

THE PROGRAMMING OF HERPES VIRUS MULTIPLICATION IN DOUBLY-INFECTED AND IN PUROMYCIN-TREATED CELLS*

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For a given host, virus, and physiologic environment, the events which take place during infection at the cellular level are reproducible to a fine detail. This reproducibility is remarkable considering that within a short interval the virion becomes dismantled, the nucleic acid is transcribed, then duplicated, and new virions are assembled. Concomitantly the cell is phenotypically altered. At the present time the nature of known events is to some extent understood; what is largely unknown, however, is the mechanism by which each sequential event is ordered. For example, what determines the onset of the duplication of viral nucleic acid?

The objectives of our initial studies using HEp-2 cells and herpes simplex (HSV), a DNA virus, are to delineate specific events occurring during the infection, and to determine to what extent the experimental manipulation of one event exaggerates or inhibits the succeeding event. This paper describes the patterns of viral multiplication in cells treated with puromycin and in cells infected 3 hr apart with marked strains of HSV. It may be inferred from the results of these experiments that one and possibly more cycles of protein synthesis take place during the eclipse phase and that the duplication of viral DNA does not begin until some of the products of the eclipse phase reach a specific critical concentration in the host cell.

Materials and Methods.—Solutions and media: *PBS-A*: phosphate buffered saline¹ containing 0.2% bovine albumin (Fraction V, Armour). *PBS-A-γG*: made by adding 0.4 ml of pooled human γ globulin (Lederle) to 100 ml of *PBS-A*. *MM-S*: maintenance medium for suspended cells contains 7.17 gm NaCl, 0.40 gm KCl, 0.20 gm MgSO₄·7H₂O, 0.125 gm NaH₂PO₄·H₂O, 0.20 gm CaCl₂, 20 ml of 100× concentrate of amino acids,² 20 ml of 100× concentrate of vitamins,² 4.72 gm of tryptose phosphate broth powder (Difco), 3.0 gm glucose, 0.70 gm NaHCO₃, 0.60 gm glutamine, 20 mg of streptomycin sulfate, 2 × 10⁴ units of penicillin G, and water to make 1.3 liters.

Viruses: The MP strain of HSV causes the formation of polykaryocytes in infected HEp-2 cell cultures; it is scored in terms of polykaryocyte-forming units (PoFU). The mP strain causes rounding and clumping of cells, and is scored in terms of microplaque-forming units. The properties of the two strains and procedures for their preparation and simultaneous scoring were reported elsewhere.^{3, 4}

Experimental procedure: The method for infecting and suspending cells described by Lwoff and Lwoff⁵ was modified to suit our needs. Approximately 10⁶ HEp-2 cells in monolayer culture were washed with *PBS-A* and exposed to 2 × 10⁸ PoFU of virus. The inoculated cultures were attached to an arm of a rotary shaker and submerged for 20 min in a 37°C water bath. At that time the inoculum was aspirated, the cells washed with *PBS-A-γG*, scraped off, separated from each other by vigorous pipetting and, after 2 cycles of centrifugation, suspended in sufficient *MM-S* to yield approximately 3 × 10⁴ cells per ml. Ten-ml amounts of the cell suspension were added to siliconized Erlenmeyer flasks equipped with a gas-tight glass cap. The flasks were attached to an arm of a rotary shaker (60 rpm) and partially submerged in a 34°C water bath. At that time, and whenever opened subsequently, the flasks were thoroughly flushed with air containing 5 per cent CO₂. Drugs were added directly to the flasks. To withdraw the drugs, the cells were sedimented by centrifugation and resuspended in fresh *MM-S*. At times indicated in the text, samples consisting of half-milliliter amounts of cell suspensions were withdrawn and frozen at -60°C. Prior to assay the samples were frozen and thawed three times.

This procedure was modified for the experiments in which some cells were infected at 0 time with mP-HSV and 3 hr later with an equivalent amount of MP-HSV. In these experiments, the cells were suspended after they were superinfected.

The procedures outlined above were designed to fulfill two experimental requirements: (1) all or nearly all cells should be infected, and (2) the interval of exposure of cells to virus should be brief. It was determined that 10–20 per cent of input virus, i.e., 20–40 polykaryocyte or microplaque-forming units per cell were removed from the inoculum. However, allowance must be made for thermal inactivation, and for virus which adsorbed but did not penetrate into cells at the time that the cells were washed with antibody (PBS-A- γ G).

The pattern of reproduction of MP-HSV in suspended HEp-2 cells has the following features: (1) Despite repeated washing with PBS-A- γ G and MM-S, infected suspended cells yield infectious virus during the eclipse phase. Similar amounts of virus could be demonstrated in cell suspensions in which viral multiplication was prevented by phenethyl alcohol.⁶ This residual, cell-associated virus fraction amounts to approximately 0.1 PoFU per cell and represents the base levels in the assays for newly formed virus. (2) New virus is first apparent 5–6 hr after infection. Since the process of infection and virus multiplication in a population of cells is at best only parasynchronous, the time at which an average of 1 PoFU per cell could be detected (6–7 hr) was arbitrarily designated as the end of the eclipse phase. (3) Virus multiplication appears to follow an exponential rate until about 13–15 hr after infection. Thereafter it tends to level off.

Two basic procedures were employed in the studies with puromycin dihydrochloride (N. B. Co.). First, puromycin was added immediately after infection or at other specified times, and allowed to react with cells for intervals ranging from 2–6 hr, i.e., during the eclipse phase only. Second, puromycin was added immediately after infection or at specified times and allowed to react for the remainder of the experiment. The final concentration of the drug was 5 μ g/ml. The sensitivity of HSV multiplication to puromycin was as follows: No new progeny could be detected in cells suspended after infection in MM-S containing 10 or more μ g of puromycin per ml. The yields from infected cells treated with 1 and with 3 μ g/ml were 6 per cent and 0.6 per cent, respectively, of the yield obtained from untreated suspensions. With 5 μ g/ml, in some experiments the base virus level doubled or tripled reaching 0.2 PoFU per cell; in others no newly formed virus could be detected.

Results.—HSV multiplication in cells exposed to puromycin during the eclipse phase: Puromycin added for any interval between 0 and 6 hr after infection prolonged the eclipse phase by an interval no less than the duration of exposure to the drug. However, once the multiplication began, the rate of reproduction of the virus was the same in both treated and untreated cell suspensions (Fig. 1 A).

Treatment with puromycin between 0 and 2 hr or between 4 and 6 hr after infection prolonged the eclipse by 2 hr. In accord with this formula, treatment between 0 and 4 hr or between 0 and 6 hr after infection prolonged the eclipse phase by 4 or 6 hr, respectively. Since in no experiment was the prolongation of the eclipse phase less than the duration of the treatment, it may be concluded that (1) puromycin affects events taking place during each of the three successive 2-hr intervals of the eclipse phase, (2) the events affected by puromycin cannot take place concurrently, that is, they are in an ordered sequence, and (3) processes inhibited by puromycin during the first and third interval resume at usual rates once the drug is withdrawn. Probably the most significant finding is that treatment with puromycin between 2 and 4 hr caused a 4-hr delay in the termination of the eclipse period. It seems likely that once the successive events of the first and second 2-hr intervals are interrupted, they cannot resume immediately and the events of the first 2-hr period must take place again. It could be that a product of the first 2-hr interval becomes diverted or degraded if it is not utilized for the synthesis of a product made at a later interval.

HSV multiplication in cells exposed to puromycin after the eclipse phase: Trivial yields were obtained from cells suspended after infection in MM-S containing 5 $\mu\text{g}/\text{ml}$ of puromycin. Sizeable yields, however, could be obtained if the puromycin was added at 2, 4, and particularly 6 hr after infection (Fig. 1 B). In general the later the puromycin was added during the eclipse phase the sooner multiplication was detected, the greater the yield, and the higher the rate of apparent multiplication of the virus. The data support the findings reported in the preceding section that puromycin-sensitive events take place in each of the 2-hr intervals during the first 6 hr after infection.

Acceleration of the eclipse phase of the superinfecting virus in cells infected 3 hr apart with marked strains: Inhibition by puromycin of protein synthesis in mammalian cells has been demonstrated repeatedly.⁷⁻¹¹ However, there is no direct

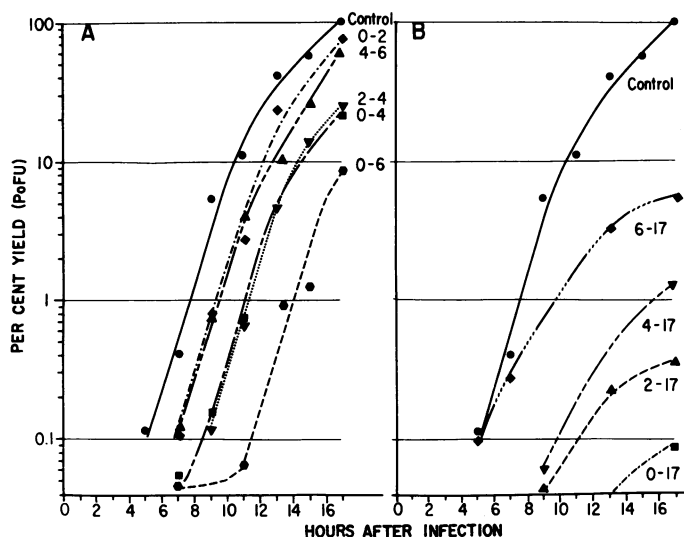


FIG. 1.—Effect of 5 $\mu\text{g}/\text{ml}$ of puromycin dihydrochloride on multiplication of HSV. The first and second numbers on the right of each curve refer to the time of addition and removal of the drug, respectively, in hours after infection.

evidence that puromycin inhibits HSV protein synthesis during the eclipse phase or, in fact, that any other synthetic processes ordered by HSV and prerequisite to its multiplication take place during that phase. To determine whether the virus directs the synthesis of some product prerequisite to its multiplication, HEp-2 cells were infected first with mP virus (0 hr) and 3 hr later with MP virus. Controls consisted of cells infected with mP virus alone at 0 hr, cells infected with MP virus at 0 hr and cells infected with MP virus at 3 hr. The results of two experiments are summarized in Figure 2. It is evident that in cells infected at 0 time with mP virus, the duration of the eclipse phase of the superinfecting MP virus was reduced by 80–100 minutes in relation to that of appropriate controls. The remarkable feature of these experiments is that in the doubly-infected cells, the superinfecting MP virus followed the pattern of development of mP virus. The results indicate that (1) the synthesis of a product prerequisite to viral reproduction takes place during the eclipse phase, (2) the product is not an accretion of the

virus itself; it can serve either mP or MP virus, and (3) the eclipse phase is not an immutable interval; it can be reduced if the products of the eclipse phase are present in the cell at the time of infection.

Discussion.—Some speculative, but perhaps significant, aspects of this study emerge from considerations of the rates of apparent multiplication of HSV in cells treated with puromycin during the eclipse phase and during the phase of viral maturation. It is evident that the presence of the drug during any portion of the eclipse phase does not ultimately affect the subsequent rate of multiplication of the virus. However, virus matures at a reduced rate if puromycin is present during the phase of active multiplication.

The emphasis on "rate" of multiplication derives from the consideration of the factors that govern it. When all precursors are available, it may be deduced that

(1) the apparent exponential rate of maturation of a DNA virus should reflect the exponential rate of duplication of the DNA, and (2) assuming the presence of the primer and of abundant substrates, the rate of duplication of the DNA should be a function of available polymerizing enzyme.

These arguments cannot be used indiscriminantly to explain the data obtained in this study because the results are based on assays of infectious virions rather than assays of viral DNA. It could be argued that in cells exposed to puromycin at 6 hr DNA multiplied at the same rate as in untreated cells, but fewer virions were formed because the synthesis of capsomeric protein was inhibited. Since puromycin may act by stripping peptides off the ribosome before the protein is completed,⁸⁻¹⁰ the small yield obtained in the presence of puromycin could be accounted for

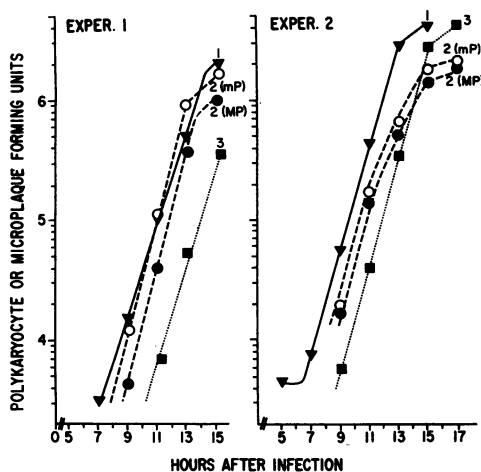


FIG. 2.—Viral multiplication in HEp-2 cells infected 3 hr apart with marked strains of HSV. (1) Cells infected at 0 hr with MP virus. (2) Cells infected with mP at 0 hr and MP at 3 hr. (3) Cells infected with MP at 3 hr. The pattern of multiplication of mP virus in cells singly infected at 0 hr coincided with mP virus development in the doubly-infected cells. Numbers on ordinate are exponents of 10.

by assuming that the capsomeric protein is itself a polymer of small molecular weight proteins and that the synthesis of a small amount of the monomer was completed in the presence of the drug.

These arguments can be used to explain some features of viral development observed in cells treated with puromycin during the eclipse phase only. It could be asked why viral reproduction follows the same rate as the controls but only after a lag. Why does not reproduction begin at 6 hr at a reduced rate? The hypothesis that puromycin "freezes" all activities of the cell may be rejected because it is incompatible with the known effects of the drug and because puromycin-insensitive events in the eclipse phase of an animal virus have been described.¹¹ One plausible hypothesis is that the duplication of viral DNA does not begin until a specific event takes place. The probability of the completion of such an event would be a

function of the concentration of enzymes and precursors involved. Another way of stating this is that for a given physiologic state of the infected cell, critical concentrations of enzymes and precursors of DNA synthesis prevail in the cell at the time DNA duplication begins. If enzyme synthesis were arrested below this critical level, the specific event would be considerably delayed, and the rate of reproduction would be reduced.

There is no direct evidence to support this hypothesis and, moreover, very little is known concerning the requirements and determinants of the duplication of DNA of animal viruses. A considerable body of information has accumulated for DNA bacterial viruses. In bacteria infected with T-even phages the synthesis of proteins concerned with intermediary DNA metabolism begins almost immediately after infection and ends shortly after the onset of DNA synthesis.¹²⁻¹⁴ In "non-permissive" bacteria infected with certain amber mutants of phage T₄¹⁴ and in bacteria exposed to irradiated phage¹³⁻¹⁵ the synthesis of apparently functional early proteins may be exaggerated but duplication of DNA does not ensue. Of particular interest is the fact that the DNA polymerases obtained from uninfected and T₂-infected *E. coli* differ in functional as well as physical properties.¹⁶ *In vitro* the "T₂" polymerase unlike the *E. coli* enzyme appears inert with native DNA and seems to react best with heat-collapsed or "single-stranded" DNA regardless of source. Moreover, the net amount of DNA synthesized by the T₂ polymerase does not exceed the amount of the primer added.^{16, 17} The net amount of DNA synthesized by the *E. coli* polymerase may exceed 10- to 20-fold the amount of native or heat-collapsed DNA added as a primer.^{18, 19} The problems involved have been stated.¹⁴⁻¹⁶ To paraphrase, in the reproductive cycles of phage and animal viruses there are two intervals of transcription of DNA separated by a period of encapsidation and extracellular existence. Very likely some regulatory process or event determines the end of the last transcription interval and the beginning of duplication of DNA. The regulatory process could entail physical participation of the DNA itself or a physical modification of the enzyme molecules (tautomerization, monomerization, or polymerization). It seems likely, though not yet proved, that (1) termination of early protein synthesis and onset of duplication of DNA are interdependent, (2) the process terminating the last transcription period involves a physical modification of DNA, (3) the DNA is "primed" by a distinct, possibly labile, enzyme formed when other enzymes and precursors necessary for DNA synthesis attain critical concentrations.

The concept that a "critical concentration" and not just any small number of enzyme molecules sets off DNA duplication is based on the net duration of the eclipse phase and apparent rate of viral multiplication in cells exposed to puromycin for any interval during the first 6 hr after infection. It should be noted that two regulatory systems, one operating at the genetic level, the other at the level of the "enzyme molecules," have been postulated.²⁰ Alterations of the existing state in one system are triggered by "effectors" binding to products of regulatory genes; in the other system inhibitors or activators bind to allosteric sites of "enzyme molecules." Implicit in both systems are "critical concentrations" of the effectors and of the allosteric inhibitors or activators, respectively.

Finally, essentially similar considerations have led to the conclusion that the onset of poliovirus RNA duplication is triggered by a specific event.²¹ It should

be noted that there is a striking correlation between cell volume and virus yields,²² an observation which extends to multinucleated giant cells produced by X-irradiation.²³ Puromycin delays poliovirus reproduction if administered briefly during the eclipse phase.^{24, 25} Moreover, following withdrawal of puromycin the rate of viral multiplication in treated and untreated cultures is the same.²⁵ It could follow that poliovirus reproduction follows an event actuated at critical concentrations of rate and yield-limiting enzymes or precursors. Interestingly enough a thermosensitive event occurring late in the eclipse phase of certain poliovirus mutants appears to determine subsequent rate of viral multiplication.²⁶ It has been tentatively concluded²¹ that the thermosensitive event involves actuation of an RNA polymerase through polymerization of inactive protein monomers. This hypothetical polymerization could be determined by an alteration of the "milieu intérieur" of the infected cell or by some product synthesized late in the eclipse phase.

Summary.—Suspensions of HEp-2 cells infected with herpes simplex virus (HSV) were treated with puromycin during the eclipse phase and at various times during the reproductive cycle. Puromycin (5 μ g/ml) treatment confined to the period of the eclipse prolongs the phase but does not alter the apparent rate of subsequent reproduction of the virus. Treatment between 0 and 2 hr, 2 and 4, or between 0 and 6 hr after infection causes equivalent delay in the termination of the eclipse phase. Treatment between 0 and 4 or between 2 and 4 hr after infection causes a 4-hr delay. Puromycin treatment beyond the end of the eclipse phase causes a decrease in the apparent rate of virus formation and decreases the yield.

The formation of a product prerequisite to viral reproduction could be inferred from an analysis of viral multiplication on HEp-2 cells infected 3 hr apart with two marked strains of HSV. The eclipse phase of the superinfecting virus was reduced. Moreover multiplication of the superinfecting virus followed the pattern of development of the first infecting virus rather than that of the homologous virus.

It is concluded that during the eclipse phase of HSV there is at least one and possibly more periods of synthesis of proteins required for the initiation of virus multiplication. It is tentatively inferred that the duplication of viral DNA does not commence until the precursors and enzymes required for its synthesis are present in the cell at a critical concentration.

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*STEPS IN THE NEOPLASTIC TRANSFORMATION OF HAMSTER
EMBRYO CELLS BY POLYOMA VIRUS**

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Cultures of hamster embryo cells infected with polyoma virus undergo a characteristic transformation within several weeks after infection. The transformed cultures are constituted by highly atypical cells which have an abnormal morphology, grow rapidly *in vitro*, and give rise to progressively growing tumors when inoculated subcutaneously into the adult hamster.¹

In the previous experiments, the transformed cells arose in mass cultures and their properties were only studied many cell generations after the original virus-cell interaction had taken place. The experiments to be reported in this communication were undertaken to get some information on the properties of transformed cells at earlier stages after infection with polyoma virus. The experiments show that the atypical cells are produced in two main steps; moreover, they reveal some new characteristics of the transformed cells which may contribute to a clarification of the mechanism of transformation.

Material and Methods.—The polyoma virus used was of the "large-plaque" type. Its origin and the preparation of its stocks have been described.² Tissue cultures of whole embryos of Golden hamsters were prepared according to a technique already described.³

A rich growth medium especially favorable for the cloning of mouse embryo cells (Weisberg, R., personal communication) and for the titration of polyoma-induced foci (Bayreuther, K., personal communication) was used for all experiments. It consisted of reinforced⁴ Eagle's medium (40 pts), Puck's⁵ nutrient solution N16 (40 pts), and medium NCTC 109 (4 pts); all ingredients were dissolved in Earle's saline containing 0.55 per cent glucose and 0.37 per cent NaHCO₃. This medium was further supplemented with 0.3 per cent Bacto tryptose phosphate broth (10 pts) and, unless stated differently, with 10 pts of fetal bovine serum.