JOURNAL OF VIROLOGY, June 1973, p. 856-861

Vol. 11, No. 6
Printed in U.S.A.

Replication Process of the Parvovirus H-1

I. Kinetics in a Parasynchronous Cell System

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Received for publication 15 January 1973

Parasynchronous cultures of hamster embryo cells were used to study some of the events in the replication process of the parvovirus H-1. Synthesis of viral DNA, viral hemagglutinating antigen, and infectious virus were examined. It was found that initiation of DNA synthesis, on which subsequent viral hemagglutinin synthesis was dependent, occurred at a specific time in late S-phase. Production of H-1 viral protein was shown to be sensitive to inhibition by α-amanitin. The implications of these findings are discussed.

H-1 virus is a member of the parvovirus group, small viruses, approximately 20 nm in diameter, with a single-stranded (DNA) genome (6, 15, 27-29). A number of these agents, such as the adenovirus-associated viruses (AAV), are completely defective and dependent on "helper" viruses for replication; others, including RV and H-1, require replicating cells and, in some instances, helper viruses in order to propagate (5, 8, 28). The findings of Tennant et al. suggest that RV depends on cellular S-phase for expression of its viral genes as determined by viral antigen synthesis (24, 25). In preliminary studies in our laboratory we have found that H-1 virus antigen synthesis occurs at a fixed time interval after the onset of S-phase in parasynchronous cultures, independent of the time of infection preceding S-phase. Ledinko has reported that the inhibitor of DNA synthesis, arabinofuranosyl cytosine (*ara*-C), inhibits H-1 antigen synthesis and infectious virus production while the mutagen iododeoxyuridine (IUdR) inhibits infectious virus production but not antigen synthesis (11). These various observations are consistent with the hypothesis that synthesis of the viral DNA by host cell mechanisms that are restricted to cells in S-phase is a prerequisite to further events in the viral replication process. In our present study a parasynchronous cell system was employed to explore further these aspects of parvovirus replication. A second report (Rhode, manuscript in preparation) will describe the isolation of H-1 replicative form DNA.

MATERIALS AND METHODS

Cell culture. Secondary cultures of hamster embryo cells were grown to confluence in Eagle minimum essential medium containing 10% fetal calf serum, 10% tryptose phosphate (TP), and 2× concentrations of minimal essential medium (MEM) amino acids, glutamine, and MEM vitamins plus penicillin (10 μ/ml) and streptomycin (5 μg/ml). After 2 to 4 days of confluence, these topoinhibited cells were stimulated to replicate by replacing the medium as described with one in which the usual 10% serum was replaced by 40% serum dialyzed against Hanks balanced salt solution.

Virus infection. The virus stocks used were sonicates of H-1 infected secondary hamster embryo cell cultures that contained 5.10^7 to 10^8 PFU/ml. Cultures were infected at multiplicities of 5 to 20 PFU/cell. Following an adsorption period of 30 min at 37 C, virus inocula were removed, and the cultures were washed three times in Hanks balanced salt and refed with regular medium. Virus plaque titrations were carried out as previously described (11).

Autoradiography. Cover-slip cultures were incubated for 30 min in medium without TP containing ^{3}H-thymidine (1 μCi/ml; 19 Ci/mmol). For autoradiography, cultures were fixed and processed as previously described (18). In the combined autoradiography and fluorescent antibody study, cultures were washed three times in Hanks balanced salt solution and chased with medium containing TP and 10^{-5} M thymidine, or fixed immediately. Fixation consisted of 10 min in acetone at 4 C and air-drying. Cultures were stained with fluorescent hamster antisera to H-1, washed in phosphate-buffered saline, and fixed in 95% ethanol as described by McDevitt et al. (14). They were then processed for autoradiography as above. Uninfected cells exposed to immune antiserum and infected cells exposed to normal hamster serum showed no nuclear fluorescence.

Virus purification. Infected cells were collected by centrifugation and homogenized in 50 mM Tris-0.1 M NaCl, pH 7.5 (TBS). The homogenate was sonically treated at 0 C for four 15-s bursts with a Biosonik II ultrasonicator and, subsequently, sodium dodecyl

sulfate (SDS) was added to a final concentration of 1% to complete lysis of the cells. Virus was then pelleted from the cell lysate in a discontinuous sucrose gradient of 30 to 10% sucrose in TBS 0.2% SDS, in an SW25 rotor at 25,000 rpm for 5 h. The pellet, after suspension in CsCl at a final density of 1.43, was banded by equilibrium centrifugation in an SW50 rotor at 35,000 rpm for 21 h. A viral band was readily visualized by verticle light and carefully withdrawn from the tube by side puncture. CsCl and SDS were removed from the collected band by dialysis in sequence against 0.3 M TBS (0.3 M NaCl, 50 mM Tris pH 7.5, 1 mM EDTA), 0.3 M TBS with 20% acetone and finally distilled water. This method allows nearly full recovery of input infectivity.

RESULTS

Description of the system. We have determined that stimulation of contact-inhibited hamster embryo cells with serum-enriched media results in a parasynchronous wave of DNA synthesis, beginning approximately 12 h after addition of the media. Similar findings have been described previously in a number of cell systems (4). In the experiments reported herein, the hamster embryo cells were infected with virus 12 h after they had been stimulated with 40% fetal bovine serum. At this time, 50 to 60% of the cells have entered S-phase. Production of cell-associated hemagglutinating (HA) viral antigen and infectious virus in this system is illustrated in Fig. 1. The first appearance of fluorescent nuclei occurred at 10 h postinfection (PI) when hemagglutination was first detectable. Onset of infectious virus production followed the appearance of fluorescent antibody (FA)-positive nuclei and HA by an interval of 0 to 2 h. In fact, the first detectable HA at 10 h PI coincided with an increase in infectivity over the adsorbed virus at t = 0. The rate of HA increment decreased markedly or ceased after a period of 6 h, whereas the assembly of infectious virus continued at an exponential rate for at least 12 h.

In order to illustrate the kinetics of some of the important events in the viral replication process the following parameters were determined in the same experiment: (i) the number of cells in S phase (ascertained by autoradiography of mock-infected cells); (ii) hemagglutinating factor (HA) synthesis; and (iii) time of resistance of subsequent HA synthesis to inhibition of DNA synthesis with *ara*-C. The results are illustrated in Fig. 2. The mock-infected cultures showed a wave of S-phase cells that had a median G1 time (as measured from the time of medium change) of 14 to 16 h and a maximum of about 18 h. HA first occurred between 8 and 10 h PI and coincided with the decline of cells in S phase. Since HA synthesis is dependent on DNA synthesis, presumably requiring viral DNA and its transcription, inhibitors of DNA synthesis, such as *ara*-C, should prevent HA synthesis if introduced before this DNA (HA-DNA) synthesis occurs. Because the nature of this DNA has not been established it will be referred to as HA-DNA. Synthesis of HA-DNA may thus be inferred from the time of infection when HA factor production becomes insensitive to *ara*-C. Medium containing *ara*-C (10 μg/ml) was added to cultures at the times indicated (Fig. 2), and the HA of the cultures was determined at 16 h PI (28 h poststimulation, PS). The results are represented as final yield of HA of treated cultures at 16 h PI plotted at the time of the *ara*-C addition. In the infected cultures, HA synthesis first became resistant to inhibition by *ara*-C at 7 to 8 h PI; the interval from the onset of S phase to the synthesis of HA-DNA was 8 h. The synthesis of HA followed the synthesis of HA-DNA by 1 to 2 h. In a similar experiment, actinomycin D was compared with *ara*-C to determine if a detectable lag occurred between the synthesis of HA-DNA and viral mRNA for HA synthesis. Such an interval would result in a delay in the increase of final yield of HA in the actinomycin D-treated cultures compared to that obtained with *ara*-C. Addition of *ara*-C or actinomycin D (1 μg/ml) at 6 h PI (18 h PS) completely prevented HA synthesis and the appearance of fluorescent nuclei at 16 h PI. Addition of *ara*-C at 8 h PI resulted in an HA titer of 2 at 16 h PI; addition of actinomycin D at 8 h gave an HA of 3. Cover-slip cultures receiving *ara*-C or actinomycin D at the indicated time were examined for FA-positive cells at 16 h PI. The results are summarized in Table 1. The increase in FA-positive cells was nearly simultaneous for the two drugs, when they were given in the amounts cited. Low doses of actinomycin D, 0.03 to 0.05 μg/ml, did not inhibit HA synthesis.

To determine if the time for viral infection was an important parameter in these experiments, cultures infected at 10 and 12 h PS were compared for viral HA production. The results, illustrated in Fig. 3, indicated that time of infection was not a factor in delaying viral HA synthesis under our conditions. Infection of cultures 12 h earlier, just prior to stimulation, resulted in a similar wave of HA synthesis beginning 22 h PS, and cultures refed with medium containing 1% serum, so as to avoid stimulation, produced an HA titer of only 1 as late as 28 h PI.

To determine whether the plateau in cell-associated HA regularly seen at 28 h PS was due to a decrease in rate of synthesis or to loss of HA into the medium, HA was determined both for

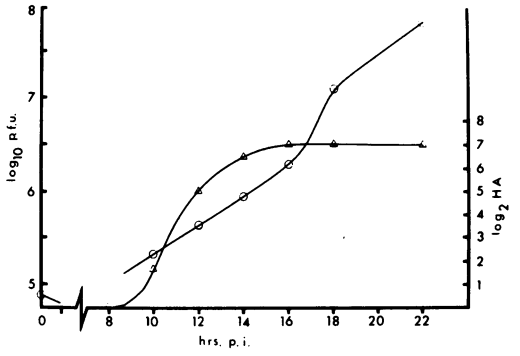

FIG. 1. *Production of cell-associated H-1 hemagglutinin (HA) and infectious virus during a parasynchronous infection of hamster embryo cells. Cultures were infected 12 h PS with 40% serum. Points are averages of replicate cultures harvested without medium into a total of 5 ml and sonically treated. Log_2 HA (Δ) and log_{10} PFU (O) per ml are indicated for hours PI.*

the infected cells as in previous experiments and also for the media plus any cells that detached from the monolayer. No HA was detected in the medium until 28 h PS, and subsequent medium HA was only a small percentage of the total up to 54 h PS. The absolute titers in this particular experiment were higher than are usually observed (Fig. 1, 2); this may have been the result of the use of a higher multiplicity of infection in this instance, about 50 PFU/cell. Thus we can conclude that HA synthesis apparently undergoes a marked reduction in rate within 8 h after its inception.

Viral DNA synthesis initiated in late S phase. The delay of 6 to 8 h between the onset of S phase and the synthesis of HA-DNA would indicate that initiation of HA-DNA synthesis is dependent on a specific event that occurs in late S phase. To examine this directly, an estimate was made of the interval that infected cells remained in S phase before HA-DNA synthesis was initiated. This was determined by establishing replicate cultures stimulated to proceed through S phase parasynchronously and infected with H-1 at 10 h PS. Cells actively synthesizing DNA at particular times after stimulation were marked by pulse labeling for 30 min with ^{3}H-thymidine at 2-h intervals, beginning 12 h after stimulation (2 h PI). The cells which first initiated synthesis of viral HA-DNA were identified by determining when *ara*-C failed to inhibit subsequent development of viral antigens, monitored by fluorescent antibody staining as in Fig. 2. The cultures were fixed and stained with FA at 26 h PS (16 h PI). The cultures which received *ara*-C at 19 h PS contained FA-positive cells (an average of 106 FA-positive cells per microscope field at ×200) while those receiving *ara*-C at 18 h PS were negative for FA-positive cells. Thus, these cultures had initiated HA-DNA synthesis between 18 and 19 h PS. To establish the length of time the cells had been in S phase before synthesizing HA-DNA, the series of cultures pulsed at different times and treated at 19 h PS with *ara*-C were analyzed by autoradiography. No FA-positive cells were found unlabeled by ^{3}H-thymidine in cultures pulsed 1, 3, or 5 h before the addition of the *ara*-C. All the cells had therefore initiated DNA synthesis at least 5 h before HA-DNA synthesis occurred. Cultures which had been pulsed with ^{3}H-thymidine 7 h before the addition of *ara*-C showed an average of 1.2 cells per microscope field (10 fields at ×200 magnification) that had no ^{3}H-thymidine labeling. These cells, representing only 1.1% of the total FA-positive cells, had initiated DNA synthesis less than 7 h before HA-DNA synthesis. The majority of the cells (98%) had allowed at least 7 h to elapse from the initiation of S phase to the time of HA-DNA synthesis. Most S-phase cells contained sufficient numbers of grains under these conditions to obscure the fluorescence so that FA-positive cells that were negative for grains received a sufficiently stringent test for the absence of DNA synthesis at the time of the pulse.

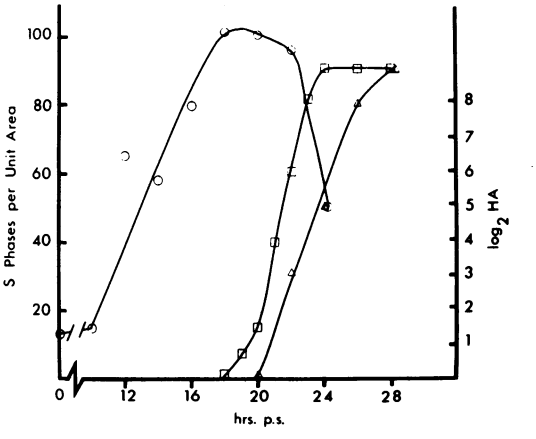

FIG. 2. *Production of cell-associated H-1 HA during parasynchronous infection of hamster embryo cells. The number of S-phase cells in mock-infected cultures was determined by autoradiography with ^{3}H-thymidine and represented by the average number of S-phase cells per high power field (O). Log_2 HA at the indicated times PS were determined as in Fig. 1 (Δ). The final yield of HA at 16 h PI after addition of ara-C (10/ml) to infected cultures at various times are indicated at the time of the addition of the drug (□).*

Effect of α-amanitin synthesis on H-1 protein. In preliminary experiments it was noted that α-amanitin was effective in inhibiting H-1 HA synthesis when applied in a medium with a pH 8.0 to 8.2 but was not effective at pH 7.2. Figure 4 illustrates such inhibition by α-amanitin in an alkaline medium at various doses when added to hamster embryo cultures at 6 h PI. It is apparent that H-1 HA synthesis is sensitive to inhibition by α-amanitin in a higher dose range than that reported by Ledinko for the inhibition of adenovirus replication in human embryonic kidney (10). The findings, however, are similar to those reported by Chardonnet et al. for adenovirus in HeLa cells (3).

Absence of virion DNA polymerase. Salzman has reported the presence of a DNA-dependent DNA polymerase in purified preparations of the parvovirus RV (21). The potential importance of this enzyme in the infectious process of the parvovirus prompted a search for this activity in our preparations. The data are summarized in Table 2. No DNA polymerase activity was found with detergent-purified H-1 virus or with RV purified with detergent or as described by Salzman (21). Activity was present in NonidetP-40 (NP40)lysates of uninfected cell cytoplasm and served as a positive control. The NP40 cytoplasmic lysate used here represented about 10^{-3} of the total enzyme in the crude infected cell harvests before viral purification. The reason for this failure to detect enzymes is not clear. Adequate quantities of virus both by HA units or infectivity were used.

DISCUSSION

In our present study a parasynchronous cell system was used to define the kinetics of H-1 replication. It was found that a DNA synthetic event on which subsequent HA synthesis is dependent, i.e., HA-DNA synthesis, is initiated only in late S phase and possibly requires a specific cell component produced at that time in addition to any requirement HA-DNA synthesis may have for the cellular DNA replication apparatus. The S phase dependence would suggest that one or more components of the host cell DNA replication system are required. Studies are in progress to determine if HA-DNA synthesis is executed solely by host cell enzymes or if a viral protein is required. A DNA polymerase has been reported by Salzman to be associated with the virions of RV (21), which might suggest the need for a virion DNA polymerase in the viral replication process. However, we have been unable to demonstrate any polymerase activity associated with H-1 or with RV using several methods of purification and em-

TABLE 1. *Effect of ara-C and actinomycin D on FA-positive cells*

Addition time PI(h)	Drug	
	ara-C	Actinomycin D
6	0	0
7	3.2	0
8	5.2	7.4
9	16.2	12.6
10	24.6	19.4

[a] Number of FA^+ cells at 16 h PI following the addition of *ara*-C (10 μg/ml) or actinomycin D (1 μg/ml) at the times indicated. Values are average number of FA^+ cells per unit area with five areas counted. Total cells were not counted because of the technical difficulty with confluent cultures. Experimental design is as in Fig. 2.

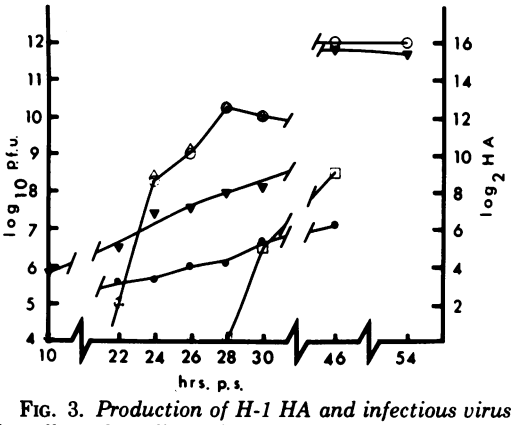

FIG. 3. *Production of H-1 HA and infectious virus in cells and medium. Cell-associated HA was determined as in Fig. 1 on replicate cultures infected 10 h PS (Δ) and 12 h PS (O). Medium HA was determined for cultures infected 12 h PS (□). H-1 PFU were determined for cultures infected 12 h PS for the medium (●) and for the cell layer (▼). All samples contained 5 ml. Since the 54 h PS cultures showed extensive cell detachment, the cells and medium were combined.*

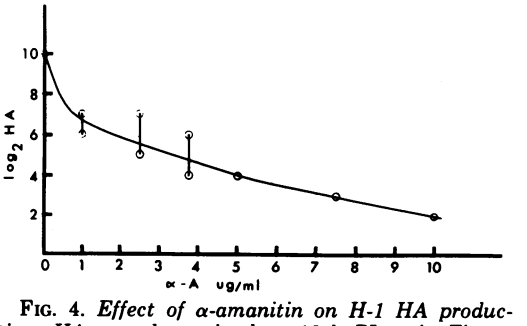

FIG. 4. *Effect of α-amanitin on H-1 HA production. HA was determined at 16 h PI as in Fig. 1. α-Amanitin in various doses was added to cultures in medium adjusted to pH 8 with bicarbonate at 6 h PI. Log_2 HA at 16 h is indicated for each dosage of α-amanitin.*

TABLE 2. *DNA polymerase assay of parvovirus virions*[a]

Virus	Method of purification	Virus HA units	Counts per min
Expt 1.			
H-1	SDS	2×10^5	222
H-1	Sarkosyl	3.7×10^3	246
H-1 background	SDS	2×10^5	227
NP40 cytoplasm			4,216
Expt 2.			
H-1	SDS	4×10^5	253
H-3	Sarkosyl	640	189
RV	Sarkosyl	3×10^5	204
RV background	Sarkosyl	3×10^5	188
NP40 cytoplasm			8,610
Expt 3.			
RV	Sarkosyl	1×10^5	145
RV	Sarkosyl	2×10^4	91
RV	Salzman (21)	0.7×10^3	135
RV	Salzman (21)	1.5×10^3	133
RV background	Salzman (21)	0.7×10^3	124
NP40 cytoplasm			5,618

[a] Complete system in a total volume of 0.2 ml contained: glycine buffer (0.02 M, pH 9.0), 0.003 M $MgCl_2$; 100 μg of calf thymus DNA; 4×10^{-5} M each of dGTP, dCTP, dATP, and in expt 1, 4×10^{-5} M ^{3}HdTTP (0.1 Ci/mM); in expt 2 and 3, 10^{-7} M ^{3}HdTTP (19.2 Ci/mM). The mixture was incubated at 37 C for 60 min. The DNA was precipitated with cold trichloroacetic acid, centrifuged, resuspended in 0.33 N NaOH and reprecipitated with trichloroacetic acid twice. The final precipitate was collected on cellulose acetate membrane filters, solubilized with an NCS solubilizer, and counted in a toluene scintillation mixture. Background was obtained by precipitating one sample immediately after adding the virus. HA units represent the inverse of the hemagglutination titer per unit volume. RV was purified as described by Salzman except that the virus was propagated on hamster embryo rather than rat embryo cells. The H-1 SDS preparation had 2×10^8 PFU/2×10^5 HA units.

ploying quantities of infectious virus or HA antigen, or both, as much as 10^3 greater than the amounts described by Salzman. In agreement, Ricceri et al. have cited evidence that no viral DNA polymerase is associated with the parvoviruses RV and X14 (19). Therefore, it seems unlikely that a virion DNA polymerase is required for infection.

The finding that S phase alone is not sufficient for initiation of HA-DNA synthesis would also suggest that a specific initiation process, such as postulated to occur for the host cell DNA, may also be required (2, 16, 23, 26). Our data indicate that HA-DNA synthesis takes place more than 5 h after the onset of S phase in all cells but less than 7 h in some cells. Since S phase is on the average 6 to 8 h in duration in hamster cells (1), HA-DNA synthesis is either a late or, possibly, a terminal S-phase event. The analysis of the control of HA-DNA synthesis may thus become an interesting probe of the specific initiation of host cell replicons. It should be noted that other viruses have been reported to have a dependence for particular events on the cell cycle. Equine abortion virus, for example, although not representative of herpesviruses in general, has a requirement for host cell S phase for its DNA replication in KB cells (9), and a recent report has indicated that synthesis of Rous sarcoma virus RNA and protein are dependent on a late G1- or S-phase function and restricted to that portion of the cell cycle (12).

A subsequent report will describe the isolation from infected cells of a double-stranded DNA which contains DNA complementary to viral DNA and is therefore a postulated viral RF-DNA. The results obtained with actinomycin D indicate that the transcription of the putative viral RF to produce viral mRNA takes place shortly after its synthesis (Table 1). In these studies it was not possible to determine if α-amanitin was inhibiting HA synthesis by inhibiting synthesis of viral DNA or by inhibiting viral mRNA synthesis, or both. Alternatively, the drug may inhibit transcription of the host mRNA for an initiator protein controlling the HA-DNA synthesis.

The experiments described here are consistent with the replication process of the parvovirus H-1 being analogous to that of the single-stranded DNA bacteriophages, ϕ174 and M13 (17). Further investigation of the replication process of parvoviruses may aid the investigation of the mechanisms and control of DNA replication in eukaryotic cells.

ACKNOWLEDGMENTS

This research was supported by U.S. Public Health Service grants CA-07826 and CA13414 from the National Cancer Institute, General Research Support Grant 1-SO1-RR05725 from the National Institutes of Health, and by a grant from the Vermont Division of the American Cancer Society.

I thank Helene W. Toolan and K. A. O. Ellem for their helpful suggestions and for critically reviewing the manuscript, and Miss Veronica Lefevre for her expert technical assistance. I am grateful to T. Wieland of Max-Planck Institute, Heidelberg, Germany, for providing the α-amanitin.

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