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Thymidine Kinaseless Revertants of Ltk⁻ Cells Transformed by Herpes Simplex Virus Type 1 Are Resistant to Retransformation by Homologous Virus

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Mouse L cells lacking thymidine kinase (Ltk⁻) that had been transformed to the thymidine kinase-positive (tk⁺) phenotype by herpes simplex virus type 1 (HSV-1) were cultured in medium containing tritiated thymidine. Six clonal lines of cells surviving this treatment were found to have the following properties: (i) the cells were tk⁻ and had spontaneous back-reversion frequencies to the tk⁺ phenotype of 10^{-5} or less, (ii) the cells contained HSV antigens, although in lesser amounts than in the parental transformed cells, and (iii) the cells were retransformable to the tk⁺ phenotype by HSV-1 at frequencies of about 1 to 13% of the frequency of the primary transformation of Ltk⁻ cells. HSV-1 plaqued as efficiently on monolayers of these cells and replicated in them to the same extent as it did in Ltk⁻ cells. These results indicate that HSV-1-transformed L cells surviving selection with tritiated thymidine are unlike the parental Ltk⁻ cells in that they are damaged in such a way that the cells are resistant to retransformation by homologous virus, although they remain fully permissive for virus replication.

L cells lacking thymidine kinase (Ltk⁻) can be transformed to the thymidine kinase-positive (tk⁺) phenotype by ultraviolet-irradiated herpes simplex virus (HSV) (13). The tk acquired by the transformed cells is the product of a viral gene (6, 14, 16). In addition, other viral genes are known to be present in transformed L cells by their expression as antigens (3, 3a) and by the demonstration that these cells contain a substantial fragment (10 to 23%) of the HSV genome in multiple copies (5, 11). Similarly, HSV antigens (7) and deoxyribonucleic acid (DNA) (9) have been shown to be present in hamster cells morphologically transformed by ultraviolet-irradiated HSV type 2 (HSV-2).

We have been interested in two related questions. First, can HSV-transformed cells accept additional HSV genetic information? Second, can tk⁻ revertants of HSV-transformed cells accept new HSV genetic information? We have recently shown (10) that cells that have accepted one HSV-1 tk gene can accept another HSV-1 tk gene in a sequential transformation and that the frequency of this second transformation is as great as for primary transformation of Ltk⁻ cells. This indicates that the presence of HSV-1 genetic information in transformed cells does not preclude the acceptance by the cell of additional HSV-1 genetic information.

In the present report we show, however, that tk⁻ revertants of transformed cells that have little or perhaps no spontaneous back-reversion rate to the tk⁺ phenotype are refractory to retransformation to the tk⁺ phenotype by homologous virus. These contrasting results suggest that it is damage to the host cell genome during selection for the revertant phenotype that lowers the retransformation frequency of revertant cells.

MATERIALS AND METHODS

Cells, media, and virus. Eagle medium (8) containing the nonessential amino acids and supplemented with 5% calf serum (EM5C) was used as the basic cell culture medium. African green monkey kidney (CV_1) cells were grown in EM5C with no additional supplement. Ltk⁻ cells were grown in EM5C supplemented with 20 μg of 5-bromodeoxyuridine (BUdR) per ml (EM5CBUdR). Transformed cells were grown in EM5C supplemented with: methotrexate, 6×10^{-7} M; thymidine, 1.6×10^{-5} M; adenosine, 5×10^{-5} M; and guanosine, 10^{-5} M (EM5CMTAG). Revertant cell lines were grown in EM5CBUdR medium. Cell lines were subcultured every 72 to 96 h. Cells were grown at 37°C in a humidified atmosphere containing 10% CO_2 and 90% air. HSV-1, strain KOS, was the virus used in all experiments reported in this paper.

Selection of tk⁻ revertant cell lines from HSV-1-

transformed (clone 139) L cells. In [^{3}H]thymidine ([^{3}H]TdR) selection, a series of tk$^-$ revertant cell lines was obtained from HSV-1-transformed (clone 139) L cells, using [^{3}H]TdR as the selecting agent. In brief, clone 139 cells were grown for two generations in EM5C medium containing thymidine, 1.6×10^{-5} M, and guanosine, 10^{-5} M. At the end of this time, 2×10^5 cells were seeded in 60-mm plastic tissue culture dishes (Lux Scientific Corp., Newbury Park, Calif.) in 2 ml of EM5C medium containing the same concentrations of adenosine and guanosine. After 1 h, when cells had attached, the medium was replaced with EM5C containing adenosine, guanosine, and 4 μCi of [^{3}H]TdR (20 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml. Dishes were incubated at 37°C until colonies appeared. Six vigorously growing colonies were picked and cultured in EM5CBUdR medium. After 10 passages in this medium, revertant lines were examined for their tk phenotype. In BUdR selection, clone 139 cells that had been maintained in EM5CMTAG medium for over 2 years were trypsinized and subcultured in EM5C containing 30 μg of BUdR per ml. Approximately 10^{-3} cells plated in this medium grew out to form colonies. Four vigorously growing colonies were picked up and grown in EM5CBUdR medium as revertant cell lines. For the purpose of this report, colonies that have been termed "vigorously growing" are those that grew as readily in BUdR or [^{3}H]TdR as in their absence.

[^{3}H]TdR uptake. [^{3}H]TdR uptake was performed according to the procedures of Breslow and Goldsby (2). Briefly, 2×10^6 cells were seeded in 60-mm tissue culture dishes. Twenty-four hours later, cells were pulse labeled for 2 h with 0.5 μCi of [^{3}H]TdR (20 Ci/mmol) per ml of EM5C. At the end of the labeling time, monolayers were washed with 5 ml of phosphate-buffered saline and then with 2 ml of 5% trichloroacetic acid for 18 h at 4°C. The trichloroacetic acid was aspirated, and monolayers were washed three times with 95% ethanol. Dishes were dried, cut into pieces, and counted in toluene-based scintillation medium.

Thymidine kinase assay. Cells were harvested by scraping them into the growth medium. The cell pellets were washed once with phosphate-buffered saline and resuspended in sonic treatment buffer (0.2 ml/10^6 cells) containing 20% glycerol, 1 mM dithioerythritol (Sigma Chemical Co., St. Louis, Mo.), and 5 mM maleate-tris(hydroxymethyl)aminomethane, pH 6.5. The cell suspension was frozen and thawed once and then sonically treated at full power for 5 s in a Branson Sonifier (model 875; Danbury, Conn.) at 4°C. The sonic extract was centrifuged in a refrigerated Sorvall centrifuge at 30,000 $\times$ *g* for 30 min, and the supernatant was used for the assay. The details of the tk assay have been described by Lin and Munyon (12).

Indirect immunofluorescent staining. The details of the indirect immunofluorescent staining procedure and of antiserum preparations have been described elsewhere by Chadha and Munyon (3).

Transformation assay. The transformation assay was carried out according to the procedure of Munyon et al. (13). Virus stock was diluted in EM5C to achieve the desired multiplicity of infection and irradiated for 2 min at 1,120 μW/cm^2 on a rotary shaker. Monolayers were infected with 0.2 ml of virus suspension containing a multiplicity of 1 plaque-forming unit/cell, calculated before ultraviolet irradiation, and incubated for 1 h at 37°C. At the end of this time, monolayers were overlaid with 2.5 ml of EM5C. Twenty-four hours after infection, EM5C was replaced with EM5CMTAG containing 0.1% human gamma globulin (Hyland, Div. of Travenol Laboratories, Inc., Costa Mesa, Calif.). This medium was replaced every 4 to 5 days until colonies appeared.

Plaque assay. Samples were titrated on confluent monolayers of CV_1 cells grown in six-well trays (35-mm diameter). After 1 h of adsorption at 37°C, the monolayers were overlaid with EM5C (without phenol red) containing 1% 4,000-centipoise methylcellulose. Plaques were visualized by overlaying with EM5C (without phenol red) containing 2% 15-centipoise methylcellulose and 100 μg of neutral red per ml.

RESULTS

Selection of tk$^-$ revertants of HSV-1-transformed L cells. Davidson et al. (4) reported that HSV-transformed cell cultures produced cells that were capable of growing in BUdR, a toxic substance for tk$^+$ cells. However, these BUdR-resistant cells apparently still contained the HSV gene for tk, because populations of such cells grown in the presence of HAT medium produced surviving clones, indicating the reacquisition of tk activity. The alteration of phenotype seemed freely reversible and did not denote loss of the viral gene or reacquisition of the tk$^+$ phenotype by an infrequent mutation. Therefore, BUdR did not seem to be a suitable selection agent for obtaining tk$^-$ revertants that could then be used in retransformation studies.

However, to determine whether stable tk$^-$ revertants could be obtained from transformed cells, clone 139 cells were cultured in medium with BUdR. About 10^{-3} of the clone 139 cells cloned in this medium and grew vigorously. Four of these cloned lines were tested for their ability to form colonies in EM5CMTAG. The data are shown in Table 1 and indicate that tk$^-$ revertants selected with BUdR are not stable and contain tk$^+$ cells at frequencies comparable to the frequency of primary transformation of Ltk$^-$ cells by HSV. Such a high background of spontaneous back-reversion to a tk$^+$ phenotype would obscure any attempt to measure HSV retransformation in BUdR revertants.

Since [^{3}H]TdR is cytotoxic for tk$^+$ cells and has been used to produce tk$^-$ revertants of HSV-transformed cells (3), this agent was also used to obtain tk$^-$ revertants from populations

TABLE 1. *Spontaneous back-reversion of tk⁻ revertant cell lines to the tk⁺ phenotype*

Revertant cell line	Selecting agent[a]	Survival in EM5CMTAG	
		Colonies/dish[b]	Frequency
139 R-2	BUdR	50	2.5×10^{-4}
139 R-3	BUdR	65	3.3×10^{-4}
139 R-4	BUdR	18	9.0×10^{-5}
139 R-5	BUdR	36	1.8×10^{-4}
139 R-8	[³H]TdR	0	$<2.5 \times 10^{-7}$
139 R-10	[³H]TdR	0	$<2.5 \times 10^{-7}$
139 R-11	[³H]TdR	1	5.0×10^{-6}
139 R-12	[³H]TdR	0	$<2.5 \times 10^{-7}$
139 R-13	[³H]TdR	0	$<2.5 \times 10^{-7}$
139 R-15	[³H]TdR	2	1.0×10^{-5}

[a] BUdR and [³H]TdR were used at concentrations of 30 μg and 4 μCi (20 Ci/mmol) per ml of the medium, respectively.

[b] The average, calculated from number of colonies in 20 dishes.

of clone 139 cells. Revertants appeared at much lower frequency (about 10^{-5}) with this selection than with BUdR. Six clonal lines of cells that grew vigorously in [³H]TdR-containing medium were isolated. These cell lines were also examined for their ability to form colonies in EM5CMTAG medium. The data are shown in Table 1 and indicate that these cells have a much more stable tk⁻ phenotype than the BUdR revertants. Four of the cell lines were not observed to produce tk⁺ back-revertants, whereas the remaining two lines had back-reversion frequencies of about an order of magnitude less than the least frequently back-reverting BUdR revertant. All of the remaining studies reported in this paper were carried out on [³H]TdR-selected revertants.

Characterization of revertant cell lines. Revertant cell lines could survive in a medium containing [³H]TdR in at least two ways. First, the cells could lack tk and could not effect the lethal synthesis of thymidine monophosphate. And, second, the cells could be impermeable to [³H]TdR but still be tk⁺. To test these possibilities, tk activity was measured in extracts of cells, and intact cells were examined for their ability to incorporate [³H]TdR into an acid-insoluble product. The results are shown in Table 2. The revertant cell lines had low levels of tk similar to that found in Ltk⁻ cells which can be associated with the mitochondrial tk possessed by these cells (1). As would be expected from this observation, revertant cells incorporated no more [³H]TdR than did Ltk⁻ cells. HSV-transformed clone 139 cells, in contrast, had more than 10 times as much tk activity and incorporated some 300 times as much [³H]TdR as did the Ltk⁻ and revertant cells. These results establish that revertant cells cannot be distinguished from Ltk⁻ cells in their tk phenotype and are quite different from the parental tk⁺ HSV-transformed cells.

All six revertant cell lines still expressed HSV-related antigens detectable by immunofluorescence, although the intensity of fluorescence was less than that observed with clone 139. Ltk⁻ cells displayed no HSV-related fluorescence. These data are shown in Fig. 1. These results support an earlier observation on a line of revertant cells selected with [³H]TdR that was lost due to bacterial contamination (3).

Retransformation of revertant cell lines by homologous HSV-1. Since the revertant cell lines were tk⁻, they were suitable substrates for transformation to the tk⁺ phenotype by ultraviolet-irradiated HSV-1 (13). Table 3 shows the results of a transformation experiment comparing the efficiencies of retransformation of revertant cell lines with primary transformation of Ltk⁻ cells. Ltk⁻ cells transformed with a frequency of 6.8×10^{-5}, whereas the revertant cell lines had retransformation frequencies that ranged from about 1 to 13% of that value. These results indicate that revertant cells are resistant to retransformation by homologous virus and, although tk⁻ in phenotype, are obviously different from Ltk⁻ cells.

One possible explanation for the refractoriness to retransformation of revertant cells is that [³H]TdR selects lines of cells in which the processes of virus adsorption and penetration are not as efficient as those in Ltk⁻ cells. If this were so, during a transformation experiment

TABLE 2. *tk activity and [³H]TdR incorporation in revertant cell lines*

Cell line	cpm[a]	
	[³H]TdR incorporation[b]	tk activity[c]
Clone 139	188,000	1,098
Ltk⁻	883	77
139 R-8	505	73
139 R-10	284	84
139 R-11	448	71
139 R-12	474	92
139 R-13	818	80
139 R-15	273	84

[a] Each number represents the average of two determinations.

[b] Counts per minute/2×10^6 cells per h.

[c] Counts per minute as thymidine monophosphate/microgram of protein per 30 min.

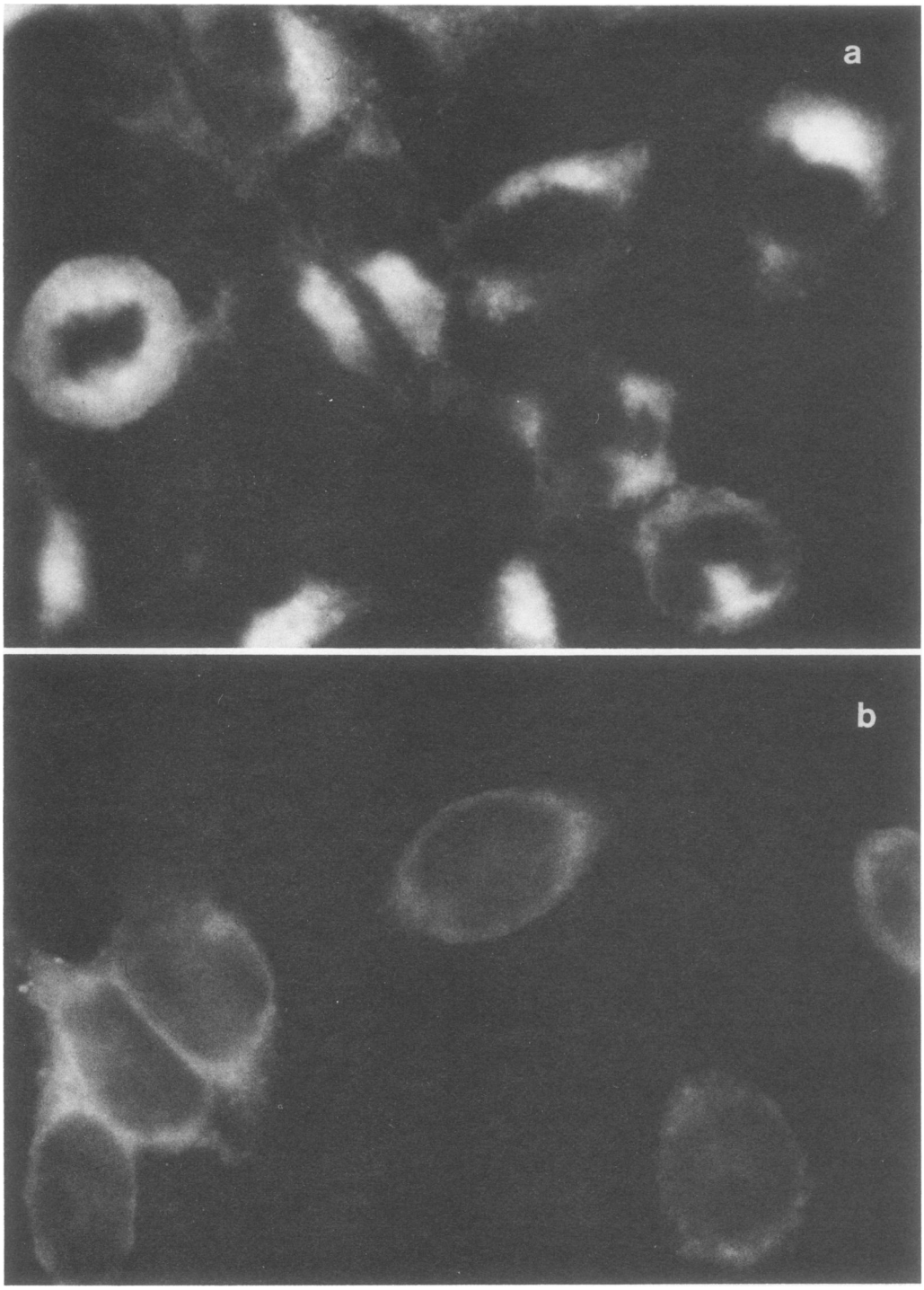

FIG. 1. *Indirect immunofluorescence test on: (a) HSV-1-transformed L cells (clone 139), (b) a revertant cell line (139R-12), and (c) nontransformed Ltk⁻ cells, using lytic antiserum. Note the bright perinuclear cytoplasmic fluorescence (a), reduced by specific cytoplasmic fluorescence (b) and negative fluorescence (c).*

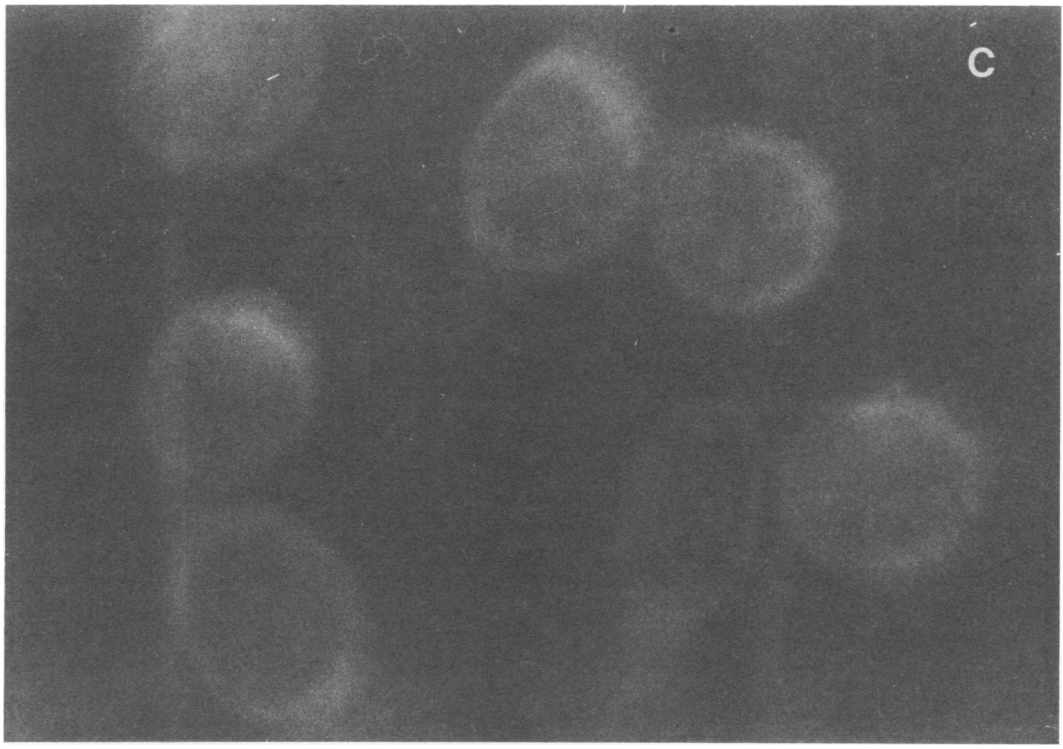

FIG. 1C.

revertant cells would not be at as high a risk for successful infection by a transforming virion as would be Ltk$^-$ cells. To examine this possibility, the efficiencies of plaque formation by HSV-1 on monolayers of revertant cells and Ltk$^-$ cells were compared. Table 4 shows that HSV-1 plaqued as efficiently on five of the revertant lines as it did on Ltk$^-$ or clone 139 cells. The virus plaqued about 50% as efficiently on 139R-8 cells as it did on other lines. These results suggest that the failure of revertant cells to transform as efficiently as Ltk$^-$ cells is not due to inefficient adsorption or penetration of HSV-1.

Another possible explanation for the inefficient transformation of revertant cells is that they are not as capable of carrying out some function(s) required for HSV formation as are Ltk$^-$ cells. If this function(s) were also required for HSV replication, such a defect would result in the cells being unable to replicate HSV to the same extent as Ltk$^-$ cells. This possibility was examined by comparing the amount of HSV produced in infected revertant cells with that in Ltk$^-$ cells. The results are shown in Table 4 and indicate that HSV-1 replicated as well in revertant cells as in Ltk$^-$ or clone 139 cells. Therefore, revertant cells are not resistant to retransformation due to the failure of any function, either viral or cellular in origin, required for full yields of progeny virus. These results do not exclude the possibility that transformation is dependent on some function not required for productive infection.

Lastly, revertant cells might be resistant to retransformation due to radiation damage from being grown in medium containing [^{3}H]TdR, irrespective of the events resulting in the establishment of the tk$^-$ phenotype from the transformed cells. This possibility was also tested by cloning Ltk$^-$ cells in [^{3}H]TdR under the same conditions in which revertants were obtained. Four vigorously growing colonies were picked and examined for their ability to be transformed. The results are shown in Table 5 and indicate that exposure to [^{3}H]TdR did not result in a decrease in the susceptibility of Ltk$^-$ cells to HSV-1 transformation. These results do not rule out the possibility that the event resulting in a transformation-resistant cell requires the action of tk on [^{3}H]TdR.

DISCUSSION

We have shown in the present paper that [^{3}H]TdR-selected tk$^-$ revertants of HSV-1-transformed Ltk$^-$ cells are resistant to re-

transformation to the tk$^+$ phenotype by homologous virus. This result stands in sharp contrast to a recent report from our laboratory (10) concerning a mutant of HSV-1 that is conditional for transformation on a high concentration of thymidine in the selection medium. Cells transformed by this mutant could be supertransformed by wild-type virus for growth in low-thymidine selection medium. It was found that the frequency of supertransformation of these conditionally transformed cells was equivalent to the primary transformation of Ltk$^-$ cells, indicating that the presence of the HSV-1 genome in conditionally transformed cells did not impair their ability to accept additional HSV-1 genetic information in a sequential transformation. Therefore, the resistance of revertant cells to retransformation is not due to the presence or expression of HSV-1 DNA per se but must be a consequence of obtaining the revertant phenotype. We have shown that resistance of revertant cells to retransformation is not due to failure of HSV-1 to adsorb and replicate in revertant cells. We have also shown that [^{3}H]TdR does not have a direct effect on Ltk$^-$ cells to induce transformation resistance.

TABLE 3. *Retransformation of revertant cell lines by HSV-1*

Cell line	Avg no. of transformed colonies/dish[a]	Transformation frequency
Lkt$^-$	13.6 ± 2.34	6.8×10^{-5}
139 R-8	1.2 ± 0.68	6.0×10^{-6}
139 R-10	1.0 ± 0.59	5.0×10^{-6}
139 R-11	0.7 ± 0.64	3.5×10^{-6}
139 R-12	0.2 ± 0.38	1.0×10^{-6}
139 R-13	1.5 ± 0.74	7.5×10^{-6}
139 R-15	1.8 ± 0.68	9.0×10^{-6}

[a] Dishes were seeded with 2×10^5 cells/dish. Each number represents the average from 20 dishes.

TABLE 4. *Ability of HSV-1 to form plaques on and replicate in revertant cell lines*

Cell line	Plaques/well[a]	PFU/ml[b] ($\times 10^6$)
Clone 139	82, 89, 95	4.5
Ltk$^-$	99, 105, 90	5.5
139 R-8	41, 56, 37	5.0
139 R-10	115, 120, 96	4.2
139 R-11	81, 90, 84	3.8
139 R-12	88, 97, 72	5.3
139 R-13	88, 80, 57	7.0
139 R-15	100, 87, 110	8.3

[a] HSV-1 was diluted and plated at a constant concentration on monolayers of the indicated cell lines.

[b] Cells were infected at a multiplicity of infection of 10 and harvested 18 h later. Virus yields were determined by titration on CV$_1$ monolayers. PFU, Plaque-forming units.

TABLE 5. *Transformation of Ltk$^-$ cells treated with [^{3}H]TdR*

Cell line	Transformed colonies/dish[a]	Transformation frequency ($\times 10^{-5}$)
Ltk$^-$	8, 9, 7, 15	4.9
Ltk$^-$ [^{3}H]TdR^{1}[b]	10, 9, 7, 11	4.6
Ltk$^-$ [^{3}H]TdR^{2}	12, 12, 16, 12	6.5
Ltk$^-$ [^{3}H]TdR^{3}	8, 9, 11, 10	4.8
Ltk$^-$ [^{3}H]TdR^{4}	7, 9, 14, 13	5.4

[a] Each dish initially seeded with 2×10^5 cells.

[b] Superscript number indicates clonal lines of Ltk$^-$ cells selected for vigorous growth in 4 μCi of [^{3}H]TdR per ml.

Although it is known that HSV-1-transformed Ltk$^-$ cells contain a fragment of 10 to 23% of the HSV-1 genome (5, 11), the nature of the association of this fragment with the host cell genome is still obscure. It is tempting to speculate that these fragments are integrated into sites in the mouse cell genome, perhaps a small number of specific sites as has been shown to be the case for simian virus 40 transformation of mouse cells (15). The observations in this paper suggest the possibility that the selection of revertant cells with [^{3}H]TdR results in damage to a site in the host cell genome, so that a succeeding viral genome cannot be accepted or can be accepted there or at another site with a lower probability than at an undamaged primary site. This hypothesis is strengthened by the observation that these [^{3}H]TdR-selected revertants have no HSV-1 DNA detectable (<3% of the HSV-1 genome) by DNA-DNA reassociation kinetics (D. Kingsbury, personal communication), although immunofluorescence data indicate that some HSV-1-related genetic information is still being expressed. Interestingly, Kraiselburd et al. (11) report a BUdR revertant that has also lost all detectable HSV-1 DNA.

In any event, it is clear from the results presented that [^{3}H]TdR-selected revertants are unlike Ltk$^-$ cells with respect to transformation by HSV-1, although they remain fully permissive for HSV-1 replication. This difference suggests that there is at least one cell-specific function required for transformation that is not required for productive infection.

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