

## Biochemical Transformation by Temperature-Sensitive Mutants of Herpes Simplex Virus Type 1

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Biochemical transformation assays of herpes simplex virus type 1 temperature-sensitive (*ts*) mutants distinguished three groups of mutants with regard to their thymidine kinase (TK) transforming ability: those incapable of transferring the TK gene at either the permissive or restrictive temperatures (group I); those resembling the wild-type virus, and therefore able to transform at both the permissive and nonpermissive temperatures (group II); and those that failed to transform or exhibited very low transformation frequencies at the permissive temperature but were able to transform at the nonpermissive temperature (group III). Two mutants in group II exhibited greatly enhanced transformation efficiency at the permissive temperature. The *ts* lesions in the majority of the mutants tested map between 0.30 and 0.60 units on the viral genome. Mutants with TK-positive (TK<sup>+</sup>), but DNA-negative, phenotypes at the nonpermissive temperature produced no TK<sup>+</sup> transformants at the permissive temperature and only unstable transformants at the nonpermissive temperature. This suggests that a function which is required for viral DNA synthesis is also required to obtain stable expression or to transfer the TK<sup>+</sup> gene or both when transfer is mediated by the entire viral genome.

Since the original observations of morphological (10) and biochemical (18) transformation of mammalian cells, a variety of methods have been used to demonstrate the transforming ability and oncogenicity of herpes simplex viruses (HSV). To destroy the lytic activity of HSV, viruses have been inactivated by UV irradiation (2, 10, 18, 21), heat (21), and treatment with various dyes followed by photoinactivation (12, 14, 24).

The use of thymidine kinase (TK) as a marker for biochemical transformation has been an important tool for studying genetic transfer, integration, and expression of the HSV genome in mammalian cells (3, 7, 8, 15). TK-negative (TK<sup>-</sup>) cells transformed to the HSV TK-positive (TK<sup>+</sup>) phenotype contain a small number of copies (1 to 5) of only part of the HSV genome (9, 11) as determined by DNA-DNA hybridization studies. A contiguous stretch of DNA encompassing 40% of the HSV type 2 (HSV-2) genome and located between 0.15 and 0.57 fractional map units has been found in one of our TK<sup>+</sup> transformants, clone 33A<sup>+</sup> (13). Various quantities of this fragment were present in subclones derived from this cell line.

The development of restriction endonuclease technology has facilitated cloning of the TK gene (6) and TK transformation by purified HSV DNA fragments (1, 6, 16, 17, 20, 25; F.

Rapp and N. Turner, unpublished data). Mouse cells biochemically transformed by an isolated DNA fragment containing the TK gene contain a single copy of this gene per cell (20). DNA isolated from TK<sup>+</sup> transformants is capable of transforming the original TK<sup>-</sup> mouse cells with great efficiency (17, 20, 25) and of complementing certain DNA-negative (DNA<sup>-</sup>) HSV temperature-sensitive (*ts*) mutants (17).

Morphological transformation of hamster embryo fibroblasts has been demonstrated with fragments of the HSV type 1 (HSV-1) and HSV-2 genomes. The transformed cells express at least one viral gene product; however, in one study (4), the TK gene was not expressed, suggesting that TK gene expression is not required for maintenance of morphological transformation.

A great deal remains undetermined with regard to the mechanism of gene transfer and integration of viral and other DNAs into mammalian cells. Recently, investigators have co-transformed mammalian cells with genes from procaryotes and eucaryotes to which the gene for HSV TK had been attached. By using these hybrid DNAs these investigators demonstrated the high frequency with which foreign DNA can be taken up and expressed by mammalian cells (26).

The present study was undertaken to identify

HSV genes and gene products which influence biochemical transformation. Viral functions required to stably integrate and express the TK gene are also likely to influence integration of other viral transforming genes. Since integration of transforming genes from intact viral genomes and their subsequent expression are presumed to be the events responsible for transformation in the natural host, we felt it was necessary to investigate the influence of individual viral functions on the integration and expression of viral sequences when the entire viral genome is used as the gene donor.

#### MATERIALS AND METHODS

**Cells and growth media.** Flow 5000 cells, human embryonic fibroblastoid cells obtained from Flow Laboratories (Rockville, Md.), were grown in Corning plastic roller bottles containing Dulbecco medium (Dulbecco modified Eagle medium) supplemented with 10% fetal calf serum, 0.075% NaHCO<sub>3</sub>, 50 U of penicillin per ml, and 50 µg of streptomycin per ml. Vero cells were grown in Bello glass roller bottles in Dulbecco growth medium supplemented with 0.03% glutamine.

An inbred Swiss mouse 3T3-derived cell line (N cIA cl10 TK<sup>-</sup>) lacking TK activity was originally obtained from R. Goldberg (National Institutes of Health, Bethesda, Md.). These cells were passed routinely every 3 to 4 days in Falcon 100-mm plastic tissue culture plates containing Dulbecco medium supplemented with 10% fetal calf serum, 0.225% NaHCO<sub>3</sub>, 50 µg of gentamicin, 2 µg of amphotericin B, and 20 U of mycostatin per ml. To repress the growth of TK<sup>+</sup> revertants, 5-bromodeoxyuridine (50 µg/ml) was added to the growth medium. The reversion frequency of the N cIA cl10 TK<sup>-</sup> cells was negligible (not detectable).

**Viruses.** HSV-1 isolate KOS was originally obtained from W. Rawls (McMaster University, Hamilton, Ontario, Canada), and *ts* mutants were isolated from KOS and characterized as described (5, 22; J. Jofre, R. Courtney, and P. Schaffer, unpublished data). Virus stocks were grown in Flow 5000 cells infected with 0.02 to 0.1 PFU per cell and incubated at 34°C in a 5% CO<sub>2</sub> humidified incubator with temperature variations of ±0.2°C. At 2 to 5 days postinfection, virus was harvested by two cycles of quick freezing in a dry ice-alcohol bath. After sonication, virus was clarified by centrifugation, quick frozen, and stored at -76°C.

The permissive and nonpermissive temperatures for HSV-1 *ts* mutants were 34 and 39°C, respectively. All mutant viruses used in transformation assays exhibited efficiencies of plating (EOP) equal to or less than 10<sup>-4</sup>: EOP = PFU per milliliter assayed at 39°C/PFU per milliliter assayed at 34°C.

**Virus assays.** Viruses were assayed in Vero cells. Confluent 60-mm plates were infected with 0.2 ml of virus diluted in Tris buffer (pH 7.4). Virus was allowed to adsorb for 1 h at room temperature, after which cultures were overlaid with 5 ml of Eagle growth medium containing 1% methylcellulose. Cells were incubated for 4 (39°C) to 6 (34°C) days in a humidified

CO<sub>2</sub> incubator, fixed with 5% Formalin, and stained with 0.7% crystal violet.

**UV survival studies.** Virus was diluted in Tris buffer to approximately 10<sup>7</sup> PFU/ml and irradiated with a General Electric G8T5 UV bulb in 60-mm plastic dishes (1.5 to 2.0 ml per dish) for periods ranging from 15 s to 30 min. A dose of 46 ergs/s per mm<sup>2</sup> was measured by a Black-Ray UV dosimeter model I-225 (Ultraviolet Products, Inc., San Gabriel, Calif.). Dishes were swirled constantly to establish uniform irradiation, and surviving virus was assayed in Vero cells.

**Biochemical transformation assays.** Biochemical transformation assays were performed by the method of Rapp and Turner (21). A 1-ml amount of N cIA cl10 TK<sup>-</sup> cell suspension (6 × 10<sup>6</sup> cells) plus 1 ml of UV-inactivated virus in Tris buffer were placed in a 100-mm plastic tissue culture dish containing 2 ml of 2% fetal calf serum-Dulbecco medium. Plates were allowed to stand at room temperature for 30 min and then placed at 34°C for 1 h for further adsorption; plates were shaken occasionally. After adsorption, cultures were flooded with nonselective TAGG medium (Dulbecco growth medium containing thymidine [4 µg/ml], adenosine [13 µg/ml], guanosine [14 µg/ml], and glycine [7.5 µg/ml]).

After incubation for 72 h at either the permissive or the nonpermissive temperature, nonselective medium was replaced with selective MTAGG medium (TAGG medium containing 0.28 µg of methotrexate sodium per ml). Cultures were again incubated at 34 or 39°C. After 3 weeks, foci were observed. Representative TK<sup>+</sup> transformants from cultures infected with each virus were picked with a Pasteur pipette and grown in selective Dulbecco-MTAGG medium. Remaining foci were fixed with 5% Formalin and stained with 0.7% crystal violet; all colonies containing more than 50 cells were counted.

#### RESULTS

The *ts* mutants used in these studies are listed in Table 1. Both functions which are required for synthesis of viral DNA and those which are not required are represented among the mutants employed. Two mutants, *ts* A1 and *ts* D9, which contain a temperature-independent mutation in the TK gene in addition to a *ts* lesion in the A or D cistron, respectively, were included as negative controls for biochemical transformation. Mutants whose physical map positions are known are located between 0.30 and 0.60 units on the physical map of HSV-1 (KOS) (5, 19). As mentioned, these sequences have been identified as encoding possible transformation-linked genes (4, 16).

Before transformation assays all mutants were examined for altered sensitivity to UV irradiation. Inasmuch as these assays were mediated by UV-irradiated virus, it was important to detect any alterations in UV sensitivity caused by mutant selection. No significant differences between the survival curves of the mutants and

TABLE 1. *Properties of HSV ts mutants*

<i>ts</i> mutant <sup>a</sup>	Complementation group <sup>b</sup>	Phenotype				Location on physical map <sup>c</sup>
		39°C			34°C (TK)	
		DNA	DNA polymerase	TK		
A1	1-1	-	+	-	-	0.339-0.464
D9	1-4	-	-	-	-	0.400-0.418
P23	1-15	-	ND <sup>d</sup>	±	+	ND
J12	1-9	+	ND	+	+	0.339-0.464
M19	1-12	±	ND	+	+	ND
T36	1-27	±	ND	+	+	0.530-0.594
K13	1-10	±	ND	+	+	ND
V37	1-28	+	ND	+	±	0.530-0.594
O22	1-14	±	±	+	+	ND
U35	1-28	+	ND	++	++	ND
A15	1-1	-	+	+	+	0.339-0.464
C7	1-3	-	-	+	+	0.386-0.397

<sup>a</sup> The complementation groups of mutants in the KOS series are designated by capital letters (22). Mutants in 11 groups were tested, including two members of KOS group A, *ts* A1 and *ts* A15 (standard group 1-1).

<sup>b</sup> Complementation groups are those described by Schaffer et al. (23) and Chu et al. (5).

<sup>c</sup> Map locations were determined by marker rescue; all mutants tested lie between coordinates 0.30 and 0.60 map units on the physical map of the HSV-1 genome.

<sup>d</sup> ND, Not determined.

that of wild-type virus were noted (Fig. 1).

Quantitative biochemical transformation experiments were executed by the previously described protocol (21). We had observed that diversity among HSV types and strains exists with regard to the conditions required for maximum transformation frequency (21). Therefore, optimal doses of irradiation and multiplicities of infection were first established for each virus so that maximum transforming potential would be obtained.

The wild-type KOS strain exhibited maximum transforming efficiency when irradiated for 5 min at 46 ergs/s per mm<sup>2</sup>. *ts* C7 and J12 required a smaller UV dose (2.5 min) for maximum TK<sup>+</sup> focus production. *ts* P23, which was unable to produce TK transformants, displayed extensive cytopathic effects when irradiated for 5 min. Irradiation for 7.5 to 10 min was required to reduce the cytotoxic effects of this virus. Altered sensitivity to irradiation was not indicated by the survival curves of any of the *ts* mutants.

UV-irradiated virus may retain sufficient biological activity to lyse cells, and such activity would be reflected as a diminished ability to transform cells; therefore, cells infected with UV-irradiated virus were counted 72 h postinfection before the addition of selective medium. Little cytotoxic effect by irradiated viruses was demonstrated at 34 or 39°C when compared with that of mock-infected controls (Table 2). A slight increase in cell numbers was noticed at 39°C, probably due to an increased cell division

rate. This increase was not paralleled in each case by an increase in the number of TK<sup>+</sup>-transformed foci at that temperature (Table 2). We concluded, therefore, that an increase or decrease in the number of TK<sup>+</sup> transformants at 34 or 39°C was not simply a direct result of an increase or decrease in the number of cells in the population.

Each mutant was tested four times for biochemical transforming ability. The results of a typical test are shown in Table 2. Transformation frequencies in the other three tests were in good agreement with those presented in Table 2. Based upon these tests, mutants were divided into the following three groups (Table 3) according to their transforming ability.

**Group I.** *ts* P23 produced no TK<sup>+</sup> transformants at either temperature. Although *ts* P23 was TK<sup>+</sup> at the lower temperature, we did not observe any TK<sup>+</sup> transformants at that temperature.

**Group II.** *ts* J12, M19, T36, V37, and K13 produced TK<sup>+</sup> transformants at both 34 and 39°C. *ts* J12, M19, and T36 (group IIA) produced similar numbers of transformants as the wild-type virus at 34 and 39°C. Thus, in this group, transformation-related events did not appear to be depressed by the *ts* lesion. *ts* V37 and K13 (group IIB) exhibited a markedly enhanced transforming ability at the permissive temperature.

**Group III.** Mutants in this group were capable of TK transformation at 39°C but, in most cases, not at 34°C. *ts* O22 and U35 (group IIIA)

produced rare TK<sup>+</sup> transformants at 34°C and stable transformants at 39°C. *ts* A15 and C7 (group IIIB) transformed at low frequencies only at 39°C; however, only unstable transformants were produced.

### DISCUSSION

The studies described herein have utilized HSV *ts* mutants in an effort to delineate the influence of individual genes on biochemical transformation. The mutants chosen possess lesions within viral sequences retained by morphological transformants. The gene for HSV-1 TK has been located between 0.27 and 0.35 map units on the HSV genome (17). Results of biochemical transformation assays with *ts* mutants mapping within coordinates 0.30 and 0.60 demonstrated that the mutants can be subdivided into three major groups with regard to their TK transforming ability.

Mutants A1 and D9 are unable to transform

at either temperature due to the absence or temperature sensitivity of the TK gene and thus served as negative controls.

**Group I.** *ts* P23 is a dominant lethal mutant thought to be defective in an early gene function necessary for virus DNA synthesis (Jofre, Courtney, and Schaffer, unpublished data). The failure of *ts* P23 to transform at 34°C could be due to a delayed lethal effect on the cells at that temperature or possibly to an additional mutation affecting the transforming region. At present, however, there is no reason to believe the latter. It is also possible that this mutant, in addition to its defect at 39°C, has a defect expressed at 34°C in a gene not required for lytic growth.

**Group II.** Like the wild-type virus, group IIA mutants transformed at both the permissive and restrictive temperatures; therefore, their *ts* lesions have no apparent effect on either stable integration of DNA from the viral genome or on expression of integrated sequences. Group IIB mutants *ts* K13 and V37 should prove useful in further investigations of HSV-induced transformation since they exhibited markedly increased frequencies of transformation when compared with that of the wild-type virus (>10-fold for K13; ~4-fold for V37). This implies that two distinct *ts* gene functions required for replication are not essential for, but can affect, the frequency of transformation. This observation merits further investigation. Other mutants in the K and V cistrons are being tested for biochemical transformation to determine whether there is a consistent increase in transformation at 34°C with temperature-sensitive inactivation of these genes or whether the differences found in this study are mutant specific, possibly due to secondary mutations. At present little is known about the nature of the *ts* defects in *ts* K13 and V37.

**Group III.** The very existence of mutants that exhibit transforming phenotypes such as those in group III was unexpected. In these mutants the TK gene does not contain a *ts* lesion. Thus, the experimental results reflect the effect of viral products outside the TK gene on the stable integration of viral sequences or on their continued expression. We observed rare TK transformation after infection with *ts* O22 and U35 (group IIIA) at 34°C, but this could be attributed to the leakiness of the mutants. It is also possible that, by virtue of their mutations, these mutants are generally poor transformers because they are highly lethal at 34°C, but their lytic functions are repressed at 39°C and thus, their transforming function can be observed. However, this would imply differences between these *ts* mutants and the wild-type virus at 34°C.

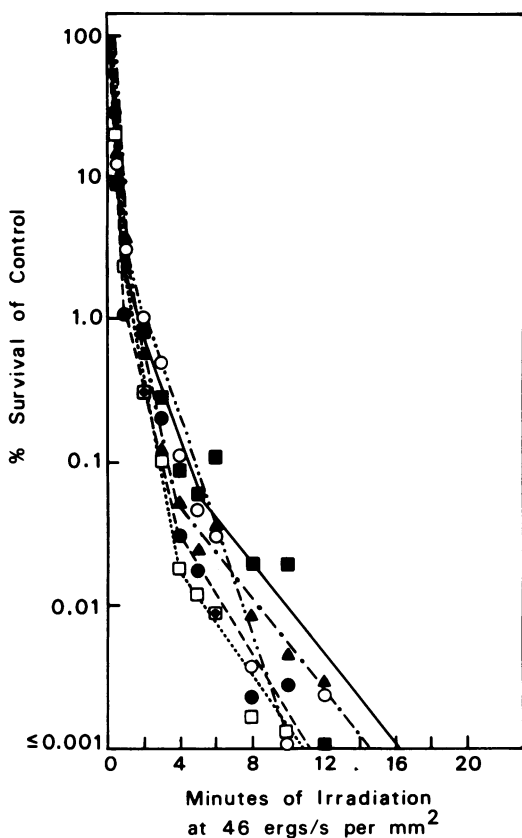


FIG. 1. Inactivation of HSV-1 *ts* mutants exposed to UV irradiation. The inactivation rates shown are representative of all HSV-1 *ts* mutants used in our experiments. Symbols: ■, KOS (wild-type); ●, *ts* T36; ▲, *ts* K13; □, *ts* V37; ○, *ts* P23.

A second explanation could be the presence of a *ts* repressor mechanism that prevents transformation at 34°C. For instance, a functional O or U gene product may, in the infectious cycle, halt expression of the gene regions including the TK gene. The presence of this same functional product at 34°C may similarly lessen expression of the integrated TK gene. At 39°C, in the absence of the functional product, the TK gene could then be expressed. If this possibility is correct, one would expect that shift of cells transformed at 39°C by *ts* O22 or *ts* U35 to 34°C would result in apparent reversion to TK<sup>-</sup>. If this is indeed the case, these mutants should be valuable for future studies of transformation regulation.

Group IIIB mutants *ts* A15 and C7 (DNA<sup>-</sup> mutants) produced only unstable TK<sup>+</sup> transformants at 39°C, and cells transformed by these two viruses survived only one or two passages when cultured in TK-selective medium. Since these transformants are unstable, they were probably not produced by revertant virus. This instability suggests that the genetic lesion responsible for temperature sensitivity for lytic

growth has a secondary effect which results in the inability to achieve stable transfer of the TK gene at 34°C. The results imply that at least two functions which are required for replication of viral DNA are required for stable integration or expression of the TK<sup>+</sup> genome.

With regard to the correlation between the ability to transfer the TK phenotype and viral DNA synthesis, only DNA<sup>+</sup> or DNA<sup>±</sup> mutants were able to transfer TK activity. DNA<sup>-</sup> mutants *ts* P23, *ts* A15, and *ts* C7 either failed to transform or produced unstable transformants. Therefore, at least some viral DNA synthesis appears to be required for stable transfer of the TK gene when the entire genome is used as the transforming agent.

The results obtained in this study indicate that the stable integration and continued expression of viral DNA sequences from an intact genome are complex events influenced strongly by the functioning of several regions of the genome. Further characterization of the *ts* mutants whose lesions prevented stable TK transformation and analysis of members of additional complementation groups will be required to clarify

TABLE 2. Biochemical transformation of mouse cells by HSV-1 *ts* mutants<sup>a</sup>

Group	Virus <sup>b</sup>	No. of cells per plate 72 h postinfection (×10 <sup>6</sup> ) at:		No. of TK <sup>+</sup> transformants per 100-mm plate at:	
		34°C	39°C	34°C	39°C
	HSV-1 (KOS)	3.6	3.2	49, 45, 49, 42 (46) <sup>c</sup>	33, 42, 43, 30 (37)
Controls	<i>ts</i> A1 <sup>d</sup>	ND <sup>e</sup>	ND	0, 0, 0, 0 (0)	0, 0, 0, 0 (0)
	<i>ts</i> D9 <sup>d</sup>	ND	ND	0, 0, 0, 0 (0)	0, 0, 0, 0 (0)
I	<i>ts</i> P23 <sup>d</sup>	3.2	5.5	0, 0, 0, 0 (0)	0, 0, 0, 0 (0)
II	<i>ts</i> J12	1.8	3.9	50, 56, 51, 54 (53)	38, 49, 41, 47 (44)
	<i>ts</i> M19	2.9	4.8	24, 35, 25, 19 (26)	20, 26, 5, 7 (15)
	<i>ts</i> T36	2.2	3.4	66, 61, 8, 43 (45)	42, 45, 30, 32 (37)
	<i>ts</i> K13	1.9	4.7	170, 163, 254, 195 (196)	2, 24, 20, 15 (15)
	<i>ts</i> V37	3.1	4.6	276, 250, 245, 230 (250)	67, 80, 48, 65 (65)
III	<i>ts</i> O22	3.1	2.9	0, 0, 0, 0 (0) <sup>f</sup>	51, 69, 46, 55 (55)
	<i>ts</i> U35	3.2	3.4	0, 0, 0, 0 (0) <sup>f</sup>	52, 65, 101, 57 (69)
	<i>ts</i> A15 <sup>d</sup>	2.9	3.8	0, 0, 0, 0 (0)	10, 12, 12, 10 (11) <sup>g</sup>
	<i>ts</i> C7 <sup>d</sup>	2.6	4.5	0, 0, 0, 0 (0)	2, 5, 6, 4 (4) <sup>g</sup>
	Mock-infected control	3.1	3.2	0, 0, 0, 0 (0)	0, 0, 0, 0 (0)

<sup>a</sup> Data are the results of a single experiment representative of a total of four experiments for each virus.

<sup>b</sup> Virus was irradiated for 2.5 to 7.5 min at 46 ergs/s per mm<sup>2</sup>. The optimum irradiation time for the wild-type virus was 5 min; however, *ts* C7 and J12 yielded maximum numbers of foci after 2.5 min of irradiation. *ts* P23 produced extensive cytopathology after 5.0 min of irradiation; therefore, P23 was irradiated for 7.5 min.

<sup>c</sup> Numbers in parentheses represent the average number of foci per plate.

<sup>d</sup> DNA<sup>-</sup> mutants.

<sup>e</sup> ND, Not determined.

<sup>f</sup> The average number of foci in a total of four experiments was four for *ts* O22 and four for *ts* U35 at 34°C.

<sup>g</sup> Transformants survived for only one or two passages in selective medium.

TABLE 3. Transformation subgroups of HSV-1 *ts* mutants

Sub-group	Transformation characteristics	<i>ts</i> mutants
I	Transformation negative at 34 and 39°C	P23
IIA	Equal to wild type at 34 and 39°C	J12, M19, T36
IIB	Greater at 34°C than at 39°C (supertransformers)	K13, V37
IIIA	Negative or rare at 34°C <sup>a</sup> and positive at 39°C	O22, U35
IIIB	Negative at 34°C and reduced at 39°C <sup>b</sup> (only unstable TK <sup>+</sup> transformants produced)	A15, C7

<sup>a</sup> Rare TK<sup>+</sup> transformants observed at 34°C. Average of four per experiment for each virus.

<sup>b</sup> TK<sup>+</sup> transformants survived only two passages or fewer in selective medium.

the role of individual viral genes in transformation.

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