium contained hypoxanthine (15 μg/ml), aminopterin (1 μg/ml) and thymidine (5 μg/ml) (HAT) (12). The medium was changed every 2–3 d. Colonies of cells resistant to HAT were scored at 14 and 21 d and isolated by the use of cloning cylinders soon thereafter. The resultant transfectant cell lines were maintained in DMEM-HAT.

HSV TK Assay

HSV TK was identified by a method described by Kit et al. (8).

Isoelectric Focusing

5% polyacrylamide gels containing 1% bis, 0.002% riboflavin, 2% Bio-lyte 3/10 ampholytes (Bio-Rad Laboratories, Richmond, Calif.) 10% glycerol, 2.5 mM ATP, 10 mM mercaptoethanol, 1.2 mM $MgCl_2$, and 0.2 mM thymidine were prepared. Polymerization was achieved by shining incandescent light.

Extracts prepared from 5-8 × 10^6 cells were mixed with sample buffer containing 50% sucrose, 12.5 mM $MgCl_2$, 25 mM ATP, and 4% Biolyte 3/10 ampholytes were applied to gel slots. The cathode buffer was 0.02 M NaOH and the anode buffer was 0.01 M H_3PO_4. The gel was run at 4°C at 150 V for 6–8 h.

TK Assay

The gel was cut into vertical strips and each strip was sliced into 0.5- to 1-cm pieces. Each piece was incubated in 35 μl of reaction mixture (0.1 M Tris-HCl, pH 8.5, 1 mM $MgCl_2$, 2 mM NaI, 5 mM ATP, and 3 μCi [^{3}H]thymidine) at 37°C for 10–15 h. Samples were spotted on DEAE paper, washed successively in 1 mM ammonium formate, distilled H_2O, 100% ethanol, then dried, and counted in a scintillation counter.

Heat Inactivation Studies

Cell extracts were prepared by homogenization of cells in 20 mM Tris-HCl buffer, pH 8.0, and centrifugation at 15,000 rpm for 45 min. The supernates were assayed for TK activity.

Cell extracts were added to 200 μl of reaction mixture containing 100 mM Tris-HCl, pH 8.5, 1 mM $MgCl_2$, 2 mM NaI, 5 mM ATP, and 3 μCi of [*methyl*-^{3}H]thymidine and incubated at 37°C for an appropriate interval. The reaction was terminated by the addition of 1 M EDTA and samples counted for thymidine monophosphate by methods described above. In these experiments the cell extracts were incubated at 55°C for 0, 5, or 7 min before assay.

Isozyme Analysis

Cell homogenates from the cell lines were tested by gel electrophoresis and specific enzyme assays for the enzyme markers listed in Table I. The methods of preparation, electrophoresis, and enzyme detection have been described elsewhere (3, 18, 19, 22). GALK was determined after starch-gel electrophoresis by modifications of the general procedures of Nichols et al (20), and esterase-D was determined by a modification of the method of Hopkinson et al. (6).

TABLE I

Isozymes Tested in Transfectant Cell Lines

Markers tested in each line			Human chromosome assignment
PEPC	3.4.11	Peptidase C	1
IDH1	1.1.1.42	Isocitrate dehydrogenase	2
ACY1	3.5.1.4	Aminoacylase-1	3
PEPS	3.4.11	Peptidase S	4
HEXB	3.2.1.30	Hexosaminidase B	5
ME1	1.1.1.40	Malic enzyme 1	6
GUS	3.2.1.31	β-Glucuronidase	7
GSR	1.6.4.2	Glutathione reductase	8
AK1	2.7.4.3	Adenylate kinase 1	9
GOT1	2.6.1.1	Glutamate-oxaloacetate transaminase 1	10
LDHA, ESA4	(1.1.1.27) (3.1.1.1)	(Lactate dehydrogenase A) (Esterase A4)	11
LDHB	1.1.1.27	Lactate dehydrogenase B	12
ESD	3.1.1.1	Esterase D	13
NP	2.4.2.1	Purine nucleoside phosphorylase	14
HEXA, PKM2, MPI	(3.2.1.30) (2.7.1.40) (5.3.1.8)	(Hexasominidase A) (Pyruvate kinase M2) (Mannose phosphate isomerase)	15
APRT	2.4.2.7	Adenine phosphoribosyltransferase	16
GALK	2.7.1.6	Galactokinase	17
PEPA	3.4.11	Peptidase A	18
GPI	5.3.1.9	Glucose phosphate isomerase	19
ADA	3.5.4.4	Adenosine deaminase	20
SOD1	1.15.1.1	Superoxide dismutase 2	21
G6PD	1.1.1.49	Glucose 6-phosphate dehydrogenase	X

Procedures for assays of each of the isozymes are described in Materials and Methods. Human chromosome assignments for each marker have been reported (23). The human chromosome assignments do not correspond to mouse or Chinese hamster chromosome assignments for the homologous markers, but indicate that genes spread throughout the rodent genomes have been examined.

RESULTS

Secondary Transfer

The purpose of the studies reported here was to determine the feasibility of transferring single copy genes from one mammalian cell to another and to determine the extent of the genetic material that is transferred. Three sources of DNA containing single copy genes were utilized in these experiments. The first was DNA from a mouse L cell transfected with UV-inactivated HSV1 and which contained and expressed the HSV TK gene (Fig. 1). This DNA was precipitated with calcium phosphate and presented to mouse LTK$^-$ cells. The cells were grown in medium containing HAT which selects for the expression of TK. Six HAT-resistant colonies emerged from this treatment (Table II). They are referred to as secondary transfectants. The TK$^+$ phenotype of these cells is either caused by reversion of LTK$^-$ cells to a TK$^+$ phenotype or they have acquired the HSV TK. LTK$^-$ cells are not known to revert to TK$^+$ phenotype. Thus, it is likely that they have acquired the HSV TK. To ascertain whether we have transferred the HSV TK from one mouse cell to another, the HAT-resistant colonies were isolated, expanded, and their cell extracts were tested for HSV TK. Profiles of TK activity in slices of polyacrylamide gels are presented in Fig. 1. Mouse A9 cells which are TK$^+$ exhibit a broad band of activity near the origin. A small peak of activity corresponding to the mitochondrial TK was observed at an R_f value of 0.55. LTK$^-$ cells were devoid of the cytosol activity and contained mitochondrial TK. Mouse TK$^-$ cells infected with HSV1 show a peak of activity at an R_f of

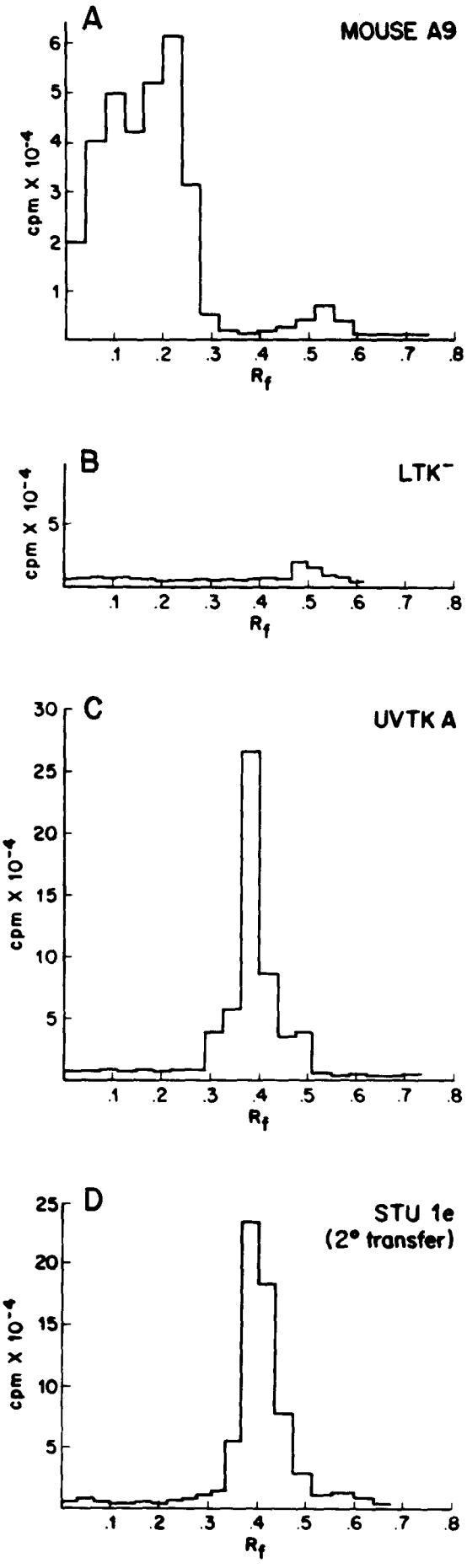

TABLE II

Frequency of Transfer of Single Copy Genes in Mouse Cells

Donor DNA	No. of exp	No. of cells $\times 10^{-6}$	No. of TK gene equivalents/cell	Total colonies
LTK	2	10	0	0
UVTK*	1	4	10	6
LGU‡	1	5	8	5
LCH§	1	5	18	9
HeLa	3	14	10-15	13
HeLa	5	25	10	13
CHO	2	10	10-15	6
CHO	3	15	10-15	20

* UVTK is a cell line obtained by transfer of TK from UV-treated HSV.
‡ LGU is a cell line obtained by transfer of purified HSV TK using a 5.1 kb fragment obtained from Kpn-digested HSV DNA.
§ LCH is a cell line derived from-transfer of CHO TK.

0.4. Cell extracts from the secondary transfectants had a peak of TK at R_f 0.4. All of the six transfectant cell lines obtained from this experiment exhibited an identical phenotype. Cell lines obtained from the use of DNA from LTK$^-$ cells transfected with a purified fragment (5.1 Kb Kpn I) of HSV DNA carrying TK gave similar results (Table II). LTK$^-$ L cells transfected with LTK DNA did not yield any colonies.

Transfer of Human and Hamster TK Gene

For transferring the human TK to mouse cells, we have isolated DNA from human HeLa cells and calcium phosphate precipitates of this DNA were presented to exponentially growing cultures of LTK$^-$ cells. The yield of HAT-resistant colonies from several experiments is shown in Table II. We were also able to obtain HAT-resistant colonies when the donor DNA was obtained from CHO cells (Table II). Even though the average yield of HAT-resistant colonies is approx. one colony/10^6 recipient cells per 20 μg of donor DNA, we observed some variation in the yield of transfectants in different experiments. These variations could not be correlated with any specific aspect of the experimental procedure.

Origin of TK in HeLa Transfectants

As shown in Fig. 1, mouse and HSV TK can be distinguished by polyacrylamide gel electrophoresis. Such a method failed to distinguish between human and mouse TK. As such, isoelectric focusing was performed on cell extracts from parental and transfected cells to determine whether HAT-resistance in these cells is caused by the acquisition of the TK gene from the heterologous cell DNA. The results are presented in Fig. 2. Mouse cell extracts (A9) show two distinct peaks of activity: the slower migrating form corresponding to the cytosol, and the faster to the mitochondrial form of TK. Both of these forms

FIGURE 1 TK activity in polyacrylamide gel slices from different cell extracts. Cell extracts were subjected to electrophoresis in polyacrylamide. The gels were cut into strips 0.5-1 cm long and assayed for TK activity. The results are plotted as a histogram. (*A*) Mouse A9 (TK$^+$) cells. (*B*) Mouse LTK$^-$ cells (small peak at R_f 0.55 corresponds to mitochondrial TK.) (*C*) UVTKA, a cell line obtained from transfection of LTK$^-$ with UV-inactivated virus. (*D*) STU1e, a cell line obtained by transfection of LTK$^-$ cells with DNA from UVTKA.

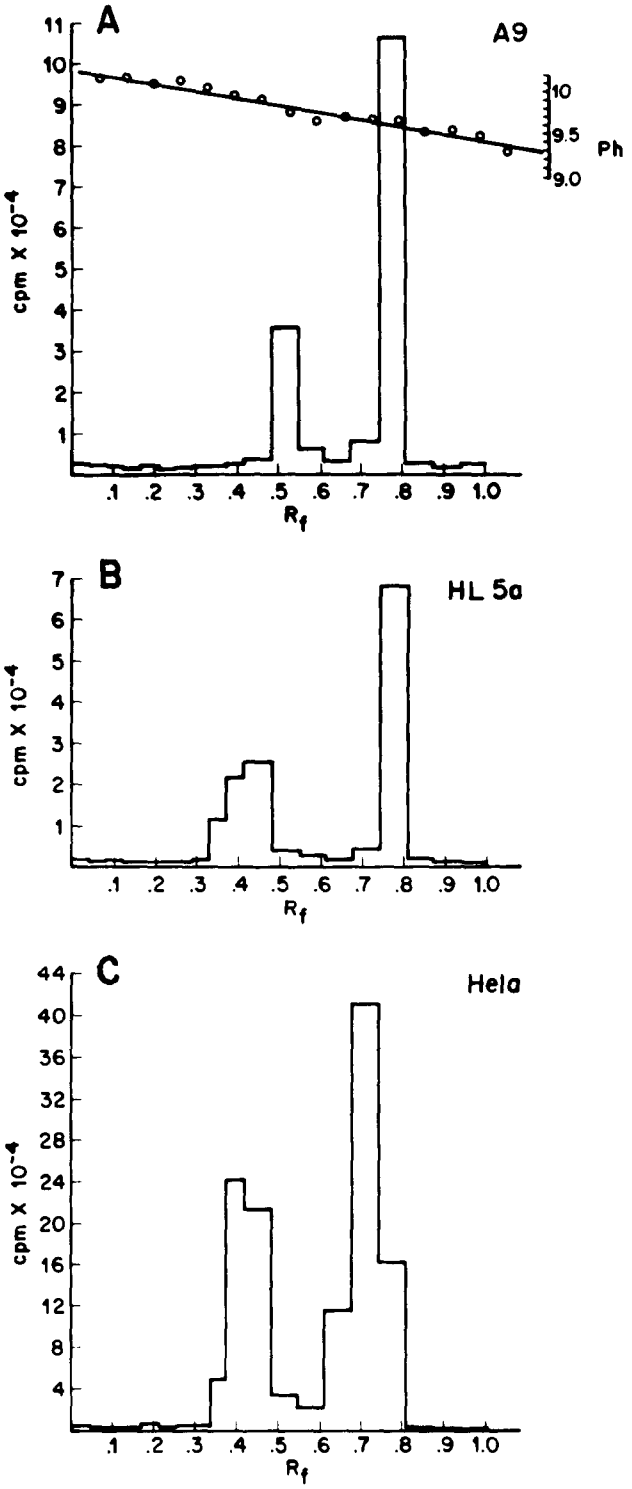

FIGURE 2 TK activity profiles of different cell extracts determined by isoelectric focusing. Isoelectric focusing of cell extracts and TK assay are described in Materials and Methods. (*A*) TK⁺ mouse A9 cells. Peak at 0.50 corresponds to cytosol TK, and peak at 0.75 corresponds to mitochondrial TK. (*B*) HL5a, a transfectant obtained using HeLa DNA. (*C*) HeLa cell extract. Note the differences in mobility of mouse and human cytosol and mitochondrial TK.

are distinguishable from their corresponding human forms. Mouse LTK⁻ cells contain the mitochondrial TK but are devoid of the cytosol enzyme. Cells transfected with HeLa DNA exhibited two forms of TK, one corresponding to the human cytosol form and the other to the mouse mitochondrial enzyme (Fig. 2 *B*). This result indicates that the human gene coding for cytosol TK has been transferred to mouse L cells.

Origin of TK in CHO Transfectants

The mouse and hamster forms of TK are not distinguishable by the polyacrylamide gel electrophoresis or isoelectric focusing methods. The cells transfected with DNA from CHO cells contained TK. Because LTK⁻ cells are not known to revert to TK⁺ phenotype, it is likely that the TK activity observed in the hamster DNA transfectants is derived from CHO cells. To confirm this, the heat stability of the parental and transfectant cell lines was tested. The results from this experiment are shown in Table III. The TK activity of mouse L cells is reduced by >95% when the cell extract is treated for 7 min at 55°C before assay. Comparable treatment of CHO cell extracts resulted in <5% reduction in TK activity. The CHO DNA transfectants behaved more like the CHO cells than the mouse cells. The somewhat higher heat sensitivity of some of these transfectants compared to CHO cells might be ascribed to differences in the dosage of the TK gene between these cell types. These results are consistent with transfer of the TK gene from CHO cell DNA into mouse L cells.

Cotransfer of an Unlinked Marker

In mouse cells transfected with intact metaphase chromosomes, linked nonselectable markers are occasionally transferred (9, 13, 17). To ascertain whether a similar transfer of nonselectable genes occurs in DNA transfection, we have assayed our HeLa and CHO DNA transfectant cell lines exhibiting the donor TK, for 25 additional enzymes which have electrophoretic differences between the recipient and donor. These enzyme markers are listed in Table I. Of these markers, genes coding for TK and GALK are known to be closely linked in human (4) and in hamster (9). To test for the cotransfer of closely linked markers we have tested all our DNA transfectants expressing TK for GALK. All of the cell lines assayed contained mouse GALK but did not contain human or Chinese hamster forms. Detailed analysis of these cell lines for additional markers which are not linked to TK revealed that one cell line, CHL 1a, expressed the hamster phenotype of esterase-D, in addition to the mouse isozyme (Fig. 3). No other heterologous forms of enzymes were detected in these transfectants. In total we have tested 15 different independently isolated cell lines for 25 different isozymes.

TABLE III

Heat Sensitivity Profiles of TK from Parental and Transfected Cells

Cell line	TK activity remaining after treatment at 55°C for: 5 min*	7 min*
	%	
A9 (mouse TK⁺)	63.7 ± 3.7	4.20 ± 3.3
CHO (hamster)	92.8 ± 3.0	95.9 ± 2.9
CHL 1a	90.5 ± 3.2	92.4 ± 3.4
CHL 1b	88.7 ± 21.6	77.5 ± 12.1
CHL 1c	83.5 ± 13.4	69.0 ± 7.2
LCH 6a	ND	77.0 ± 3.4
LCH 6d	ND	92.0 ± 11.2

ND, Not done.

* Average of three reactions.

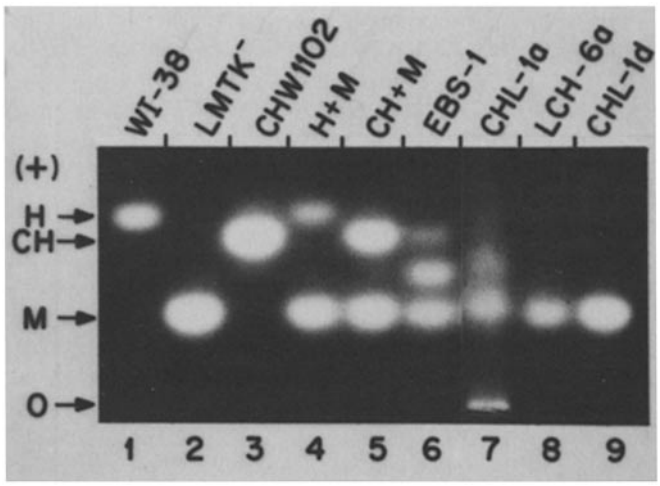

FIGURE 3 Esterase-D isozyme profiles in cell extracts from Chinese hamster DNA transfectants. Esterase-D electrophoretic phenotypes for human (channel *1*), mouse (channel *2*), and Chinese hamster (channel *3*) cell homogenates. Mixtures of human and mouse (channel *4*) and Chinese hamster and mouse (channel *5*) are at a 1:1 concentration. A mouse-Chinese hamster somatic cell hybrid clone (channel *6*) retains both rodent esterase-D genes and demonstrates the three-banded pattern with a heteropolymeric interspecific form characteristic for a dimeric enzyme. Mouse cell homogenates transfected with Chinese hamster DNA are in channels *7–9*. CHL-1a (channel *7*) demonstrates a characteristic mouse-Chinese hamster interspecific heteropolymer. This phenotype, which exhibits diminishing activity for the molecular forms of esterase D in the order mouse > heteropolymer > Chinese hamster (not seen photographically), is characteristic for a population of cells not expressing Chinese hamster esterase D in every cell. Expression of multimeric forms of enzymes in somatic cell hybrids has been discussed (11).

Because only one cell line expressed one additional isozyme, the frequency of cotransfer of an unlinked marker in these experiments is much less than 1%.

DISCUSSION

We have demonstrated that the gene for TK, which constitutes a single copy gene, can be transferred into mouse cells using purified DNA. The sources of the DNA were mouse cells transfected with HSV DNA, human HeLa cells, and CHO cells. Wigler et al. (25) have shown that is is possible to obtain HAT-resistant colonies using DNA from similar sources. The frequency with which this transfer occurs is approx. one colony/10^6 cells, using 10–15 TK gene equivalents/cell. There are three lines of evidence which support the view that the TK expressed in these transfectants is derived from the donor DNA. First, the recipient cells were not known to revert (15, 26) and, as such, any cell that grows in HAT and exhibits TK activity should have received the TK gene from the donor. All of the transfectants we have obtained behaved in this fashion. Second, in HeLa transfectants several independently derived cell lines contained the human cytoplasmic TK and no other human marker. Hence the mouse cells have acquired the human gene for TK. Third, in the CHO transfectants the heat-sensitivity profile of TK from several cell lines is similar to that of hamster cells. Thus TK from three different sources can be efficiently transferred to mouse TK^- cells.

The transfer of single copy genes by chromosome-mediated transfer varies from 10^{-7}–10^{-5}, (14, 17, 28). The efficiency of transfer reported here compares favorably with the chromosome-mediated transfer.

Genes for TK and GALK are closely linked in human (4) as well as in Chinese hamster (13). All of the cell lines we have tested for the heterologous GALK proved to be negative. Tests for transfer of any of 25 other isozymes, representing all of the human chromosomes except chromosome 22, revealed no other transfer. A similar assay of hamster TK transfectants revealed that a single marker, esterase-D, has been transferred to a single cell line. Thus the frequency of cotransfer is $<1/100$.

It has been shown that in chromosome-mediated transfer, closely linked markers are transferred at a high efficiency (2) and under certain circumstances even unlinked markers can be transferred (17). The intrinsic differences in the sizes of DNA (chromosomal size in one case and isolated DNA of varying sizes in another) and the conditions of transfection can explain the differences in these cotransfer frequencies.

Using purified DNA, and TK as the selectable marker, it has been possible to transfer specific, defined fragments of chromosomal DNA at high efficiencies (7, 27, and our unpublished observations). But in these instances the relative proportion of TK and the nonselected markers was as high as 1:1,000 or more. In the current experiments the ratio of TK and any other gene is ~1:1. This ratio might also explain the relatively low frequency of cotransfer.

The ability to transfer single copy genes from one eukaryote to another enables detailed functional mapping of the gene and might provide a method of eventually purifying these sequences from the rest of the DNA.

We acknowledge Ginny Grosse for technical assistance and Noël Mann for manuscript preparation.

The work is supported by grants from American Cancer Society, National Science Foundation, and The National Foundation—March of Dimes. H. Warrick is a National Institutes of Health predoctoral trainee.

Received for publication 1 March 1980, and in revised form 24 April 1980.

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