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Transfer of the gene for thymidine kinase to thymidine kinase-deficient human cells by purified herpes simplex viral DNA

(transformation/gene expression/somatic cell genetics)

SILVIA BACCHETTI* AND FRANK L. GRAHAM*†

Departments of *Pathology and †Biology, McMaster University, Hamilton, Ontario, Canada L8S 4J9

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ABSTRACT Transformation of human cells from a thymidine kinase (ATP:thymidine 5′-phosphotransferase, EC 2.7.1.75)-negative to a thymidine kinase-positive phenotype has been achieved by using purified DNA from herpes simplex virus type 2. The specific activity of the DNA was in the range 0.5 to 2.0 transformants per μg and the efficiency of gene transfer was up to 1 transformant per 10^5 recipient cells. Several transformed lines able to grow continuously in medium selective for thymidine kinase-positive cells have been established. All of these lines express a thymidine kinase activity of viral origin but they differ from each other in the stability of enzyme expression. Subclones derived from a given transformed line inherited the degree of stability of the parental line.

The ability to use pure DNA to transfer genes between cultured mammalian cells would be of considerable importance to studies on the genetics of higher organisms. Although the transfer of viral genes that induce oncogenic transformation of cultured cells can be achieved routinely with purified viral DNA (1) and is likely to involve events similar to those expected to occur in the transfer of other types of genes, neither the viral gene products responsible for oncogenic transformation nor their functions are well defined. Consequently, oncogenic transformation is not a suitable model system for studying the characteristics of gene transfer in general. However, it is well established that herpes simplex viruses (HSV) code for a thymidine kinase (ATP:thymidine 5′-phosphotransferase, EC 2.7.1.75) (TK) (2) and that this enzyme can be transferred to thymidine kinase-deficient (TK^-) cells by transformation with UV-inactivated HSV-1 or HSV-2 (3) or with temperature-sensitive mutants of these viruses (4). The viral TK is well characterized and can be easily distinguished from most mammalian cell TKs (2). Moreover, a number of TK^- mammalian cell lines are available as recipients for the viral enzyme. Thus, the transfer of TK would appear to be an excellent model system for studying gene transfer between mammalian cells.

Our aim is to learn how viral genes are processed by recipient mammalian cells—i.e., whether and how they become integrated into the recipient cell genome, how their expression is controlled, and what determines the efficiency of these processes. Studies such as these hopefully may lead to DNA-mediated transfer of genes between mammalian cells as well as to a better understanding of some of the processes involved in oncogenic transformation. In this article we describe the transfer of the HSV-2 TK to human TK^- cells by infection with purified HSV-2 DNA and the preliminary characterization of the resulting transformed lines.

Abbreviations: HSV, herpes simplex virus; TK, thymidine kinase; α-MEM, α minimal essential medium; TK^-, TK-deficient; α-HAT, α-MEM containing hypoxanthine, aminopterin, and thymidine; α-BrdUrd, α-MEM containing BrdUrd at 30 μg/ml.

MATERIALS AND METHODS

Cells and Virus. A line of TK^- human cells, 143, derived from the murine sarcoma virus-transformed line R970-5 (5) by selection with BrdUrd at 60 μg/ml (K. Huebner and C. Croce, personal communication) was used as recipient for the HSV-2 TK gene. (We are grateful to K. Huebner and C. Croce for providing both cell lines to us.) The lines were routinely grown in monolayer cultures in α minimal essential medium (α-MEM) (6) (nonselective medium) plus 10% fetal calf serum. Transformed lines were grown in α-MEM containing 0.1 mM hypoxanthine, 1 μM aminopterin, and 40 μM thymidine (α-HAT medium), selective for TK^+ cells (7). For experimental purposes the transformed lines sometimes were grown or plated in counterselective medium–α-MEM containing BrdUrd at 30 μg/ml (α-BrdUrd)–selective for TK^- cells; in this case, the lines were first either extensively washed free of HAT medium or were cultured for at least one passage in α-MEM. All lines were routinely subcultured twice weekly with a split ratio of 1:6 to 1:8. Periodic checks of cultures for mycoplasma contamination (8) were negative.

HSV-2, strain 219 (9), was propagated in Vero cells (multiplicity of infection = 0.5 plaque-forming units per cell) and harvested 24 hr after infection. Virus suspensions prepared from sonicated cell pellets were stored at −70°.

DNA Purification. Total DNA (cellular plus viral) was extracted from HSV-2 infected Vero cells (multiplicity of infection = 0.5 plaque-forming units per cell) at approximately 24 hr after infection by lysis of the cells with 0.2% (wt/vol) sodium dodecyl sulfate and digestion with Pronase (0.5 mg/ml in 10 mM Tris·HCl, pH 8.0/10 mM EDTA) followed by two phenol extractions and precipitation of the nucleic acids with ethanol. The viral DNA was then purified by two cycles of buoyant density centrifugation in NaI gradients containing ethidium bromide (10). DNA extracted from infected cells usually contained 20–30% viral DNA, as determined by analytical buoyant density centrifugation in CsCl (Fig. 1). After one cycle of centrifugation in NaI gradients the percentage of viral DNA was increased to about 80%, and after the second cycle it was more than 95%. After removal of the ethidium bromide (11), the DNA was dialyzed extensively against 10 mM Tris·HCl, pH 7.5/10 mM NaCl/1 mM EDTA and stored at −70°.

Cell Transformation. Transformation of subconfluent monolayers of TK^- 143 cells (approximately 5×10^5 cells per 60-mm dish) with HSV-2 DNA was carried out by using the calcium technique (12) as modified (13). To eliminate infectivity, viral DNA preparations were sheared through a 19-gauge needle to a molecular weight of about 3×10^7 as measured by gel electrophoresis in 0.4% agarose (14). After treatment with the viral DNA, the cultures were incubated for 24 hr in α-MEM,

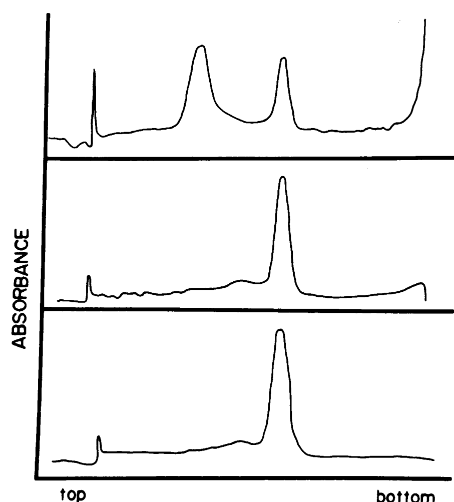

FIG. 1. Analytical ultracentrifugation in CsCl of DNA extracted from Vero cells lytically infected with HSV-2. *Top Panel.* Before purification in NaI gradients. *Middle Panel.* After the first cycle of purification. *Bottom Panel.* After the second cycle of purification.

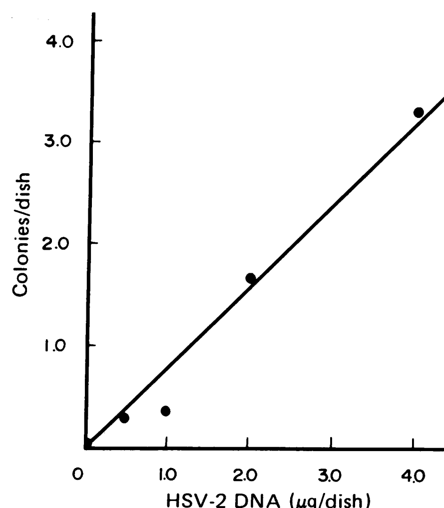

FIG. 2. Dose-response for transformation of human 143 cells from TK^- to TK^+ by sheared HSV-2 DNA.

then switched to α-HAT to select for TK^+ transformants, and refed every 2–3 days. Untransformed TK^- cells died and became detached within a few days, and colonies of transformed cells became visible in 7–10 days. By day 12, colonies were isolated to establish cell lines in α-HAT or cultures were fixed and stained for counting.

For purposes of comparison, cell cultures were also transformed with UV-inactivated HSV-2, according to Munyon *et al.* (3), and cell lines growing in α-HAT were established from the resulting surviving colonies.

Assay for TK Activity and Gel Electrophoresis. TK activity in crude cell extracts was assayed according to Munyon *et al.* (15) except that cells were disrupted by sonication. Electrophoresis in 5% (wt/vol) polyacrylamide was carried out in cylindrical gels (0.6 × 7 cm) according to Davis (16) except that, following Kit *et al.* (17), the gels and cathode buffer contained 0.2 mM thymidine and 2 mM ATP. After electrophoresis for 100 min at 3 mA per gel and 4°, the gels were fractionated into 2-mm-thick slices that were assayed for TK activity as above (15). The rate of migration (R_F) of the TK was calculated relative to that of the bromophenol blue tracking dye.

Autoradiography. Cells were labeled for 24 hr with [^{3}H]-thymidine (specific activity 20 Ci/mM) at 0.1–0.25 μ Ci/ml in α-MEM, fixed in Carnoy's fluid, washed in ice cold 5% (wt/vol) trichloroacetic acid, rinsed extensively in water, dried in air, and coated with Kodak NTB-3 nuclear track emulsion. After 24–48 hr at 4° the autoradiographs were developed and the cells were stained with Giemsa stain.

RESULTS

Transformation with Viral DNA and Isolation of Transformed Lines. Infection of 143 TK^- cells with HSV-2 DNA resulted in the appearance of colonies within 7–10 days of incubation in α-HAT. The dose-response was approximately linear (Fig. 2) as has been described previously for oncogenic transformation by adenovirus DNA (1). The specific activity of the viral DNA, calculated from Fig. 2 (average of six experiments), was approximately 1.5 colonies per μg of DNA for up to a maximum of 5 colonies per culture, or 1 colony per 10^5 treated cells. From experiment to experiment the specific activity of the same DNA preparation varied between 0.5 and 2 colonies per μg of DNA. In untreated cultures or in cultures treated with DNA in the absence of Ca^{2+} or with DNA predigested with DNase, no surviving colonies were observed. The spontaneous reversion rate (TK^- to TK^+) of cell line 143 was estimated to be less than 10^{-8} (in agreement with findings of K. Huebner and C. Croce, personal communication). Several colonies were isolated from DNA-treated cultures, and lines growing continuously in α-HAT were established from all of them.

TK Activity of the Transformed Lines. Two types of study were carried out to verify that the TK expressed by the transformed cells was of viral and not cellular origin. First, the thermal lability of the TK in all of the DNA-transformed lines was found to be similar to that of the TK expressed in 143 cell line cells lytically infected with HSV-2 and clearly distinguishable from the lability of the enzyme in human R970-5 (TK^+) cells. Second, polyacrylamide gel electrophoresis (Fig. 3) indicated that the TK from crude extracts of DNA-transformed cells migrated at a rate similar to that of the viral enzyme (R_F = 0.6) and significantly greater than that of the enzyme from R970-5 cells (R_F = 0.2). All of the clones tested gave similar results. From the properties of the TK expressed in transformed cells as well as from the fact that spontaneous revertants of 143 cell line cells were never observed, we conclude that the transformed cells expressed an enzymatic activity coded for by the HSV-2 genome.

Stability of Expression of TK Activity in Transformed Lines. The stability of expression of the viral TK in cell lines derived from DNA-transformed colonies was examined in a number of ways. When the lines were maintained in α-HAT (selective for TK^+ cells) they all continued to grow (up to approximately 120 cell doublings at the present time) without indication of a "crisis" or significant reduction in their growth rate. However, distinct differences between cell lines became apparent when plating efficiencies in counterselective medium (α-BrdUrd) were assayed for each line growing continuously in α-HAT or in nonselective α-MEM (Table 1). Among the most stable lines were AC3 and AC4, in that early after isolation as well as after prolonged culture in α-HAT or α-MEM they contained a fairly low (less than 5% and 1%, respectively) and constant fraction of BrdUrd-resistant cells (i.e., cells that had presumably lost or were not expressing TK activity). On the other hand, in the AC5 line the fraction of BrdUrd-resistant cells remained low (0.1–0.2%) as long as the line was kept under selective pressure but increased progressively up to 80% upon

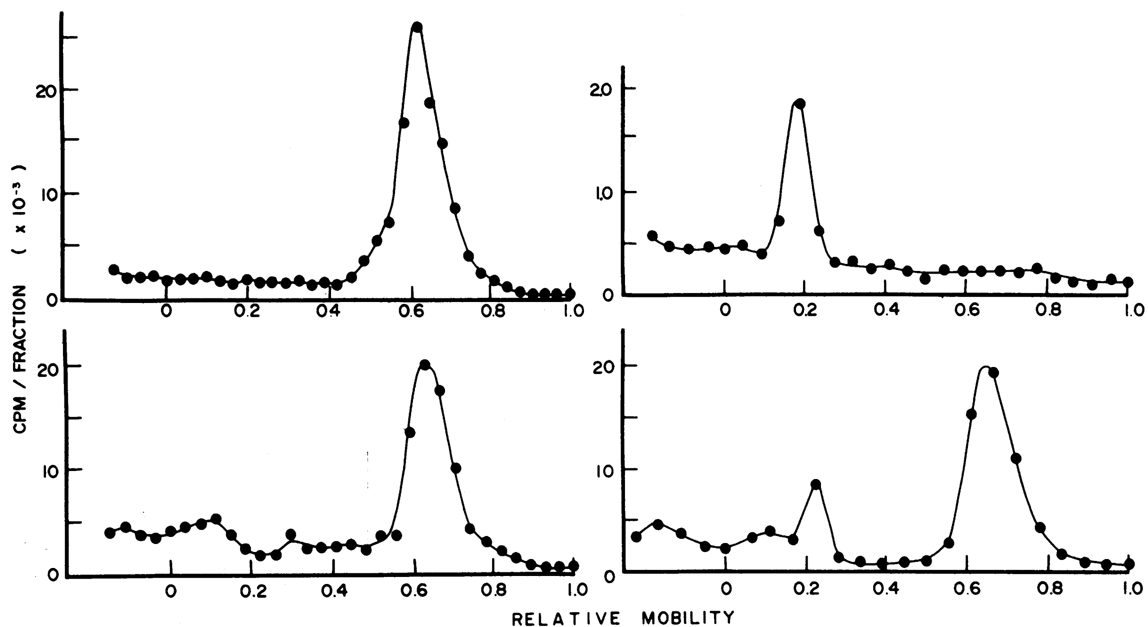

FIG. 3. Polyacrylamide gel electrophoresis of TK activity. *Upper Left.* 143 (TK$^-$) cell line cells lytically infected with HSV-2. *Upper Right.* R970-5 (TK$^+$) cells. *Lower Left.* AC3 cells transformed by HSV-2 DNA. *Lower Right.* Mixture of R970-5 (TK$^+$) and HSV-2 infected 143 (TK$^-$) cells.

subculture in nonselective medium. The behavior of the AC1 line appeared to be more complex: at early times of growth in α-HAT (before passage 20), about 25% of the cells were BrdUrd-resistant but by passage 39 in selective medium this fraction had decreased to 4%. Removal of the selective pressure increased the fraction of cells surviving the BrdUrd challenge to about 45% independently of the length of subculturing in α-MEM. Somewhat similar behavior was observed for the AC2 line.

Qualitatively similar results were obtained with lines transformed with UV-inactivated HSV-2. No colonies (less than 0.001%) were observed when R970-5 (TK$^+$) cells were plated in α-BrdUrd (data not shown).

Subcloning of the transformed lines in α-HAT usually resulted in sublines with characteristics similar to those displayed by the parental line at the time of subcloning. Sublines derived from lines relatively variable in their expression of TK generally retained this inherent instability, whereas more stable parental lines gave rise to equally stable subclones (Table 1). This behavior held true even when the sublines were recloned and was again observed for subclones derived from lines transformed with UV-inactivated virus (data not shown). Four subclones (derived from lines AC1 and AC5 at passage 5 in α-HAT) were isolated and maintained in α-BrdUrd. After four to six passages they were tested for their ability to plate in α-HAT, and no colonies (or less than 0.0001%) were observed, suggesting that these clones were stable in their lack of expression of TK.

To examine further the expression of TK in transformed lines, experiments were carried out in which the cells were labeled with [^{3}H]thymidine and the uptake of radioactivity was determined by autoradiography. Transformed lines growing under various conditions were plated at low density; after 7–10 days, when well isolated colonies had developed, the cultures were switched to α-MEM containing [^{3}H]thymidine and incubated for a further 24 hr before fixation and autoradiography. The colonies were then examined for the presence of cells containing labeled nuclei.

Colonies derived from control R970-5 (TK$^+$) cells or 143 (TK$^-$) cells were 100% labeled or 100% unlabeled, respectively. In contrast, the labeling patterns of colonies derived from transformed cells were variable and complex. When transformed lines, growing either in α-HAT or α-MEM, were plated in α-HAT prior to being labeled, essentially all of the resulting colonies were 100% labeled, with rare instances of colonies containing one or two unlabeled cells. When the same lines were plated in α-BrdUrd prior to labeling, the majority of the resulting colonies were unlabeled but a significant fraction contained cells that had incorporated radioactivity although to a lower extent than colonies growing in α-HAT. The percentage of BrdUrd-resistant labeled colonies and the extent of their labeling varied from cell line to cell line. For example, when AC1 was plated after 39 passages in α-HAT or 34 in α-MEM, 1–2% of the colonies contained a few lightly labeled cells, whereas close to 50% of the colonies derived from AC5 (at the same passages) had relatively large patches of lightly labeled cells. Thus, it would appear that cells expressing a low level of TK activity survive the BrdUrd challenge and consequently the plating efficiency in α-BrdUrd is likely to represent an overestimate of the fraction of the cells in a population that are unable to express TK. When four sublines, derived by subcloning AC1 and AC5 in α-BrdUrd and maintained in this medium for four to six passages, were plated in α-MEM and labeled after 10 days of growth, no labeled cells were observed in any of the colonies, suggesting that prolonged exposure to BrdUrd had resulted in irreversible loss of the ability to express TK. These observations are consistent with the finding (previously mentioned) that these same lines were unable to form colonies in α-HAT.

The most interesting results emerged when transformed lines growing in α-HAT or α-MEM were plated directly in the latter prior to being labeled. Under this condition both TK$^+$ and TK$^-$ cells could form colonies and, as expected, totally positive and totally negative colonies were observed (Fig. 4A). Their relative percentages were in approximate agreement with the plating efficiency values obtained in α-HAT and in α-BrdUrd (Table 1). In addition to these colonies, however, a significant percentage of mixed colonies (colonies only partially labeled) was observed, especially in the least stable lines. Their labeling

Table 1. Survival of DNA-transformed lines in selective and counterselective media

Cell line	No. of passages in*		% survival in†	
	α-HAT	α-MEM	α-HAT	α-BrdUrd
Parental lines				
AC1	5	0	76	24
	12	0	74	26
	20	0	83	17
	28	1	84	16
	39	0	96	4
	5	7	55	45
	5	15	55	45
	5	34	59	41
AC2	10	0	88	12
	5	5	52	48
	5	15	68	32
AC3	10	0	99	1
	4	5	96	4
	4	15	97	3
AC4	10	0	100	0.06
	19	2	99	1
	4	5	100	0.5
	4	15	99	1
AC5	5	0	100	0.2
	12	0	100	0.1
	20	0	100	0.1
	30	2	98	2
	39	0	100	0.3
	5	7	68	32
	5	15	54	46
	5	28	15	85
	5	34	20	80
Sublines‡				
AC1-21	(30)6	2	100	0.2
AC1-22	(30)6	1	100	0.4
AC2-20	(11)2	2	48	52
AC2-21	(11)2	2	57	43
AC2-23	(11)2	2	4	96
AC2-24	(11)2	2	67	33
AC3-20	(12)2	2	99	0.8
AC3-21	(12)2	2	99	1
AC3-23	(12)2	2	97	3
AC4-20	(12)2	2	100	0.4
AC4-21	(12)2	2	100	0.4
AC4-22	(12)2	2	100	0.5
AC4-23	(12)2	2	100	0.1
AC4-24	(12)2	2	99	1

* Each cell line was cultured in α-HAT and then in α-MEM for the indicated number of passages.

† Expressed as the ratio of the plating efficiencies in α-HAT or α-BrdUrd to the sum of the plating efficiencies in both media, this latter value being approximately equal to the plating efficiency in nonselective medium.

‡ Derived from parental lines at the passages in α-HAT indicated in parentheses, then cultured in α-HAT followed by α-MEM as indicated.

patterns (Fig. 4*B–D*) were quite variable and tend to suggest that the ability to express TK can be suppressed or reactivated within the limited number of cell divisions occurring during colony formation in nonselective conditions. Some of the more clearly sectored colonies also indicate that the modulation of TK expression is not a random process and that a limited stability of the suppressed or activated condition is maintained in the progeny of a given cell. Control experiments in which cocultivated R970-5 (TK^+) and 143 (TK^-) cells were plated at similarly low densities indicated that clumps of two or more cells could account for only a minority of the mixed colonies observed with the transformed lines.

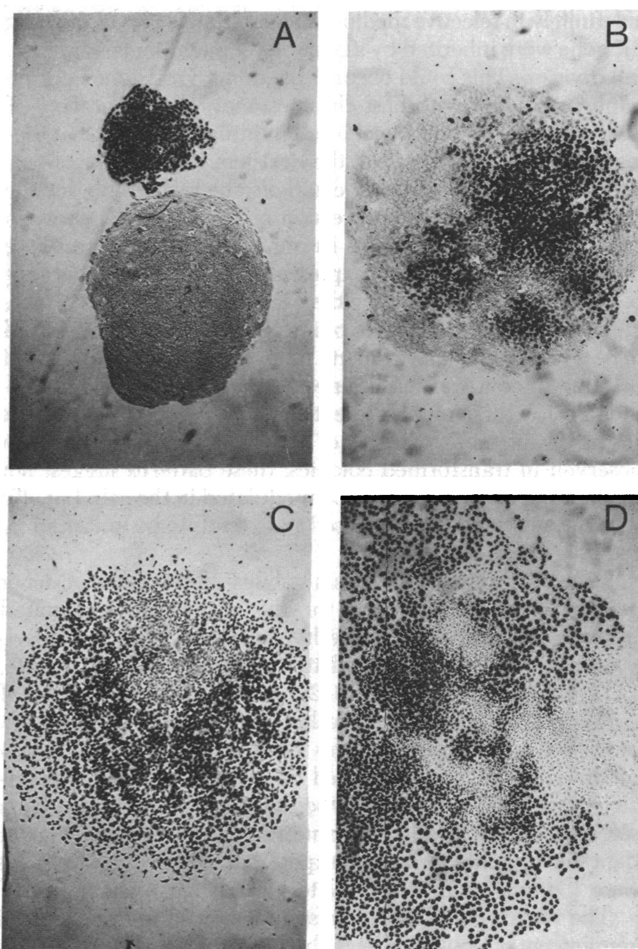

FIG. 4. Autoradiographs of colonies from DNA-transformed lines grown in α-HAT or α-MEM and cloned in the latter. See *text*. (Magnifications: *A*, *B*, and *C*, ×8; *D*, ×33.)

DISCUSSION

Our results have shown that DNA-mediated transfer of the HSV-2 TK gene to TK^- human cells can be readily achieved with an efficiency of up to 1 transformant per 10^5 recipient cells. The specific activity of the DNA was approximately 1.5 colonies per μg of viral DNA or per 6×10^9 viral DNA molecules, in the same range as values obtained for oncogenic transformation by simian virus 40 or adenovirus DNA with the calcium technique (1). This is not unexpected if one assumes that similar events, in terms of uptake, expression, and stabilization of donor genes, occur for both types of transformation. While this work was in progress we learned that other investigators had succeeded in transforming TK^- mouse cells with sheared HSV-1 or HSV-2 DNA (A. C. Minson, G. R. Darby, and P. Wildy, personal communication) or with restriction enzyme fragments of HSV-2 DNA (N. Maitland and J. McDougall, personal communication). Thus, DNA-mediated transfer of the TK gene is not restricted to the particular system used in our studies.

The observation that all TK^+ transformed colonies isolated could be established as cell lines and maintained apparently

indefinitely in selective medium suggests that copies of the viral TK gene were inherited by a majority of daughter cells at each cell division. This could occur if the donor viral gene became stably associated with host chromosomes or, alternatively, if such a gene is present in each cell in multiple copies that segregate randomly. The state of the viral gene in the recipient cells and its mode of inheritance could also be responsible for the observed variability of expression of the viral TK between different transformed lines. In our studies, some lines were relatively "committed" to expressing the TK while others appeared to be able to switch to the TK^- phenotype at a relatively high frequency. The degree of instability, however, appeared to be a relatively stable property of each independently derived cell line since it was maintained on repeated subcloning. Although it is difficult at present to draw detailed conclusions from the different patterns of $[^3H]$thymidine incorporation observed in transformed colonies, these patterns suggest not only that TK expression can be modulated in the cells but also that the activated or suppressed state tends to be inherited by the progeny of a given cell.

Somewhat similar findings on stability of expression of donor genes have been reported for the chromosome-mediated transfer of human or murine hypoxanthine phosphoribosyltransferase (IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) to mouse cells (18–20) as well as for the transfer of the HSV-2 TK gene to mouse cells by UV-inactivated virus (21). However, at variance with this latter report, that the activity of the viral TK can be turned on and off repeatedly at efficiencies higher than 10% when the cells are switched from selective to counterselective conditions and vice versa, are our data showing that prolonged exposure to BrdUrd results in less than 1×10^{-6} cells being able to survive in HAT medium.

The variability of TK expression observed in our studies is reminiscent of position effects observed in other genetic systems in which variegated or unstable phenotypes result from translocation of a gene into proximity with heterochromatin (22). Such a mechanism could account not only for the instability of TK expression in some of our lines but also for the fact that this instability is inherited by subclones of such lines. The observations reported by Lin and Munyon (23) suggesting that TK expression in virus-transformed lines might not be under control of the mechanism that normally regulates expression of the cellular enzyme are not in disagreement with such a model.

One of our interests in carrying out the present study was to evaluate the feasibility of DNA-mediated gene transfer between mammalian cells, akin to that used in bacterial systems. If we assume that the efficiency of transfer for the HSV TK gene can be extrapolated to the case of DNA-mediated gene transfer between mammalian cells (with a genetic complexity 2×10^4-fold greater than that of HSV) one might hope to obtain about 1 transformant from 2×10^9 recipient cells exposed to 10 mg of cellular DNA. Although the amount of DNA required presents no obstacle, the detection of such a low transformation frequency would be difficult, even if spontaneous reversion did not occur. Considerations such as these indicate that improved transfection techniques and DNA fractionation procedures may be necessary before gene transfer with purified mammalian cell DNA becomes a reality.

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