Method for Isolation and Selection of Temperature-Sensitive Mutants of Herpes Simplex Virus

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Received for publication 5 February 1974

A method for induction and selection of temperature-sensitive mutants of herpes simplex virus is presented. After the infected cells were treated with 5-bromodeoxyuridine, the virus was extracted by repeated freezing and thawing and cloned in microcultures which were then incubated at permissive temperature until viral plaques appeared. The microcultures were then replicated at nonpermissive temperature. Clones not forming plaques in these latter were further purified and examined for temperature-sensitive characteristics. Viral clones mutated in plaque-forming ability or in yield were obtained and preliminarily characterized.

Temperature-sensitive (TS) mutants are conditional lethal mutants, able to grow at low temperatures (permissive), but not at high temperatures (nonpermissive).

In virus research, these mutants have been used for the establishment of genetic maps, the identification of the function(s) of genes, the determination of unknown stages of viral replication, and the correlation between TS phenotype and virus pathogenicity (5, 6).

To isolate viral TS mutants, different methods have been used. Initially, the mutagenized stocks were clonally plated on cell monolayers and incubated at permissive temperature until cytopathogenicity appeared. Since that time, three procedures have been used alternatively, which are as follows. (i) The cultures are shifted to a nonpermissive temperature and clones are isolated from plaques which do not increase in size. (ii) The plaques are picked and replated at nonpermissive temperature and clones are isolated from plaques of the master plate which are unable to give rise to an infective progeny. Or (iii) the entire culture (obtained in microtest plate) is replicated and the replica plate is transferred to nonpermissive temperature. Clones are selected on the master plate from wells unable to give rise to an infective progeny on the replica plate.

Methods (i) and (ii) have been used to obtain TS mutants of herpes simplex virus (HSV), after treatment with 5-bromodeoxyuridine (BUDR) (1, 9, 10, 12, 13), nitrosoguanidine (10), or ultraviolet light (10). Method (iii) has been used under conditions of low cytopathogenicity with simian virus 40 (7) and murine leukemia virus (11).

In this laboratory, method (iii) has been successfully adapted to selection of TS mutants of HSV. In this paper I present the technical details and the preliminary characterization of some HSV mutants obtained by this method.

MATERIALS AND METHODS

Cell cultures and solutions. Human epidermoid (Hep-2) cells were grown in monolayers in Eagle solution (minimum essential medium) (3) supplemented with 10% fetal bovine serum as growth medium, and in Eagle minimal essential medium supplemented with 2% fetal bovine serum (EFBS) as maintenance medium. Penicillin (150 international units/ml) and streptomycin (150 μg/ml) were added to the media. The confluent cultures were dissociated with versene and subcultured.

Virus and virus assay. Strain 13 (2) of subtype 1 was used as wild-type virus. It had been isolated on human KB cells from a case of labial herpes, passed five times on KB cells, cloned three times at 34 C and once at 40 C on human Hep-2 cells on Microtest II plates (Falcon Plastics, Los Angeles, Calif.), and passed three times at 40 C on the same cells to eliminate preexistent TS mutants. Its infective titer did not vary greatly between 34 and 40 C.

Viral plaques were obtained on Hep-2 cells overlaid with EFBS with 0.1% gamma globulin (4). Plaques were scored by the aid of a binocular microscope at ×50 magnification. The infective titer was expressed in plaque-forming units (PFU) per milliliter (4). A
phosphate-buffered isotonic solution with 1% serum (PBS-A/SV) was used to wash monolayers.

Temperature of selection and characterization of TS mutants. Temperatures of 35 and 40°C were used as permissive and nonpermissive temperatures, respectively, for screening potential mutants. Plaque-forming ability was directly assayed at 34, 35, 38, and 40°C in 30-ml, tightly-stopped Falcon flasks in water-jacketed incubators; the temperature variations did not exceed 0.2°C. Yield of infectious virus from one cycle of growth at 34, 35, 38, and 40°C was determined by titration at 36°C in 35-mm Falcon petri dishes in a water-jacketed CO₂ (5%) incubator. This latter temperature is routinely used in our laboratory for incubating petri dishes and the PFU of mutants did not vary consistently between 34, 35, and 36°C.

Mutagenic treatment. Monolayers consisting of 5 × 10⁶ cells in 200-ml bottles were infected with 1 PFU per cell, shaken for 2 h at room temperature, washed three times, refed with EFBS₂ containing the desired concentration of BUdR (Nutritional Biochemicals Corp., Cleveland, Ohio), and incubated at 34°C in the dark. (A stock solution of BUdR was prepared by dissolving 3 mg/ml (10⁻² M) in sterile, twice-distilled water. This solution was kept at −70°C and used for further dilution.) After 48 h, the cultures were gently washed, refed with EFBS₂, frozen and thawed three more times, and titered for infectivity at 35°C.

Microcultures and replica-plating device. Microtest II tissue culture plates (Fig. 1A), consisting of 96 flat-bottomed wells of a capacity of 0.3 ml each, were employed as master plates (for obtaining viral clones) and as replica plates. Sterile transparent polystyrene covers were used to keep cell microcultures in a CO₂ atmosphere at 36°C. The plates sealed with transparent adhesive film were further incubated at different temperatures.

A multiple aspirator (Fig. 1B) was designed to aspirate the medium from the microtest plate, thus avoiding cross-contamination among wells. It consists of a 1-cm-thick Plexiglas plate (11 by 9 cm). Ninety-six stainless-steel needles (1.0 K51 gauge), corresponding in position to the 96 wells, protrude 1 cm and extend 0.4 cm inside the plate to flow into 12 internal canals (0.3 cm in diameter). These canals lead to a main canal (0.5 cm in diameter) which is connected to a vacuum pump.

A replicator (Fig. 1C) was used to transfer a part of the contents of each well from one plate to another. It consisted of a metal plate (12 by 8 by 1.2 cm) with 96 stainless-steel screws (5 cm long and 25 mm thick) placed in positions corresponding to the wells of the plate.

The replicator and the multiple aspirator were sterilized by immersion in 95% ethanol. Before use the replicator was also flame-sterilized.

Isolation of TS mutants: (i) cloning. The virus stocks treated with BUdR, as well as untreated virus, were titered, diluted appropriately, added to cell suspensions, and distributed in microtest plates (master plates). Each well received a volume of 0.1 ml, consisting of approximately 3 × 10⁶ cells, and 0.5 PFU of virus per well. Twenty-nine of the 96 wells (30%) should have received one infective unit. (According to the Poisson distribution, the fraction 〈k〉 of wells infected with k particles is given by the following equation: 〈k〉 = (e⁻ᵐ⁻²/(k!)), where m is the number of PFU per well and e is the base of natural logarithms. The 96 wells are classified into three groups: (i) noninfected (k = 0); (ii) infected with a single virus particle (k = 1); (iii) infected with 2 or more virus particles (k > 2). At a multiplicity of infection of 0.5 PFU per well, of 96 wells 58 should not have been infected, 29 should have been infected with 1 PFU, and 9 should have been infected with more than 1 PFU.)

The master plates were placed for 2 h at 36°C, covered with polystyrene to allow cell attachment, sealed, and further incubated at permissive temperature (35°C). The cell sheets reached confluency within 24 h and retained a normal morphology for no less than 5 days without need for renewal of the medium. The wells were inspected daily by aid of an inverted microscope. If about 30% of the wells exhibited an initial cytopathic effect (CPE) on the 3rd to the 5th day, the medium was renewed and plates were further incubated until CPE was complete.

![Fig. 1. Microcultures and replica-plating system. (A) Microtest II plate with adhesive film; (B) multiple aspirator; (C) replicator.](http://aem.asm.org)
Plates were then frozen and thawed twice and kept at -70°C.

(ii) Replica plating. Microtest plates (replica plates) inoculated with uninfected cells were incubated for 24 h at 36°C until cells were confluent. The master plates (see above) were thawed, replicated on the replica plates, and refrozen. The replica plates were sealed, incubated for 3 days at nonpermissive temperature (40°C), and observed for CPE with an inverted microscope. The clones which did not produce a CPE at 40°C were considered potential mutants.

RESULTS

Efficiency of the method. The method was checked for (i) the efficiency of viral transfer through replica-plating and (ii) cross-contamination between wells.

A cell-free master plate was inoculated with graded dilutions of virus in alternate rows, whereas the remaining rows contained only the diluent. This plate was immediately replicated and the replica plate was incubated at 40°C and examined for CPE. The results (Table 1) show the following. (i) All the wells of the replica plate were positive when the corresponding wells in the master plate contained no less than 100 PFU. (ii) No wells corresponding to those containing only the diluent were positive. It was concluded that no cross-contamination had occurred and that a reliable replica-plating had been obtained with 100 PFU per well after 72 h of incubation.

From separate titrations it was estimated that a well positive for CPE at permissive temperature contains from 10⁴ to 10⁶ PFU. Thus, the absence of CPE at nonpermissive temperature after 3 days of incubation (see Materials and Methods) should not be ascribed to an insufficient transport of virus.

Isolation of TS mutants. The yield in infective virus after mutagenic treatment for 48 h at 34°C decreased almost exponentially with BUdR concentration up to 30 μg/ml and then became asymptotic (Fig. 2). The concentrations used for mutagenic treatment (i.e., 3, 30, 90, and 300 μg/ml) pertain to different parts of the curve.

Of 77 clones of the untreated virus and 274 clones of the mutagenized virus derived from approximately 1,200 wells, 37 (10.5%) showed a CPE at 35°C on the master plates, but not at 40°C on the replica plates. All these clones were considered to be potential mutants (Fig. 2). They are distributed among the different concentrations of BUdR, the frequency rising from 5% in untreated viral stocks up to 15% in stocks treated with 300 μg of BUdR per ml.

The content of each well was removed with a Pasteur pipette and transferred into a tube of Hep-2 cells. This was incubated at 35°C until

![Fig. 2. Effect of increasing concentrations of 5 BUdR on wild-type yield (filled circles, continuous line and left ordinate) and on frequency of potential mutants (“clones 35°40°”), empty circles, dotted line and right ordinate). Hep-2 cells were infected with 1 PFU per cell, incubated for 2 h at room temperature for virus adsorption, washed three times, reinfected with appropriate concentration of BUdR, and incubated for 48 h at 34°C in the dark. They were then gently washed, reinfected with EFBS₂, frozen and thawed three times, and titrated for their content of PFU at 35°C.](http://aem.asm.org/Downloaded from)

### Table 1. Efficiency of replica-plating and check for cross-contamination among wells

| Row | Cell-free master plate (PFU/well) | Infected wells in replica plate* | 24h | 48h | 72h |
|-----|---------------------------------|---------------------------------|-----|-----|-----|
| A   | 10⁴                            | 12/12                           | 0/12| 0/12| 0/12|
| B   | 0                              | 0/12                            | 0/12| 0/12| 0/12|
| C   | 10⁴                            | 7/12                            | 12/12| 0/12| 0/12|
| D   | 0                              | 0/12                            | 0/12| 0/12| 0/12|
| E   | 10⁴                            | 0/12                            | 5/12| 12/12| 0/12|
| F   | 0                              | 0/12                            | 0/12| 0/12| 0/12|
| G   | 10                             | 0/12                            | 0/12| 0/12| 0/12|
| H   | 0                              | 0/12                            | 0/12| 0/12| 0/12|

*3 × 10⁴ Hep-2 cells/well.

* Hours of incubation at 40°C.
CPE became complete, frozen and thawed three times, and titered for infectivity at 35 and 40°C. The clones exhibiting an infective titer at 35°C at least 1,000 times greater than at 40°C were considered to be stable TS mutants. They were purified by two additional clonings in microtiter plates and used for preparation of viral stocks.

Of 37 clones isolated as potential mutants, only 6 firmly maintained the initial TS character (Table 2). Thus, the frequency of stable mutants is reduced to 1.7%. In particular, no stable mutants were detected in nonmutagenized viral stocks (Table 1). No correlation was evident between frequency of stable mutants and BUdR concentration.

**Characterization of the mutants.** In addition to the temperatures of 35 and 40°C, 34 and 38°C were also adopted for the following reasons: (i) spontaneous morphological lesions sometimes occur in cells at 40°C, (ii) by lowering the nonpermissive temperature it is possible to estimate the degree of leakiness of the mutants, and (iii) the virus yield is higher at 35 than at 34°C.

The mutants were characterized according to plaque morphology, plaque-forming ability, and yield of infectious virus.

(i) **Plaque morphology.** At permissive temperatures (34 and 35°C) the plaques of five of the six mutants were composed of clumps of rounded cells, typical of subtype 1 HSV, and were therefore similar to those produced by the wild-type virus, although smaller. The plaques of the sixth mutant, B4, were composed of polykaryocytes instead of clumps of rounded cells (Fig. 3). They differed in nuclei distribution from the polykaryocytes produced by the known MP mutant of HSV (4) (Fig. 3).

(ii) **Plaque-forming ability.** Plaque-forming ability was assayed at 34 and 35°C (permissive temperatures) and at 38 and 40°C (nonpermissive temperatures) (Table 3). The wild type showed equivalent plaque-forming ability at permissive and nonpermissive temperatures. The mutants exhibited a titer ranging from 4.0 x 10⁶ to 1.5 x 10⁸ at permissive temperatures. At nonpermissive temperatures the mutants D10 and F3 failed to produce plaques, whereas F4, G5, and C4 exhibited a titer ranging from 3.0 x 10⁶ to 5.0 x 10⁴. The polykaryocytogenic mutant B4 showed at 38°C a titer corresponding to that at permissive temperatures.

(iii) **Yield of infectious virus.** Yield of infectious virus was assayed at 36°C after a one-step growth cycle at permissive (34 and 35°C) and nonpermissive temperatures (38 and 40°C) in cultures infected with 10 PFU per cell (Table 3).

The growth of the TS mutants was partially inhibited at 38°C and completely so at 40°C. The difference between titers at these two temperatures could be due to an altered metabolism of the cells maintained at 40°C, or to leakiness resulting from the decrease in temperature from 40 to 38°C, or to both factors. Both the yield of the wild type and the cell multiplication were greatly reduced at 40°C, thus supporting the first hypothesis. (The cell multiplication experiment, not presented in detail, showed that Hep-2 cells exhibited the following plating efficiencies: 30% at 34°C, 50% at 36°C, 48% at 38°C, and 19% at 40°C.) Therefore, the nonpermissive temperature of 38°C was adopted throughout.

**Stability of the mutants.** Yield and plaque ratios at 38°C/34°C and 40°C/35°C provided an estimation of the stability of the mutants. On the basis of these data, D10 and F3 showed neither leakiness nor back-mutation, whereas G5, F4, and C4 showed only slight evidence of both. The number of TS revertants to the wild type was not determined, but the low values of the yield and plaque ratios suggest that the reversion rate is very low. In addition, at 38°C (nonpermissive temperature) five of the six mutants proved defective both in yield and in plaque-forming ability, whereas one, B4, was defective in yield but not in the ability to produce plaques at this temperature.

### DISCUSSION

To obtain TS mutants, BUdR was adopted as a mutagen. It is known to replace thymidine during replication, and has been shown to be mutagenic for various deoxyribonucleic acid viruses such as poxvirus (8), adenovirus (14), and HSV (1, 9, 10, 12, 13). In the present experiments, BUdR inactivation of the HSV-1 strain used as a wild type followed the curve...

### Table 2. Temperature-sensitive mutants of herpes simplex virus obtained after treatment with 5-bromodeoxyuridine

| BUdR (µg/ml) | Survival (%) | No. of clones 35°/40°/no. of clones 35° | No. of stable TS mutants |
|-------------|--------------|----------------------------------------|--------------------------|
| 0           | 100          | 4/77                                   | 0                        |
| 3           | 0.79         | 6/66                                   | 2 (3.0)*                 |
| 30          | 0.023        | 6/57                                   | 1 (1.8)                  |
| 90          | 0.0013       | 8/63                                   | 1 (1.6)                  |
| 300         | 0.00069      | 13/88                                  | 2 (2.3)                  |
| Total       | 37/351       | 6 (1.7)                                |                          |

*35°/40°-, Clones growing at 35°C but not at 40°C. They are referred to as "potential mutants" in the text and in Fig. 1. 35°, Clones growing at 35°C. * Numbers in parentheses represent percentages.
reproduced in Fig. 2. Surviving mutagenized clones of the wild type were then scored to TS mutants by means of a method based on microcultures and replica-plating. This procedure permits an accurate evaluation of the frequency of mutation. Indeed, the possibility that the mutants derived from multiply infected clones is unlikely for three reasons. First, according to the Poisson distribution, only nine wells per plate should have contained more than one infective unit (see Materials and Methods). Second, the further purifications of mutagenized clones very likely ruled out the propagation of clones derived from multiply infected microcultures. Lastly, the clones would have been classified as mutants only if all the coexistent virus particles had mutated and had not complemented. This is unlikely, since mutation frequency is low, and wells containing more than one plaque at permissive temperature were discarded. This method also has the advantage that the microscopic observation of single microcultures allows ready identification of the wells showing a
CPE even reduced at nonpermissive temperatures. The result is a fairly high difference in mutant titer at permissive and nonpermissive temperatures (Table 3).

Our results suggest that the procedure described selects against spontaneous TS mutants. Sister mutants are thus unlikely to appear because, as suggested by other investigators (8), they probably originate from preexisting mutant clones. From the mutagenized virus, provisional mutants were isolated with a frequency of 10.5% (Fig. 2). However, the majority of them were unsatisfactory as they had an efficiency of plating at 40 C/35 C greater than 10^-2 and were discarded. The frequency of mutants thus dropped to 1.7%. This figure expresses the incidence of stable mutants and is of the same order of that found by other investigators (9, 13). A weak correspondence was found between BUdR concentration and potential mutant frequency (Fig. 2). No such correspondence is evident with stable mutant frequency. Thus, the present results do not allow for an estimate of mutation rate, particularly as the number of mutants is small.

The method of cloning reported here permits screening and analyzing a large number of viral clones, and the replica-plating system makes the isolation and purification of mutants relatively easy and inexpensive. The characters of the mutants which were screened included plaque morphology, efficiency of plaquing, and virus yield. Attempts are currently underway to isolate additional TS or host-dependent mutants.

ACKNOWLEDGMENTS

I thank E. Calef, Department of Genetics, University of Camerino, and M. Terni, Department of Microbiology, University of Ferrara, for helpful suggestions and revision of the manuscript. B. Roizman and P. G. Spear, Department of Microbiology, University of Chicago, for gift of the replicator, technical advice, and for providing Hep-2 cells and the mutant MP, and Wilma Rossi for technical assistance.

This research was supported by grant no. 71.00762.04 from Italian Consiglio Nazionale delle Ricerche.

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**Table 3. Plaque-forming ability and yield of wild type and TS mutants of herpes simplex virus at permissive and nonpermissive temperatures**

| Virus | BUdR concn (μg/ml) | Ratio of plaque-forming ability* | Ratio of yield* |
|-------|-------------------|----------------------------------|-----------------|
|       |                   | 38 C/34 C | 40 C/35 C | 38 C/34 C | 40 C/35 C |
| WT    | 0                 | 1.1       | 4.9 x 10^-1 | 1.2       | 5.0 x 10^-2 |
| F4    | 3                 | 6.7 x 10^-4 | 3.3 x 10^-7 | 4.1 x 10^-4 | <1.1 x 10^-4 |
| G5    | 3                 | 5.2 x 10^-4 | 9.0 x 10^-7 | 2.9 x 10^-5 | <3.3 x 10^-4 |
| F3    | 30                | <2.5 x 10^-1 | <7.7 x 10^-8 | <5.0 x 10^-4 | <2.2 x 10^-4 |
| D10   | 90                | <1.7 x 10^-1 | <4.0 x 10^-8 | <8.3 x 10^-4 | <3.3 x 10^-4 |
| C4    | 30                | 1.8 x 10^-1 | 3.5 x 10^-5 | 1.4 x 10^-4 | <1.4 x 10^-4 |
| B4    | 300               | 4.0 x 10^-1 | 3.3 x 10^-5 | 2.1 x 10^-4 | <1.1 x 10^-4 |

* Monolayers of 2 x 10^4 cells in 30-ml Falcon flasks were inoculated with appropriately diluted virus, shaken for 2 h at room temperature, refed with EFBS, with gamma globulin, and incubated at the indicated temperatures for 48 h. The infective titer was expressed in PFU. Values represent the average of two to three determinations.

* Monolayers of 4 x 10^4 cells in tubes were infected with 2 PFU per cell, shaken for 2 h at room temperature, washed three times, and incubated with 2 ml of EFBS, at the indicated temperatures. After 24 h, cultures were frozen and thawed three times and the yield in infectious virus was titered in petri dishes at 36 C.
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