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Genetics

Cotransfer of thymidine kinase and galactokinase genes by chromosome-mediated gene transfer

(gene mapping/somatic cell genetics/gene linkage/eukaryotic genes)

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ABSTRACT **The Chinese hamster genes for thymidine kinase (ATP:thymidine 5′-phosphotransferase, EC 2.7.1.75) and galactokinase (ATP:D-galactose 1-phosphotransferase, EC 2.7.1.6) have been cotransferred to mouse cells by chromosome-mediated gene transfer. Hamster metaphase chromosomes were incubated with mouse B_{82} cells and 22 independent colonies were isolated in a selective medium. All of the 12 colonies analyzed expressed the donor form of thymidine kinase; the hamster form of galactokinase was also expressed in 2 of these colonies, indicating cotransfer with a frequency of about 20%. There was coordinate loss of both transferred genes from each colony when selection was applied for the loss of thymidine kinase alone. Comparison of the regional localization of these two linked genes with the frequency of cotransfer suggests that the transgenome is probably not larger than about 0.25% of the donor genome.**

Functional chromosomal donor genes can be stably transferred to eukaryotic cells when isolated mammalian metaphase chromosomes are incubated with recipient cells *in vitro* (1–8). This transferred genetic information (transgenome) represents only a fragment of a donor chromosome (3–5, 9). Knowledge of the size of the transgenome is required to evaluate the utility of chromosome-mediated gene transfer for various potential applications, including genetic mapping in eukaryotic cells.

Previous studies (3–5) have demonstrated that the transgenome can represent no more than about 1% of the haploid human genome. This estimate is based on the regional localization of three gene loci, hypoxanthine phosphoribosyltransferase (*hprt*), glucose-6-phosphate dehydrogenase (*gpd*), and phosphoglycerate kinase (*pgk*) on the long arm of the human X chromosome. When human chromosomes were incubated with rodent *hprt*$^-$ cells and transformed cells (transgenotes) expressing the human *hprt* gene were isolated in a hypoxanthine/amethopterin/thymidine (HAT) selection system, the nonselected human flanking markers, *gpd* and *pgk*, were never detected.

Subsequently, another gene transfer system has become available which provides a much better estimate of the size of the transgenome. This system involves the selectable thymidine kinase (TK) locus (*tk*) which has been shown to be tightly linked to the galactokinase (GalK) locus (*galk*) in man (10). Both loci have been assigned to a very small region on the long arm of human chromosome 17, representing no more than 0.2% of the haploid human genome. Demonstration of the close linkage between these two loci in humans led us to determine the species of GalK in extracts of transgenotes isolated in HAT medium after incubation of Chinese hamster metaphase chromosomes with mouse *tk*$^-$ cells. The chromosomal donor species of GalK was detected in 2 of 11 such extracts (3). Initially, the hamster donor form of TK could not be distinguished from the mouse recipient form of this enzyme by various criteria including chromatography and electrophoresis. Nevertheless, the transfer of the nonselected *galk* gene in this system with a frequency of 20% indicated that cotransfer of these two genetic markers must have occurred.

Recently, studies in two other laboratories (6, 7) have indicated that the cotransfer of human *tk* and *galk* genes also occurs, with a high frequency, by chromosome-mediated gene transfer. This paper compares the frequency of cotransfer of these two loci from Chinese hamster chromosomes with results of the two other studies using human donor chromosomes. These results also provide evidence for conservation of the *tk/galk* linkage in species as evolutionarily divergent as primates and rodents. Preliminary reports of these results have been presented (3, 11, 12).

MATERIALS AND METHODS

Cell Cultures. Cells used were: (*i*) wild-type Chinese hamster fibroblasts (V-79) as chromosome donors; (*ii*) mouse fibroblasts (L_{929}); and (*iii*) mouse L, cells, B_{82} and A_9, deficient in TK and hypoxanthine phosphoribosyltransferase (IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8), respectively (13). Culture conditions and media have been described (1, 3). All lines were free of mycoplasma as determined by cultivation on agar (14).

Metaphase Chromosome Isolation and Purification. Chromosomes were isolated under sterile conditions from [^{3}H]dT-labeled cells (0.2 mCi/liter) by slight modifications of the procedure of Maio and Schildkraut (15) in 0.02 M Tris·HCl, pH 7/3 mM $CaCl_2$/1% Triton X-100. The chromosomes were purified by differential and unit gravity sedimentation as described (2) but isopycnic centrifugation was omitted to minimize chromosome aggregation. The molecular weight of the dissociated, single-stranded, chromosomal DNA was 20–21 × 10^6 as determined by velocity sedimentation in alkaline sucrose density gradients, with ^{14}C-labeled simian virus 40 DNA I and II as markers (2).

Chromosomal Gene Transfer. Metaphase chromosomes (5 × 10^7 cell equivalents) isolated from Chinese hamster cells were dispersed in a sterile, siliconized glass culture tube with B_{82} mouse fibroblasts (5 × 10^7) in 8 ml of complete Eagle's minimal

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Abbreviations: TK, thymidine kinase (ATP:thymidine 5′-phosphotransferase, EC 2.7.1.75); GalK, galactokinase (ATP:D-galactose 1-phosphotransferase, EC 2.7.1.6); *tk* or *galk*, gene directing synthesis of TK or GalK, respectively; HAT, hypoxanthine/amethopterin/thymidine.

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essential medium containing 10% fetal bovine serum and poly-L-ornithine (molecular weight, 70,000) at 12 μg/ml and were incubated for 2 hr at 37° (1). Aliquots (5×10^5 cells) were transferred to 100-mm plastic dishes containing 10 ml of complete minimal essential medium. The medium was replaced with HAT medium (16) after 3 days, and the plates were re-fed twice weekly with this selective medium for 6 weeks. Colonies that appeared during this interval were cloned in metal cylinders, removed by treatment with trypsin, and recultured in HAT medium. All colonies that were analyzed appeared in separate plates.

Enzyme Extracts and Assays. Cells were maintained in exponential growth in selective medium in suspension cultures, and cytosol TK extracts were prepared as described by Kit *et al.* (17). Washed cells were disrupted in a Dounce homogenizer in cold 0.01 M KCl/1 mM $MgCl_2$/0.01 M Tris·HCl, pH 7.4/0.2 mM dT/50 mM ε-aminocaproic acid; KCl (0.15 M) and 2-mercaptoethanol (3 mM) were added prior to centrifugation at $9500 \times g$ for 10 min. Nonidet P-40 (0.5%, vol/vol) was added to the supernatant fluid which was then centrifuged for 1 hr at $105,000 \times g$. The high-speed supernatant fluid was stored at −90° after addition of glycerol (10%, wt/vol).

The assay for TK activity was a modification of the method of Kit and Dubbs (18). Aliquots of enzyme extracts (40–250 μg of protein) were incubated at 38° for 10–60 min in 100 μl containing 100 mM Tris·HCl (pH 8.0), 10 mM ATP, 13 mM $MgCl_2$, 11.5 mM 3-phosphoglycerate, and 0.12 mM [^{14}C]thymidine (8 mCi/mmol). The reaction was terminated by addition of 1 ml of cold 1.5 mM EDTA/1 mM Tris·HCl, pH 7.0, and the incubation mixture was applied to DEAE-cellulose disks (Whatman DE-81) and washed with four aliquots (10 ml each) of water. The disks were dried and the [^{14}C]TMP product was quantitated by scintillation counting. Appropriate corrections (usually ≤0.2%) were made for binding of radiochemical impurities to the DE-81 disks in the absence of enzyme or incubation.

GalK activity was assessed by incubating enzyme extracts (10–25 μg of protein) for 10–30 min at 38° in 100 μl containing 100 mM Tris·HCl (pH 7.4), 5 mM ATP, 10 mM $MgCl_2$, and 0.12 mM D-[^{14}C]galactose (4 mCi/mmol). The [^{14}C]galactose 1-phosphate was collected on DEAE-cellulose disks as described above.

Heat Inactivation of TK. Extracts were thawed at 20°, dialyzed against 40 volumes of 10 mM potassium acetate, pH 5.0/0.5 mM dithiothreitol/0.05 mM dT/0.1% Triton X-100 at 5° for 7–12 hr, and centrifuged for 20 min at $10,000 \times g$ (5°) to remove a large white precipitate. The clear supernatant fluid was dialyzed for 8–12 hr at 5° against 40 volumes of 60 mM Tris·HCl, pH 6.7/2 mM EDTA/0.5 mM dithiothreitol/0.1 mM dT/0.1% Triton X-100/10% sucrose. Aliquots (50 μl) of the dialyzed extracts were transferred to 10×70 mm glass tubes, covered tightly with Parafilm, and incubated in a water bath for intervals at 55° or 65°. Heating and assay of each extract was performed in triplicate, and the standard deviation was usually less than 5% of the mean. All extracts were incubated together to ensure that there were no variations in the interval of heating for different extracts. Tubes were plunged into an ice-water bath after heating and a 2-fold concentration of the TK reaction mixture (50 μl) was added to each tube. TK activity was determined by incubation of the tubes for 15 or 30 min at 38°. Residual enzyme activities were determined by comparison with identical extracts that had not been heated.

Gel Electrophoresis. Electrophoresis of GalK was performed at 4° in 12% starch gels at 150 V for 3–15 hr; the gels were treated with D-[^{14}C]galactose substrate and the [^{14}C]galactose 1-phosphate product was precipitated with $LaCl_3$ and detected by autoradiography (19).

Table 1. Heat inactivation of cytoplasmic TK

Temp., °C	Time, min	% residual activity* CH	M
50	1	55	21
	5	45	16
	10	35	12
55	1	50	18
	5	29	7
	10	19	6
60	1	49	15
	5	11	1
	10	3	0
65	1	27	4
	5	3	1
	10	0	0

* Extracts were: CH, Chinese hamster (CH-V-79); M, mouse (L_{929}).

Karyotypes. Cells were trypsinized from monolayers after mitotic arrest with Colcemid (0.2 g/ml) for 4 hr, swollen in 0.075 M KCl at 37° for 10 min, and fixed in three changes of methanol/acetic acid, 3:1 (vol/vol). Mitotic spreads were prepared by air drying, immersion in 0.07 M NaOH/3 ethanol (vol/vol), rinsing in 7 ethanol (vol/vol) and then water, and staining with Hoechst 33258 (0.05 μg/ml) (20). The spreads were mounted in citrate/phosphate buffer (pH 4) and photographed under a fluorescence microscope. The total number of chromosomes in each metaphase was determined, and mouse chromosomes were identified by differential centromeric fluorescence. Treatment of mitotic spreads with alkali increased the differential centric fluorescence of mouse chromosomes (20); use of 30% ethanol both provided adequate protection against chromosome degradation and diminished the subsequent rate of quenching of fluorescence.

RESULTS

Gene Transfer to B_{82} Cells. Colonies appeared in 22 of the 100 plates that were inoculated after incubation of mouse B_{82} cells with Chinese hamster chromosomes. They were first detected 10 days after the shift to selective medium, and 17 colonies were found during the interval between 10 and 17 days in HAT medium. The five other colonies appeared at irregular intervals throughout the remainder of the experiment. The frequency of colonies was similar to that reported in other heterologous chromosome-mediated gene transfer systems (12). The spontaneous reversion frequency of the recipient B_{82} cells was about 10^{-8} (2 colonies per 2.2×10^8 cells).

Heat Inactivation of TK. Both mouse and Chinese hamster TK are progressively inactivated by heating at 50°–65°, but the mouse enzyme was inactivated more rapidly than the hamster enzyme at each temperature tested (Table 1). At least 50% of the Chinese hamster TK activity remained under conditions in which 80% of the mouse TK activity was lost. This differential heat sensitivity provides a reliable means of distinguishing mouse and hamster TK.

The heat-inactivation profile of TK of 12 transgenotes was compared with that of Chinese hamster and mouse extracts (Table 2). The TK activity in all 12 transgenotes had a heat-inactivation profile similar to that of Chinese hamster extracts and markedly different from that of mouse extracts. After 1–2

Table 2. Heat inactivation of TK at 55°

Source*	% activity remaining† 1 min	2 min	5 min	1 min‡	2 min‡
CH	46 } 49	(69) } 57	34 } 35	(36)	(15)
CH	52 }	51 }	36 }	(32)	(10)
L_{A9}	12 } 17	18 } 20	11 } 12	(7)	(2)
L_{929}	21 }	22 }	14 }	(9)	(2)
R-1	7	(12)	7	(3)	(2)
R-2	4	6	3	3	2
1	58	(70)	52	(48)	(14)
3	47	(52)	37	(32)	(7)
4	56	54	44	39	14
5	53	(55)	32	(28)	(5)
8	56	(54)	39	(24)	(17)
9	(69)	(68)	(52)	(56)	(18)
13	(73)	(72)	(57)	(56)	(22)
14	55	(42)	39	(34)	(11)
15	43	(54)	44	(28)	(23)
17	63	62	51	45	18
18	(71)	(72)	(57)	(55)	(26)
20	70	66	52	46	21

* CH, Chinese hamster; L_{A9}, mouse L cell; L_{929}, mouse fibroblast. Transgenote clones are indicated numerically and the two B_{82} revertant clones are denoted as R-1 and R-2. Protein concentrations varied from 2.0 to 6.1 mg/ml.

† Mean values from three separate experiments; parentheses denote single experiments.

‡ Extracts heated at 65°.

min at 55°, 50–70% of the Chinese hamster or transgenote TK activity remained, whereas only 20% of the mouse activity remained. Similarly, after 5 min at 55°, 35–50% of the hamster or transgenote TK activity remained, compared with only 10–15% of the mouse activity. About 30–55% and 10–25% of hamster or transgenote TK activity persists after 1 and 2 min, respectively, at 65°, whereas almost all mouse TK activity was lost under these conditions. Two revertant B_{82} lines exhibited much greater thermal lability of TK activity than did the wild-type mouse line. Increased thermolability of a mutant gene product has also been reported by others (21, 22).

The heat-inactivation of TK in these extracts did not follow first-order kinetics. The activity initially declined rapidly,

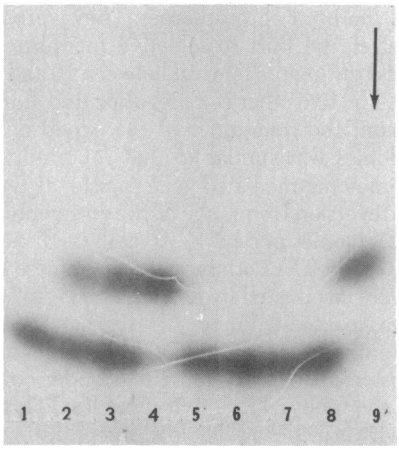

FIG. 1. Starch gel electrophoresis of GalK activity. The extracts in channels 1, 2, 3, and 8 were from gene transfer clones 14, 13, 5, and 18, respectively; two separate mouse (L_{B82}) revertant extracts are shown in channels 6 and 7. Mouse (L_{A9}) extract was in channel 5 and Ch-V-79 extract was in channels 4 and 9. Electrophoresis was carried out for 15 hr at 150 V; the arrow indicates the direction of migration.

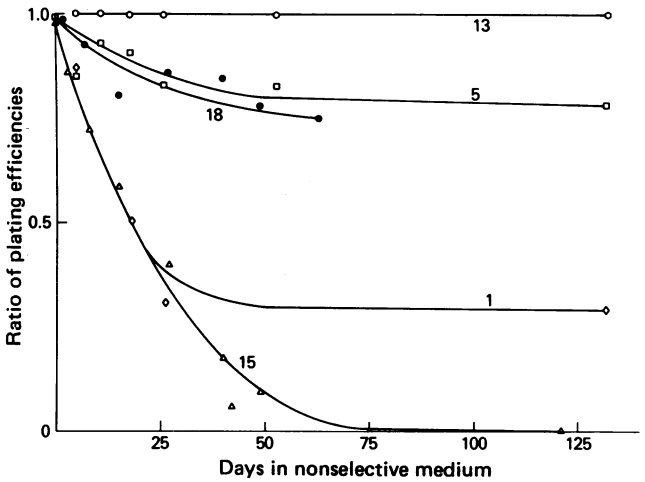

FIG. 2. Stability of tk^+ phenotype in transgenotes. ◇, △, The fraction of cells retaining the tk^+ phenotype (number in HAT medium/number in nonselective medium) plotted as a function of the time interval after the cells were switched from selective medium. The corresponding ratio of plating efficiencies (BrdUrd/nonselective medium) is plotted for clones 5 (□), 13 (○), and 18 (●).

followed by a subsequent slower rate of inactivation. This suggests the presence of two, or more, forms of the enzyme in the extracts. However, only one form was observed on analysis by polyacrylamide gel electrophoresis (not shown). These extracts apparently contained little of the mitochondrial species of TK.

Characterization of GalK Activity in Transgenotes. The GalK in extracts of 14 transgenotes was examined by starch gel electrophoresis with autoradiography; Fig. 1 shows a typical result. The mouse form of GalK was present in all extracts; in addition, the Chinese hamster chromosomal donor form was present in two of the extracts (clones 5 and 13). In these two clones, the activities of hamster and mouse GalK were about equal.

Stability of Transgenote tk^+ Phenotype. Transgenotes were cultured in selective medium for several weeks to months and then shifted to nonselective minimal essential medium. At intervals after the shift, plating efficiencies in selective HAT and BrdUrd media were determined (Fig. 2). The transgenote clones exhibited various degrees of stability of the transformed tk^+ phenotype (Table 3), ranging from clones with all unstable cells (clone 15) to those with less than 1% unstable cells (clones 3, 8, 9, 13, 14, and 17).

Concordant Segregation of GalK and TK. The two transgenote lines expressing the chromosomal donor species of GalK were selected for cells that had lost TK activity by growth in 0.1 mM BrdUrd. BrdUrd-resistant lines were obtained from both transgenotes, although surviving cells of transgenote clone 13 were rare in this medium. Extracts prepared from these lines contained no TK activity. Electrophoresis of GalK in these extracts indicated that the recipient (mouse) species was still present but the donor (hamster) form of this enzyme could no longer be detected (Fig. 3).

Karyotypes. Mouse chromosomes exhibit differential centromeric fluorescence after staining with Hoechst 33258 and thus can be distinguished from hamster chromosomes which stain uniformly and less intensely (Fig. 4). In metaphase spreads of B_{82} cells, three chromosomes regularly failed to exhibit centromeric fluorescence, and they differed in size and staining pattern from Chinese hamster chromosomes. One of these three chromosomes was biarmed and had bright teleomeres. The other two were acrocentric; the smaller one had bright teleo-

Table 3. Properties of transgenotes and revertants

Clone	TK*	GalK*	Chromosomes, no. Total†	Dull centromeres‡	Phenotype, % unstable§
1	CH	M	51	3–4	70
3	CH	M	50	3	<1
4	CH	M	49	3	—
5	CH	CH + M	50	3–4	22
8	CH	M	51	3	<1
9	CH	M	51	3	<1
12	—¶	M	—	—	30
13	CH	CH + M	49	3–4	<1
14	CH	M	51	2–3	<1
15	CH	M	49	4	100
17	CH	M	50	3	<1
18	CH	M	50	3	25
19	—	M	50	3	—
20	CH	M	52	3	—
R-1	M	M	48	3	0
R-2	M	M	—	—	0
B_{82}	O	M	52	3	—
CH	CH	CH	23	23	—

* Form of TK or GalK present in extracts: CH, Chinese hamster; M, mouse.
† Total number of chromosomes.
‡ Number of chromosomes per metaphase lacking bright centromeric heterochromatin fluorescence after staining with Hoechst 33258.
§ Percentage of cell population that loses the ability to survive in HAT medium after prolonged culture in nonselective medium. These values were determined from data similar to those in Fig. 2.
¶ —, Not analyzed.

meres and the larger one had a fluorescent band at the middle of its arms. When mitotic spreads of transgenote lines were examined after staining with Hoechst 33258, no hamster chromosome or recognizable donor chromosome fragment was detected. No biarmed chromosome with dull centromeric fluorescence was found in metaphases of most transgenote lines or the B_{82} revertant but it was replaced by an extra acrocentric chromosome with a dull centromere. This extra chromosome always resembled one of the two acrocentric chromosomes in B_{82} metaphases which lacked differential centromeric fluorescence. Occasionally, additional copies of these two distinctive chromosomes were present in metaphases. Transgenote lines 5 and 15 did contain the biarmed chromosome lacking centromeric fluorescence that was observed in B_{82} cells. A summary of all data relating to the transgenotes and revertants is presented in Table 3.

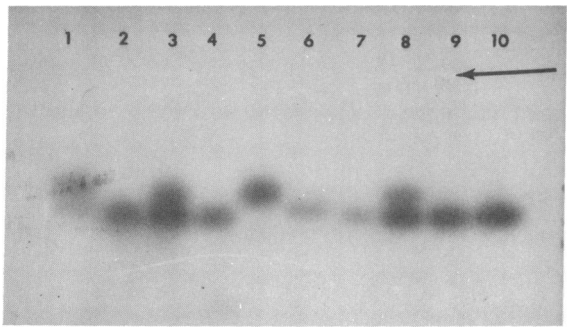

FIG. 3. Starch gel electrophoresis of GalK activity. The extracts depicted are: 1, Ch-V-79 + L_{A9}; 2, clone 15; 3, clone 5 (HAT); 4, clone 5 (BrdUrd); 5, CH-V-79; 6, L_{A9}; 7, clone 13 (BrdUrd); 8, clone 13 (HAT); 9, clone 3; 10, clone 17. The sample origins are indicated by the arrow. Electrophoresis was for 3⅓ hr at 150 V.

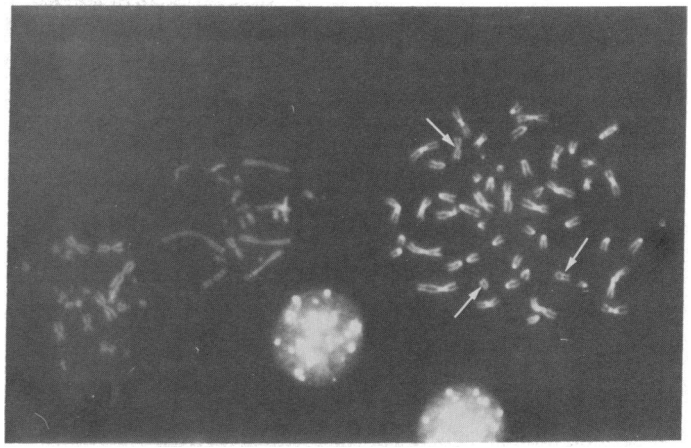

FIG. 4. Fluorescence photomicrograph of Hoechst 33258-stained metaphases. Mitotic Chinese hamster (CH-V-79) and mouse (L_{B82}) cells were mixed, and air-dried spreads were stained with Hoechst 33258. A mouse mitotic cell is shown in contiguity with two mitotic CH-V-79 cells and two mouse interphase cells. Note the intense centromeric fluorescence of mouse chromosomes and more uniform fluorescence of Chinese hamster chromosomes. The arrows indicate the three mouse chromosomes that do not exhibit differential centromeric fluorescence.

Absence of Other Gene Markers. Crude extracts of 11 transgenotes were also analyzed by starch gel electrophoresis (23) for the presence of the chromosomal donor species of nine other enzymes including peptidase B (EC 3.4.11.1), mannose-phosphate isomerase (EC 5.3.1.8), glucosephosphate isomerase (EC 5.3.1.9), nucleoside phosphorylase (EC 2.4.2.1), malate oxidoreductase (EC 1.1.1.37), 6-phosphogluconate dehydrogenase (EC 1.1.1.44), glutathione reductase (EC 1.6.4.2), acid phosphatase, and esterase D. Only the mouse form of each of these enzymes was detectable. All of these genetic markers are nonsyntenic with *tk* and *galk* in humans; the chromosomal location of these markers in Chinese hamster cells is currently unknown. The recipient B_{82} mouse cells lack adenine phosphoribosyltransferase (EC 2.4.2.7) activity and none was detected in transgenotes.

DISCUSSION

After incubation of Chinese hamster chromosomes with TK-deficient mouse cells, colonies were isolated under conditions selecting for transfer of the *tk* gene. Although Chinese hamster and mouse TK are indistinguishable by various chromatographic and electrophoretic procedures, their heat sensitivities are sufficiently different to provide a reliable means of distinguishing the two species. The thermal inactivation profile of TK in extracts of all 12 transgenotes examined was similar to that of Chinese hamster extracts, indicating that transfer of the Chinese hamster *tk* gene had occurred. This conclusion is supported by the phenotypic instability of *tk* expression in several transgenotes (i.e., clones 1, 5, 15, and 18). Although numerous factors unrelated to the primary structure of this enzyme could have influenced its inactivation on heating of crude extracts, none would explain the appearance of an enzyme with properties of the hamster donor form in mouse extracts. Moreover, the property acquired was one of increased heat stability whereas the TK in authentic revertants exhibited decreased heat stability, as expected.

The simultaneous transfer of the *galk* gene to 20% of the transgenotes selected for *tk* transfer indicates that linked genes are cotransferred by this procedure. The frequency of transfer of any gene by chromosome-mediated gene transfer is about 10^{-7} (12); thus, the nonselected donor *galk* marker was detected

in transgenotes with a frequency 10^6-fold greater than predicted on the basis of chance alone (e.g., 2×10^{-1} rather than 10^{-7}). The segregation of *galk* under conditions selecting only for the loss of *tk* provides further evidence for cotransfer of these two genes.

Willecke *et al.* (6) observed a similar frequency (2/8) for cotransfer of human *tk* and *galk* whereas Wullems *et al.* (7) reported cotransfer of these human gene markers in all nine transgenotes that they examned. One other difference was also noted among these three studies. Both of our transgenotes that exhibited cotransfer of *tk* and *galk* also demonstrated persistence of this linkage after transfer. *galk* segregated with *tk* in a transgenote that was phenotypically stable (clone 13) and in one that was moderately unstable (clone 5). The former clone presumably represents one in which these genes have become integrated into the genome of the recipient cell (8, 24) whereas the transgenome is probably not integrated in unstable transgenotes of the latter clone. Those cells that had lost both *tk* and *galk* probably originated from the rare spontaneous loss of a chromosome or chromosome fragment bearing the integrated transgenome from clone 13 or from the more frequent loss of the nonintegrated transgenome from clone 5. Hence, linkage of these two genes persisted in both situations.

In contrast, one of the clones reported by Willecke *et al.* (6) exhibited an unstable phenotype for *tk* and expression of donor *galk* at low levels; the donor form of GalK could no longer be detected in subclones after stabilization of the *tk* gene. Willecke *et al.* (25, 26) reported that *tk* and *galk* could be serially transferred by chromosome-mediated gene transfer, indicating that these genes were closely linked in the other transgenote that showed stable expression of the *tk* gene. A possible explanation for the independent segregation of two transferred genes in an unstable transgenote is fragmentation of an ingested chromosome during initial processing and retention of two or more separate fragments (transgenomes) containing functional genes. However, this appears quite unlikely because there should be apparent cotransfer of any two genes on a chromosome with constant frequency. The absence of cotransfer of syntenic genes that are not closely linked on the X chromosome (3–5) contradicts this hypothesis. Thus, the results of Willecke *et al.* suggest that further fragmentation of the transgenome can occur during, or prior to, its integration.

Demonstration of the cotransfer of these linked genes has several implications. It indicates that chromosome-mediated gene transfer provides a useful method for identifying close gene linkages and for mapping linked genes, as previously indicated (11, 12, 25). It also affords a rough estimate of the size of the transgenome that appears to be no more than about 0.25–0.3% of the donor genome (12). Finally, cotransfer of *tk* and *galk* indicates conservation of the close linkage of these genes in species as evolutionarily divergent as man and rodent.

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