JOURNAL OF VIROLOGY, June 1976, p. 867-872

Vol. 18, No. 6
Printed in U.S.A.

Mutant of Herpes Simplex Virus Type 1 Conditionally Able to Transform Thymidine Kinaseless L Cells to a tk$^+$ Phenotype

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Received for publication 6 November 1975

After nitrous acid mutagenesis of herpes simplex virus type 1 (HSV-1), a mutant, 1093, was isolated which, during productive infection, induced very low levels of thymidine kinase (tk). The mutant virus was found, after UV irradiation, to be unable to transform L cells lacking tk (Ltk$^-$) to a tk$^+$ phenotype as characterized by growth of the cells in a modified HAT-selective medium containing 1.6×10^{-5} M thymidine. Cells transformed by wild-type virus grew vigorously under the same conditions. The mutant was able to transform Ltk$^-$ cells if the medium contained 10^{-3} M thymidine. These transformed cells maintained their conditional character and would not grow in low concentrations of thymidine in selective medium. Therefore, this mutant is conditional on the thymidine concentration in the selection medium in its ability to transform Ltk$^-$ cells to a tk$^+$ phenotype. The conditionally transformed cells could be supertransformed with wild-type UV-irradiated HSV-1 to a phenotype which would grow in low-thymidine selective medium. The frequency of supertransformation closely approximated the frequency of transformation of Ltk$^-$ cells by wild-type virus. Supertransformation at high frequency could not be effected by mutant 1093 or the tk$^-$ mutant B2006. These results indicate that the presence of HSV-1 genetic information in HSV-1-transformed cells does not preclude the acquisition by these cells of at least one additional HSV-1 gene, that for tk.

The transformation system developed by Munyon et al. (9) relies on the ability of thymidine kinaseless L (Ltk$^-$) cells that have been infected with UV-irradiated herpes simplex virus (HSV) to grow in a culture medium containing methotrexate, purine nucleosides, thymidine, and glycine (2). The ability of cells to grow in such a medium is dependent on the heritable acquisition of a thymidine kinase (tk) which is HSV specific as determined by its electrophoretic mobility (8), its type-specific thermal lability (2), and its immunological type specificity (17). Strains of HSV that do not induce tk fail to transform Ltk$^-$ cells to a tk$^+$ phenotype (6, 9).

The gene for tk is the only one known to be required for transformation of Ltk$^-$ cells to a tk$^+$ phenotype (6, 9) and no HSV gene has been shown to be essential for the morphological transformation of hamster, human (16), or rat embryo (14) cells. The gene for tk is not required for morphological transformation of hamster embryo cells (19). This report concerns a mutant of HSV type 1 (HSV-1) able to induce very low tk activity in infected cells. This property of the virus renders it conditional on the thymidine concentration in the selective medium in its ability to transform Ltk$^-$ cells to a phenotype capable of growing in methotrexate-containing medium. We also show that the conditionally transformed cells can be transformed again by wild-type HSV-1, indicating that HSV-1-transformed Ltk$^-$ cells can accept additional HSV-1 genetic information in a second transforming event.

MATERIALS AND METHODS

Media, viruses, and cells. The media, viruses, and cells used in this study were described in detail previously (6). Briefly, cells were routinely grown in a modified Eagle medium containing 5% calf serum (EM5C). This medium was modified for selection of tk$^+$-transformed cells from populations of Ltk$^-$ cells by the addition of methotrexate, purine nucleosides, and thymidine (MTAGGEM5C). Plaque-purified HSV-1, strain KOS (KOS 1.1), was the wild-type virus from which mutant 1093 was derived. Viruses were plaqued on CV-1 cell monolayers under a methylcellulose-containing EM5C as described previously (6).

Mutagenesis of KOS 1.1 by nitrous acid. The procedure for mutagenesis of KOS 1.1 was an adaptation of one described previously (7). Briefly, a high-titer stock of KOS 1.1 was diluted 10-fold into 0.1 M sodium acetate buffer (pH 4.6) that had been made 0.05 M in sodium nitrite. The solution was incubated for 10 min at 37 C and diluted in EM5C. This treatment reduced the infectivity of the virus prepa-

ration by 99.3% compared to treatment of the virus with buffer alone. Virus was plaqued and stocks were prepared in CV-1 cells from individual plaques.

tk and transformation assays. The transformation assays were carried out as described previously (6), except that virus was inactivated for 2 min at 1,120 μW/cm^2. The tk assay was similar to that described previously (6), except that the reaction mixture contained 2 μmol of ATP, 2 μmol of $MgCl_2$, and 20 nmol of [^{3}H]thymidine (specific activity, 0.1 Ci/mmol). Samples were counted in toluene containing the standard fluors (6) at an efficiency of about 4%. Sample preparation is described in Table 2.

RESULTS

Lytic properties of the mutant. In a search for mutants defective in transformation, HSV-1 strain KOS 1.1 (6) was mutagenized with nitrous acid as described above. The surviving virus was plaqued on CV-1 monolayers at 34 C. A total of 145 clonal isolates were examined for their tk phenotype by suppression of plaque formation in CV-1 cells by 5-bromo-2'-deoxyuridine and for their ability to transform Ltk$^-$ cells to a tk$^+$ phenotype after UV irradiation of the virus, by techniques described previously (6).

One mutant, 1093, was found to be unable to transform Ltk$^-$ cells although plaque formation was suppressed by 5-bromo-2'-deoxyuridine. The mutant did not have detectable defects, either temperature sensitive (ts) or constitutive, in lytic functions. This is shown (Table 1) by its ability to replicate at times approximating single cycles of growth at 34 and 39 C as well as the parental virus KOS 1.1.

Since tk is the only HSV gene that has been shown to be required for the Ltk$^-$-to-Ltk$^+$ transformation (6, 9), it was necessary to determine whether 1093 failed to transform due to inadequate tk expression or whether another gene was involved in transformation. When the mutant was examined for its ability to induce tk in Ltk$^-$ cells in a lytic infection, it was found that 1093 induced amounts of tk that could not be detected by the assay. The results of one assay, similar to one used by Summers et al. (15) for detecting low levels of tk activity, are shown in Table 2. The data indicate that 1093 induces no more tk activity than does the tk$^-$ mutant B2006. However, cells infected with either of these mutants have slightly more activity than is found in uninfected Ltk$^-$ cells. Two lines of evidence suggest that the mutant does induce some viral tk, however. 5-Bromo-2'-deoxyuridine suppressed plaque formation by 1093 but not by mutant B2006 (6). Second, the results given below showing that 1093 can transform whereas B2006 cannot suggest that the mutant is tk$^+$. Therefore, the mutant does not identify a new gene, in addition to tk, required for transformation but presumably fails to transform due to the defect in tk metabolism.

TABLE 1. *Ability of mutant 1093 to replicate 34 and 39 C*[a]

Virus	PFU/ml			EOR[b]
	E[c]	34 C	39 C	
KOS 1.1	6.4×10^6	4.5×10^8	3.7×10^8	0.8
1093	1.7×10^6	3.9×10^8	2.9×10^8	0.7

[a] CV-1 cells were infected at a multiplicity of infection of 5 PFU/cell. After adsorption for 1 h at 34 C, cell layers were washed and the dishes were incubated as indicated.

[b] EOR, the efficiency of replication, is the titer at 39 C divided by the titer at 34 C.

[c] E samples were harvested 1 h after infection. The 39 C samples were collected 24 h after infection, and the 34 C samples were collected 48 h after infection.

TABLE 2. *tk activity of Ltk$^-$ cells infected with mutant 1093*

Sample[a]	Time[b] (h)	tk activity	
		Counts/min[c]	Sp act[d]
KOS 1.1	0.33	520	9.6
KOS 1.1	0.67	1,260	23.3
KOS 1.1	1	3,400	63.0
KOS 1.1	2	4,980	92.2
B2006	23	582	8.0
B2006	48	657	9.0
B2006	72	702	9.6
B2006	96	942	12.9
1093	23	440	4.7
1093	48	780	8.3
1093	72	854	9.1
1093	96	927	9.9
Uninfected	23	398	2.8
Uninfected	48	503	3.5
Uninfected	72	498	3.5
Uninfected	96	513	3.6

[a] Ltk$^-$ cells were infected at a multiplicity of infection of 5 PFU/cell and incubated at 37 C. Samples were collected 24 h after infection. Cell pellets were suspended in 4 volumes of buffer (6), frozen and thawed, briefly sonically treated, and centrifuged at 20,000 $\times$ *g* for 20 min. The supernatant was used as the source of enzyme.

[b] Time of incubation of the reaction mixtures.

[c] Counts per minute as TMP per 50 μl of reaction mixture.

[d] Counts per minute per microgram of protein.

Conditional transformation by the mutant. Since it seemed that the inability of the mutant to transform was due to inadequate levels of intracellular tk induced in the putatively transformed cells, we reasoned that increasing the exogenous thymidine concentration might result in the tk phosphorylating enough thymidine to provide an adequate level of TMP to

sustain growth in methotrexate-containing medium.

Ltk$^-$ cells were infected with UV-irradiated KOS 1.1 (6), B2006, a tk$^-$ mutant of HSV-1 (3), or mutant 1093. Cells were also mock infected with EM5C. Twenty-four hours after infection, the cells were cultured in the tk$^+$-selective medium MTAGGEM5C (6) containing various concentrations of thymidine (Table 3). The parental, wild-type virus KOS 1.1 transformed in all concentrations of thymidine tested, ranging from 1.6×10^{-5} M (normal MTAGGEM5C) to 1×10^{-3} M. Mutant 1093, on the other hand, failed to transform in 1.6×10^{-5} M thymidine but did transform about 27% as efficiently as KOS 1.1 in 1×10^{-3} M thymidine. Mutant B2006 failed to transform at any concentration of thymidine tested. These results suggest that the failure of 1093 to transform at the usual concentration of thymidine in MTAGGEM5C was due to inadequate quantities of TMP being synthesized intracellularly.

Cells that were transformed by 1093 and growing in MTAGGEM5C containing 10^{-3} M thymidine could be cells that had reacquired the phenotype of cells transformed by wild-type virus. That is, these could be cells that would grow in MTAGGEM5C containing 1.6×10^{-5} M thymidine after the transforming event, which required a high concentration of thymidine.

Cells that had been transformed by 1093 in MTAGGEM5C containing 10^{-3} M thymidine and which were maintained in the same medium were trypsinized and seeded at 250 cells per dish in the same medium at 37 C. After 3 days, the medium was replaced with MTAGGEM5C containing various concentrations of thymidine. Cultures were incubated at 34, 37, or 39 C for 21 days. Colonies were then stained and counted (Table 4). The transformed cells failed to grow in selective medium containing 1.6×10^{-5} M thymidine but plated with an efficiency of about 50% at 37 C in medium containing 10^{-3} M thymidine. These results indicate that cells transformed by 1093 retain their conditionally transformed phenotype and are unable to grow in a selective medium that will permit the growth of cells transformed by wild-type virus.

TABLE 3. *Transformation of Ltk$^-$ cells by HSV mutant 1093*

Thymidine (M)	Transformed clones/well[a]			
	KOS 1.1	B2006	1093	Mock infected
1.6×10^{-5}	39,33,37	0,0,0	0,0,0	0
3.0×10^{-5}	41,34,37	0,0,0	0,0,0	—[b]
1.0×10^{-4}	34,38,36	0,0,0	0,1,0	0
3.0×10^{-4}	48,43,43	0,0,0	5,4,7	—[b]
1.0×10^{-3}	40,47,50	0,0,0	12,13	0

[a] Ltk$^-$ cells were infected with the indicated UV-irradiated virus and cultured in a medium containing methotrexate, purine nucleosides, and the indicated concentrations of thymidine. Details of the transformation assay were described previously (6).

[b] Not tested.

TABLE 4. *Conditional ability of cells transformed by HSV mutant 1093 to grow in medium selective for the tk$^+$ phenotype*

Thymidine (M)	Transformed clones/well at:[a]		
	34 C	37 C	39 C
1.6×10^{-5}	0,0,0	0,0,0	0,0,0
3.0×10^{-5}	0,4,4	0,1,1	0,0,0
1.0×10^{-4}	54,58,58	13,10,7	0,0,0
3.0×10^{-4}	104,106,98	136,137,128	28,40,44
1.0×10^{-3}	97,88,116	133,124,135	85,86,98

[a] Cells transformed by mutant 1093 and growing in selection medium (MTAGGEM5C) containing 10^{-3} M thymidine were cultured in selection medium containing the indicated concentrations of thymidine.

The cloning efficiency of the cells fell at limiting thymidine concentrations with increasing temperature of incubation. One explanation for this effect is that not all of the transformed cells express the same amount of tk and that, with increasing temperature, thermal inactivation reduces the intracellular levels of tk in an increasing number of cells below that necessary to permit growth at certain concentrations of thymidine in the selective medium.

Supertransformation of conditionally transformed cells by wild-type virus. Since the conditionally transformed cells were unable to grow in a medium in which cells transformed by wild-type virus would grow, it was possible to determine whether cells that had been transformed by HSV-1 could be super-transformed. That is, could cells containing HSV-1 genetic information accept new HSV-1 genetic information?

Eight clonal lines of conditionally transformed cells were isolated from Ltk$^-$ cells transformed by UV-irradiated 1093 in MTAGGEM5C containing 10^{-3} M thymidine. These clonal lines were maintained in the same medium to eliminate any cells that may have reverted to the Ltk$^-$ phenotype. These cells and Ltk$^-$ cells were infected with UV-irradiated KOS 1.1, B2006, or 1093. To select for wild-type transformants, the cells were then cultured in MTAGGEM5C containing 1.6×10^{-5} M thymidine (Table 5). KOS 1.1 transformed Ltk$^-$ cells efficiently and also transformed seven of the conditionally transformed lines with frequencies approximating that of transformation of Ltk$^-$ cells. The Ltk$^-$ mutant B2006 and mutant 1093 failed to transform efficiently any of these

TABLE 5. *Retransformation by wild-type virus of clones of conditionally transformed cells*[a]

Cell type	Transformed clones per well of cells transformed by:									Mock infected
	KOS 1.1			B2006			1093			
	4[b]	2	1	4	2	1	4	2	1	
Expt I										
Ltk⁻	53,44,68	59,50,47	27,34,29	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
Cl M1093.1	21,32	23	37,27	0,0,0	0,0,2	0,0,0	1,0,0	0,0	0	0,0
Cl M1093.4	33,38,39	34,33,46	20,23	0,0	0	0	0,0	0,0,0	0	0,0
Expt II										
Ltk⁻	ND[c]	ND	18,24,25	ND	ND	0,0,1[d]	ND	ND	0,0,0	0,0,0
Cl M1093.3	ND	ND	13,11,14	ND	ND	0,0,0	ND	ND	0,0,0	0,0,0
Cl M1093.4	ND	ND	25,27,19	ND	ND	0,0,0	ND	ND	0,0,0	0,0,0
Cl M1093.6	ND	ND	17,19,19	ND	ND	0,0,0	ND	ND	0,0,0	1,[d]0,0
Cl M1093.8	ND	ND	27,28,32	ND	ND	1,[d]0,0	ND	ND	0,0,0	0,0,0
Cl M1093.10	ND	ND	32,25,24	ND	ND	0,0,0	ND	ND	0,0,0	0,0,1[d]
Cl M1093.11	ND	ND	8,6,7	ND	ND	1,[d]0,1	ND	ND	0,0,0	5,1,6
Cl M1093.12	ND	ND	27,23,32	ND	ND	1,1,0	ND	ND	0,0,0	0,0,0

[a] Transformations were carried out in 35-mm plastic wells as described previously (6).
[b] Multiplicity of infection calculated before UV irradiation of the virus.
[c] ND, Not done.
[d] These colonies were very small and failed to grow vigorously, if at all, in selective medium. They were either abortively transformed or were due to clumps of cells present when the wells were seeded.

cell lines to the wild-type-transformed phenotype. However, in two experiments a few colonies did arise after infection with these viruses in three of the conditionally transformed lines, Cl M1093.1, Cl M1093.11, and Cl M1093.12. Two of these lines have no measurable spontaneous reversion frequency to growth in low-thymidine selection medium as shown in the mock-infected column, whereas the third line, Cl M1093.11, has a high spontaneous reversion frequency and is poorly, if at all, supertransformable by wild-type virus. The colonies arising in these cell lines after infection with 1093 or B2006 may be the result of spontaneous reversions to the wild-type-transformed phenotype or it may be that the genomes of B2006 and 1093 occasionally interact with the in situ 1093 genome to confer the wild-type-transformed phenotype on the conditionally transformed cell. No transformed colonies arose in the other conditionally transformed lines or in Ltk⁻ cells after treatment with B2006 or 1093.

These results show that supertransformation cannot be effected by a mutant, 1093, that induces little functional tk or by a mutant, B2006, that lacks tk. These data add to the already substantial body of evidence (2, 6, 8, 17) that the tk in Ltk⁻ cells transformed to the tk⁺ phenotype by HSV is of viral, not cellular, origin.

DISCUSSION

We have shown that a mutant, 1093, of HSV-1 which is unable to induce wild-type activities of tk during productive infection is also unable to transform Ltk⁻ cells to the tk⁺ phenotype under conditions permissive for wild-type HSV-1 transformation (6, 9). Permissive conditions for transformation by the mutant were found to be increased concentrations of thymidine in the tk⁺ selective medium. These properties are consistent with the explanation that cells transformed by 1093 have insufficient tk activity to support growth in selective medium containing the usual (1.6×10^{-5} M) concentration of thymidine but are able to phosphorylate sufficient thymidine for growth in selective medium containing 1×10^{-3} M thymidine.

The nature of the defect in tk metabolism of 1093 is not known. It could be a missense mutation that gives rise to a tk with a much lower specific catalytic activity than wild-type tk or the mutant could have a defect in a control element governing the synthesis of wild-type tk polypeptide. In the first case, one would expect the induction, during lytic infection, of normal or near-normal amounts of tk protein of low specific activity, whereas in the second case very little tk protein would be produced. Recently, a number of mutants of HSV-1 have been described (15) with defects in tk. These mutants were found to fall into three classes: (i) missense mutants producing amounts of tk polypeptide comparable to that of wild-type virus but with little or no enzymatic activity; (ii) chain-terminating mutants that produced only short fragments of the tk polypeptide and no enzymatic activity; and (iii) mutants that produced no detectable tk polypeptide and which may be chain-terminating mutants producing undetectably short chains, deletion

mutants of the structural gene, or mutants in an element controlling the expression of the tk structural gene.

Regardless of the nature of the mutation in mutant 1093, it has provided a means to determine whether cells once transformed by HSV-1 can be retransformed by the same virus without the necessity of first obtaining phenotypically revertant cells from the initial transformation. We have shown that cells transformed by 1093 can be retransformed by wild-type virus with frequencies approximating those of transformation of Ltk$^-$ cells by wild-type virus. Thus, cells transformed by HSV-1 can accept additional HSV-1 genetic information during another, sequential, transformation. In this regard, the HSV-1 transformation system is different from lysogeny by lambda phage, in which lysogenization of the host cell prevents the cell from accepting another lysogenizing genome, except at very low frequency, of the same sort that carried out the first lysogenization (1). Cells lysogenized by lambda are also immune from infection by another lambda phage to give rise to a lytic infection. Both of these phenomena are related and are under complex genetic control (5).

Cells transformed by HSV-1 are permissive to superinfection and the production of new virus by HSV-1 (6, 10). This quality of HSV-1-transformed cells is similar to that of cells transformed by polyoma and simian virus 40, (SV40) which can be shown not to produce immunity substances that prevent the replication of a superinfecting infectious virus either in nonpermissive cells transformed by infectious virus or in permissive cells transformed by defective virus particles (18).

Cells transformed by SV40 can be transformed subsequently by polyoma virus, and cells can be transformed in the reverse order as well (18), indicating that there is no cross-reacting immunity between these two viruses. Furthermore, cells mixedly infected with two plaque morphology mutants of SV40 give rise to clones of transformed cells from which both kinds of marked virus can be rescued by fusion with permissive cells (4). On the other hand, Scher et al. (13) were unable to transform sequentially with wild-type SV40 cells that had been transformed with a ts mutant of SV40. And SV40-transformed cells bearing a ts lesion that conferred the parental, untransformed, phenotype at the nonpermissive temperature could not be retransformed by wild-type virus at the nonpermissive temperature (12). The mutation in this case seems to be in a cellular gene since virus rescued from these cells cannot be distinguished from wild-type SV40. These seemingly disparate observations on the ability of cells transformed by SV40 to be retransformed by the homologous virus in nonrevertant cells can be reconciled by the studies of Prasad et al. (11), indicating that the mouse cell genome contains a limited number of sites available for SV40 transformation. That is, cells transformed at a low multiplicity of infection have available unoccupied sites and can be supertransformed. However, when these sites are fully occupied with transforming genomes by transformation at a high multiplicity of infection, supertransformation cannot be effected. Therefore, supertransformation by SV40 seems to be limited, not by immunity substances, as in the case of the temperate phages, but by the availability of a limited number of sites in the host cell genome.

The ability of HSV-1-transformed cells to be retransformed readily by homologous virus in nonrevertant cells could be explained by a site-specific integration of transforming genomes in tandem, integration of transforming genomes at a variety of sites in the host cell genome, or by the existence of the transforming genomes as episomes. Experiments permitting a choice among these possibilities should extend not only our knowledge of the biological capabilities of HSV but of the host cell genome as well.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants CA-13114 and CA-14801 from the National Cancer Institute.

We wish to thank Paul Hart and Paul Kurowski for their valuable contribution to this study.

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