Replication Process of the Parvovirus H-1

III. Factors Affecting H-1 RF DNA Synthesis

SOLON L. RHODE III

Putnam Memorial Hospital Institute for Medical Research, Bennington, Vermont 05201

Received for publication 21 March 1974

Replication of the single-stranded DNA parvovirus H-1 involves the synthesis of a double-stranded DNA replicative form (RF). In this study, the metabolism of RF DNA was examined in parasynchronous hamster embryo cells. The initiation of RF DNA replication was found to occur late in S phase, as was the synthesis of the DNA upon which subsequent viral hemagglutinin synthesis is dependent. Evidence is presented which indicates that initiation of RF replication requires proteins synthesized in late S phase, but that concomitant protein synthesis is not required for the continuation of RF replication. The data also suggest a requirement for viral protein(s) for progeny strand synthesis. Incorporation of 5-bromo-2'-deoxyuridine (BUdR) into viral DNA resulted in an "all-or-none" inhibition of viral hemagglutinin and viral antigen synthesis. BUdR inactivation of viral protein function was used to explore the time of synthesis of viral DNA serving as template for viral RNA synthesis and the effect of viral protein on RF replication and progeny strand synthesis. Results of this study suggest that parental RF DNA is synthesized shortly after infection, and that viral mRNA is transcribed from only a few copies of the viral genome in each cell. They also support the conclusion that viral protein is inhibitory to RF DNA replication. Density labeling of RF DNA with BUdR, allowing separation of viral strand DNA (V) from viral complementary strand (C), provided additional data in support of the above findings.

It has been shown that the replication process of the parvovirus H-1 is coordinated with the period of DNA replication of the infected cell (6, 16, 23). Previous papers in this series established that a DNA synthetic event (HA-DNA synthesis) which occurs in late S phase of the infected cell is required for the subsequent viral mRNA synthesis for capsid protein (the viral specific hemagglutinin, HA). Viral HA synthesis follows this event by 1 to 2 h (16, 17). A double-stranded replicative form (RF) viral DNA has been isolated from the nuclei of infected cells (17). It was proposed that synthesis of viral RF DNA is the first event in virus replication and may be the DNA synthetic event upon which subsequent viral hemagglutinin synthesis is dependent. In this study we will examine some of the factors that affect the synthesis and replication of H-1 RF DNA.

The system used here was confluent hamster embryo cells stimulated for 12 h with fresh medium containing 40% dialyzed fetal calf serum. After removal of this medium the cultures were infected with H-1 (12 h post-stimulation, PS).

A portion of the cells in these cultures (usu-

ally about 50 to 60%) enter S phase parasynchronously with a maximum in the number of S-phase cells at 18 to 20 h PS. The number of S-phase cells declines during 20 to 26 h PS and HA-DNA synthesis takes place in the period 8 to 12 h postinfection (PI) (20 to 24 h PS) (16). About 60% of the S-phase cells become positive for H-1 antigens by fluorescent staining at an MOI (multiplicity of infection) of 3.

MATERIALS AND METHODS

Cell culture and virus infection. Hamster embryo cultures were prepared, synchronized, and infected with H-1 at 12 h PS as previously described (16). Cell number was measured by suspending the cells in 0.25% trypsin in Tris-buffered saline containing 2.5 mM EDTA and counting an appropriate dilution in a hemocytometer. Plaque assay of H-1 was performed as noted (12).

DNA labeling and isolation of viral RF DNA. These methods were previously described (17). In many cases, cultures were labeled with [³H]thymidine in the presence of fluorodeoxyuridine (FUdR) to inhibit endogenous thymidine synthesis. By this means the specific activity of the [³H]thymidine incorporated is that of the thymidine of the medium and uncontrolled pool effects are avoided.

Chemicals. Cycloheximide (Acti-dione R, Upjohn Co.) was used at a concentration of 50 $\mu\text{g/ml}$, and actinomycin D (Sigma Chemical Co.) at 1 $\mu\text{g/ml}$. 5-Bromo-2'-deoxyuridine (BUdR) was obtained from P-L Biochemical and [^3H]BUdR (2 Ci/mmol), [^{14}C]thymidine (57 mCi/mmol), and [^3H]thymidine (20.5 Ci/mmol) were obtained from Amersham/Searle. FUDR was a gift of Hoffman-LaRoche. α -Amanitin was a gift of T. Wieland, Max-Planck Institute, Heidelberg, Germany, and was used at a concentration of 10 $\mu\text{g/ml}$ in HEPES-buffered medium, pH 8.0).

Virus purification. Virus purification was done as previously described (16). Virus for labeling with [^3H]thymidine was propagated in NB cells (20) and labeled by incubating the infected cells in medium containing FUDR (0.5 $\mu\text{g/ml}$) and [^3H]thymidine at 5×10^{-6} M (1 Ci/mmol) from 6 to 48 h PI.

RESULTS

Dependence of RF DNA synthesis on RNA and protein synthesis. To determine if the synthesis of RNA and protein were required for synthesis of RF DNA, the effects of an inhibitor of translation, namely cycloheximide, and of two inhibitors of transcription, α -amanitin and actinomycin D, on the incorporation of [^3H]thymidine into RF DNA were examined. When the drugs were separately added to culture media prior to the onset of HA-DNA synthesis, virtually complete inhibition of uptake of thymidine into RF DNA occurred (Table 1). Cells in the H-1 infected parasynchronous cultures would be in mid-S phase at the inception of drug treatment.

Under these conditions, α -amanitin reduced the H-1 HA titer at 14 h PI from 1:128 to 1:4 and actinomycin D at 1 $\mu\text{g/ml}$ inhibited HA synthesis entirely at 14 h PI when the drugs were administered at 6 h PI.

It has been established that a ribonucleotide transferase provides ribonucleotide 3'-hydroxyl termini upon which DNA polymerase initiates deoxynucleotide transfer reactions in various bacterial and eukaryotic DNA replication systems (1, 22, 25). It is thus possible that α -amanitin and, for other reasons, cycloheximide and actinomycin D have direct inhibitory effects on DNA synthesis in addition to their primary actions on RNA and protein synthesis. To test for this possibility, these drugs were added to infected cultures after the period of viral protein synthesis (10 to 16 h PI) and their effects on continued synthesis of viral RF DNA were determined.

The data in Table 2 show that all three of these drugs added at 16 h PI decreased the rate of incorporation of thymidine into RF DNA at 19 to 20 h PI. The degree of inhibition was not as profound as when the inhibitors were intro-

TABLE 1. Inhibition of RF DNA synthesis by cycloheximide, α -amanitin, and actinomycin D added before HA-DNA synthesis^a

Drug	Time added (h PI)	Method of RF DNA extraction	% Inhibition		
			RF DNA	Cell DNA	Uninfected cell DNA
Cycloheximide	6*	CPG	100		
	6	Hirt	100	88	91
α -Amanitin	5	CPG	96		
	5	CPG	98	22	7
Actinomycin D	6*	Hirt	97	50	52

^a Parasynchronous cultures of hamster embryo cells infected with H-1 were treated with cycloheximide, α -amanitin, or actinomycin D at the times indicated. The treated cultures and untreated controls were labeled with [^3H]thymidine (5 $\mu\text{Ci/ml}$) as in the table in the presence of the drugs from 8 to 10 or 9 to 10 (as indicated by *) h PI. RF DNA was extracted by the CPG or Hirt methods and yields determined from the amount of ^3H incorporated into the 17S region, after zonal sedimentation in neutral sucrose gradients as described previously (7, 17). Background was estimated by a projected continuation of the baseline counts for the regions outside of the 13 to 21S areas of the gradients. Cell DNA synthesis was estimated by using the incorporation of [^3H]thymidine into the Hirt pellets or the DNA retained by the CPG column for infected cultures and the total incorporation into trichloroacetic acid-insoluble material for the uninfected cultures.

TABLE 2. Inhibition of [^3H]thymidine labeling of RF DNA by α -amanitin, actinomycin D, and cycloheximide, added after HA-DNA synthesis^a

% Inhibition	α -Amanitin	Actinomycin D	Cycloheximide
Hirt pellet	33	42	81
Hirt supernatant	25	36	76
RF DNA	27	34	74

^a Parasynchronous cultures of hamster embryo cells infected with H-1 received (i) no drug, (ii) α -amanitin, (iii) actinomycin D, or (iv) cycloheximide, in HEPES-buffered medium, pH 8.0, at 16 h PI. At 18.5 h PI, the cultures were treated with FUDR for 30 min, and 19 h PI, [^3H]thymidine at a final concentration of 5×10^{-6} M (1 Ci/mmol) was added. This method ensures a uniform specific activity for the [^3H]thymidine incorporated. The cultures were labeled for 1 h (19 to 20 h PI), the DNA then extracted by Hirt's method and, after centrifugation, samples taken of the supernatant fraction and the pellet which was dissolved in 0.1 N NaOH, for trichloroacetic acid precipitation and measurement of radioactivity (17). The supernatants were sedimented in high salt sucrose gradients and the yield of RF DNA determined as in Table 1. The yield of RF DNA is expressed as percent inhibition relative to the untreated control.

duced at 5 to 6 h PI. In a previous study and in subsequent sections of this report, evidence is presented which indicates that asymmetric incorporation of the labeled nucleotide into the viral strand, presumably the result of progeny DNA synthesis, accounts for the bulk of the incorporation at the later times (> 16 h PI) (17). Thus the incorporation of [^3H]thymidine examined in Table 2 is largely progeny strand synthesis and not RF DNA replication. To examine the effects of these agents on continuation of RF DNA replication as distinct from progeny strand synthesis, cultures were incubated with FUdR (0.5 $\mu\text{g/ml}$) and [^3H]thymidine (5×10^{-6} M, 1 Ci/mmol) continuously from 6 h PI, which is before the time RF DNA replication can be detected (c.f. subsequent sections), to 16 h PI. At 12 h PI, drugs were added to their respective cultures which were then extracted by the Hirt method at 13 and 16 h PI; control cultures were extracted at 12, 13, and 16 h PI. The yield of RF DNA was estimated by the total activity of the Hirt supernatant, which is over 80% 17S RF DNA by zonal centrifugation under these labeling conditions (unpublished data). The results are shown in Table 3. No decrease in RF replication rates compared to the untreated cultures was evident. Actually, all three metabolic inhibitors increased the yield of RF DNA at both 13 and 16 h PI. The addition of the

inhibitor of RNA synthesis, actinomycin D, at 12 h PI was previously shown to be too late to inhibit the eventual yield of viral protein, i.e., HA (16). On the other hand, when added at 12 h PI, cycloheximide, which effects a rapid inhibition of protein synthesis, reduced the viral protein yield at 16 h PI by 75%. Thus the effect of these agents on the yields of RF DNA at 16 h PI is correlated inversely with the extent of their inhibition of viral HA synthesis. Further evidence for a negative effect of viral protein synthesis on the yield of RF DNA is cited below.

Effect of BUdR on viral replication. The treatment of infected cultures with high doses of BUdR (FUdR, 0.5 $\mu\text{g/ml}$ and BUdR, 10 $\mu\text{g/ml}$) before HA-DNA synthesis, which initiates 7 to 8 h PI, caused greater than 90% inhibition of subsequent production of HA and infectious virus (Table 4). Treatment of cultures after HA-DNA synthesis and HA mRNA synthesis has occurred (by about 12 h PI [16]) does not inhibit HA synthesis and only decreases the yield of infectious virus at 30 h PI by 50 to 60% (data not shown). The differences between cultures treated with BUdR at $\frac{1}{2}$ h PI and 6 h PI are small, and if they are significant (they differ at 90% confidence by the Student's *t* test) they may relate to the time of synthesis of parental RF DNA (c.f. below). When compared for the extents of inhibition by BUdR, the yield of FA and HA showed similar amounts. The FA+ cells in the presence of BUdR were as brightly stained as the controls, suggesting that the BUdR inhibition may be an "all or none" phenomenon.

This result was explored further by varying the relative concentration of BUdR versus TdR in the medium and determining the yield of FA and HA relative to the control receiving only TdR. This experiment was carried out with hamster embryo cells and with the NB cell line used for the plaque assay of H-1 (Table 5). Within the limitations of the HA assay, the production of HA and the yield of FA+ cells showed similar degrees of inhibition at the different dosages of BUdR. This correlation implies that the BUdR effect is "all or none" on a cell by cell basis. In experiment B the MOI used was 15 based on the plaque assay of the virus stock; however when the MOI was increased 10-fold the yield of FA+ cells increased 2.5 times to about 95% of the cells. Thus assuming a Poisson distribution of virus among the cells, the effective multiplicity used under these conditions was about 0.5. When the MOI was increased 10-fold the HA and the yield of FA+ cells increased for both the 0% BUdR and 80% BUdR-treated cultures. The effect of MOI on the survival of FA production in the presence

TABLE 3. Effects of α -amanitin, actinomycin D, and cycloheximide on RF DNA replication added during viral protein synthesis^a

Drug	Yield of RF DNA (pmol and % of control)			
	12 h PI	13 h PI	16 h PI	
None	37	59 100%	209 100%	
α -Amanitin		92 156%	304 145%	
Actinomycin D		80 136%	436 209%	
Cycloheximide		66 112%	781 374% ^b	

^a The total yield of RF DNA per culture was measured by the Hirt extraction of cultures (60-mm petri dish) labeled from 6 h PI in HEPES-buffered medium, pH 8.0, containing FUdR (0.5 $\mu\text{g/ml}$) and [^3H]thymidine (5×10^{-6} M, 1 Ci/mmol). The following drugs and final concentrations were added at 12 h PI: (i) α -amanitin, 10 $\mu\text{g/ml}$, (ii) actinomycin D, 1 $\mu\text{g/ml}$, and (iii) cycloheximide, 50 $\mu\text{g/ml}$. The Hirt extractions were carried out at the times indicated and ^3H content of the supernatant and pellet measured by trichloroacetic acid precipitation of duplicate samples. Values expressed are total yields of thymidine incorporated into DNA in the Hirt supernatant in picomoles, and the percentage of change relative to the untreated control.

^b This sample was analyzed by zonal sucrose gradient centrifugation to verify that it represented 17S RF DNA.

TABLE 4. *Effects of BUdR on H-1 FA, HA, and infectious virus synthesis^a*

Drug	Added	Removed	Culture harvested	FA	HA	PFU
FUdR-TdR	½	6	6		120	1.1×10^6
	½	12	12		480	2.8×10^6
	½	18	18	51 ± 3.8	6720	$10.0 \pm 1.08 \times 10^7$
FUdR-BUdR	½	6	18	48 ± 4.2	4800	$5.5 \pm 0.91 \times 10^7$
	½	18	18	0.1	120	
	6	18	18	0.1	120	

^a Parasyynchronous H-1-infected cultures of hamster embryo cells were treated in triplicate with FUdR-thymidine (10^{-5} M) or FUdR-BUdR (3×10^{-5} M) for the times indicated and harvested at 18 h PI for determination of HA and PFU. Cover slip cultures were similarly treated and fixed at 18 h PI for FA staining. The cultures were incubated in medium containing FUdR-thymidine at all times except during the BUdR treatments. Values expressed are average HA units per culture for duplicate cultures and average number of FA+ cells per high power field (10 high power fields counted, ± 2 standard deviations of the mean), and mean PFU per culture $\pm$ one standard deviation of the mean. The difference between the means for FUdR-TdR, 18 h and FUdR-BUdR ½ to 6 h, 18 h PI is 4.5×10^7 which was a significant difference at only 90% confidence by the Student's *t* test.

TABLE 5. *Effect of BUdR dosage on H-1 HA and FA synthesis*

Cells	BUdR % total						
	0	20	40	50	60	80	100
Secondary hamster embryo ^a							
FA (% control)	100	89	66	30	15	10	ND ^b
HA (% control)	100	50-100	50	50	50	25	ND
NB ^c							
FA (% control)	100	82.0	37.2	7.8	0.7	0	0
HA (% control)	100	50-100	25	6	0.7	0.7	0

^a Parasyynchronous cultures of hamster embryo cells were infected with H-1 at an MOI of 100 in the usual manner and treated with BUdR + TdR, 10^{-5} M, at various relative amounts of BUdR in the presence of FUdR, 0.5 μ g/ml, from ½ to 18 h PI. The numbers of FA+ cells per microscope field (10 fields counted) were counted and the HA titers determined.

^b ND, Not determined.

^c Asynchronous cultures of NB cells were infected with H-1 at an MOI of 15 and treated with various concentrations of BUdR from ½ to 24 h PI. Cultures were processed for FA and HA determinations. Values expressed are the numbers of FA+ cells or HA units per culture relative to the control (%) which received only TdR.

of BUdR was examined for 0, 50 and 80% BUdR (Table 6). At the MOI of 22 the cells were only 10% FA+ and the yield increased with increasing MOI to a maximum of 79%. As the MOI increased, the degree of inhibition at both 50 and 80% BUdR decreased. This is the expected result if BUdR acts in an "all or none" fashion on each virus. The increase in resistance to BUdR relative to the increase in MOI was disproportionately high and may represent an unknown multiplicity effect. Pretreatment of NB cells for 24 h prior to infection with 100% BUdR reduced the subsequent HA at 24 h PI by only 50%, so that this result and those discussed previously indicate that the BUdR-mediated inhibition of HA synthesis is not primarily the result of BUdR inhibition of cellular functions required by H-1.

Rate of RF DNA replication. The relative size of the RF pools and their rate of increase were determined for various times PI. Cultures were labeled continuously from 6 h PI with FUdR:[³H]thymidine or FUdR:[³H]BUdR at 5×10^{-6} M (0.4 Ci/mmol) so that absolute amounts of RF DNA could be estimated (Table 3). By comparing cultures treated with FUdR-thymidine to those receiving FUdR-BUdR, the effect of BUdR inhibition of viral protein synthesis on the rate of RF replication could be measured. Additional cultures were used to determine the cell number and the number of cells positive to fluorescent staining (FA) with fluorescein-conjugated H-1 antisera. Assuming the thymidine content of the RF DNA of H-1 to be 28% (13), each molecule contains about 2,850 thymidine molecules. Therefore, on the basis of

the specific activities of the labeled nucleotides in the medium (endogenous thymidine synthesis was inhibited with FUdR), the data are expressed as picomoles of thymidine incorporated and the equivalent number of RF molecules per culture. Yields of RF-DNA with FUdR-thymidine are shown in Fig. 1A. The cultures were extracted by the Hirt method at the respective times noted and the radioactivity in the Hirt supernatant was determined on samples. Cultures extracted at early periods were analyzed by zonal sucrose gradient density centrifugation to determine the actual amount of activity in the supernatants that represented RF DNA at the 17S position. At later times (≥ 16 h PI) the total Hirt supernatant measures the total extractable RF DNA with only a small error of 10 to 15%. The RF increased nearly exponentially 9 to 12 h PI, but showed a constantly declining rate of increase after 12 h PI, coincident with the sharp increase in HA synthesis (16). Accumulation of RF-DNA practically ceased from 20 to 24 h PI. In another experiment, the yield of RF in cells treated with FUdR-thymidine and FUdR-BUDr were compared (Fig. 1B). The FUdR-BUDr cultures had a slower rate of increase, which remained exponential up to 24 h PI, however.

The yield of RF per cell should extrapolate back to near the MOI at the time of initiation of RF DNA replication. In Fig. 1A, the samples at 7 and 8 h PI had no ^3H above the background at the 17S position. A clear peak at 17S, which represented 0.43 pmol of thymidine, was first apparent at 9 h PI. This is equivalent to 0.8×10^8 RF molecules or an average of 20 per cell for the culture. The MOI in this experiment was 14, and the yield extrapolates back to less than 1 RF per cell (0.4) at 6 h PI if all cells are infected and to 4 per FA+ cell. Data have been obtained which indicate that virus which has been inacti-

FIG. 1. (A) Kinetics of H-1 RF DNA replication. Parasynchronous cultures infected with H-1 were labeled with medium containing FUdR, 0.5 $\mu\text{g}/\text{ml}$, [^3H]thymidine, 5×10^{-6} M, 1 Ci/mmol; from 6 h PI, and were extracted by the Hirt method at various times PI. Yields of viral RF DNA were measured by assaying the trichloroacetic acid-insoluble ^3H in the supernatants. For samples at early times, 7 to 12 h PI, the extracts were sedimented in sucrose gradients and the total yield in the 17S region was calculated as in Table 1. Background was estimated in the sucrose gradients by continuation of the baseline through the 17S area. Values expressed are total yields of thymidine (TdR) per culture in picomoles for the Hirt supernatant —, and for 17S RF DNA by sucrose gradient analysis —. The symbol ‡ indicates yield was not measurable and was less than value shown. One picomole of TdR in RF DNA is equivalent to 2.1×10^8 RF molecules. (B) Kinetics of H-1 RF DNA replication. The yield of RF DNA at various times PI was determined by Hirt extraction as in A for cultures labeled with FUdR- ^3H]thymidine or with FUdR- ^3H]BUDr, 5×10^{-6} M, 0.5 Ci/mmol. Values shown are total yield of TdR or BUDr in picomoles incorporated.

TABLE 6. Effect of MOI on survival of FA after treatment with BUDr

% Control	MOI (0% BUDr %+)			
	700 (79)	222 (64)	70 (46)	22 (10)
50% BUDr	91	80	43	13
80% BUDr	73	59	9	<1

^a Asynchronous NB cover slip cultures were inoculated at various MOI and treated with BUDr-TdR and FUdR as in Table 5, $\frac{1}{2}$ h PI. At 24 h the cover slips were fixed and stained for H-1 specific FA and the percentage of + cells was determined (500 cells counted). The values shown are the percentage of + cells for 0% BUDr and the percentage of surviving + cells for 50 and 80% BUDr relative to 0% BUDr for each MOI.

vated by mutagenesis and not able to produce HA can still initiate RF replication, so the true average per cell probably lies between 0.4 and 4 (unpublished data). For a short labeling period, such as 17 to 18 h PI, the extraction recovers 30 to 40% of the total incorporation as 17S RF DNA so that it is at least that efficient. Six hours was previously determined as the minimal estimate of the latent period before HA-DNA synthesis began (16). These results are compatible with the initiation of RF replication

and HA-DNA synthesis being the same event. They also indicate a declining rate of RF replication coincident with the period of viral protein synthesis, 12 to 16 h PI. This decline in RF replication rate was shown to be inhibited by continuous treatment with BUdR from 6 h PI. Our results implicate a viral protein as the most likely cause for the decline in RF replication rate in the period 12 to 20 h PI. However, more recent data imply that the decline in rate is a time-dependent phenomenon not dependent on viral protein function (unpublished data).

Density labeling of RF DNA. It was shown previously that density labeling with BUdR in the presence of FUDr resulted in two density forms of RF DNA (17). The heavy form contained BUdR in both strands (H-H) and the relative yields of label in both strands at later stages of the infection indicated that the other form of hybrid density was predominantly or entirely substituted with BUdR in the viral strand (H-L). By convention the first letter will refer to the viral (V) strand in RF DNA. The asymmetric distribution of BUdR in the hybrid density RF probably results from the conservation of viral complementary (C) strands synthesized before the introduction of BUdR in the RF pool. Thus, by varying the time of introduction of BUdR, the extent of (C) strand synthesis prior to that time can be estimated by determining the amount of intermediate density RF DNA that is H-L. This was done by adding BUdR to one group of cultures at 7.5 h PI, just prior to the onset of HA-DNA synthesis, and then labeling the cultures with [^3H]BUdR from 8 to 10 h PI. A second group of cultures received BUdR at 13.5 h PI and were labeled with [^3H]BUdR from 14 to 16 h PI. If H-1 RF DNA initiates replication after the addition of BUdR at 7.5 h PI, then no H-L RF DNA would be detected. The RF DNA was extracted by the Hirt method and sedimented in high salt neutral sucrose gradients (Fig. 2A and B). Under these conditions, in addition to 17S RF DNA, a moiety sedimenting at about 27S is apparent and, normalized to the incorporation of ^3H into 17S RF, its synthesis is relatively decreased between the 8 to 10 h PI and the 14 to 16 h PI labeling periods. About 10 times as much incorporation into RF occurred during the 14 to 16 h PI labeling period compared to the recovery from the 8 to 10 h PI labeling period. Peak fractions of the 17S RF DNA and the 27S DNA were separately pooled, precipitated with ethanol and analyzed in Cs_2SO_4 as before (17). The results for the 17S RF DNA are shown in Fig. 3A and B. The RF from the cultures receiving

FIG. 2. (A and B) Sucrose gradient analysis of BUdR-substituted viral DNA. Parasynchronous cultures infected in the usual manner were incubated with medium containing FUDr (0.5 $\mu\text{g}/\text{ml}$) and BUdR, 3×10^{-5} M at 7.5 h PI in A and at 13.5 h in B. After 30 min the cultures were labeled with medium containing FUDr and [^3H]BUdR, 0.75×10^{-6} M, 2 Ci/mmol for 2 h, i.e., A, 8 to 10 h PI and B, 14 to 16 h PI. Viral DNA was extracted by the Hirt method and sedimented in high salt neutral sucrose gradient as in (17).

BUdR at 7.5 h PI was largely H-H ($\rho = 1.472$ g/cm 3) and showed only a slight shoulder at the area of hybrid density RF (1.444 g/cm 3). It thus contained no more than traces of RF-containing parental viral strand DNA, which would be L-H hybrid density RF DNA. On the other hand, the cultures receiving BUdR at 13.5 h PI ended the labeling period at 16 h PI with 70% of the ^3H included in hybrid density RF and the remainder as H-H RF. Since the H-L RF is labeled in only one strand this figure corrects to 82% of the total RF. To confirm that the hybrid density RF ($\rho = 1.444$) and the heavy RF ($\rho = 1.472$) are in fact H-H and H-L, respectively, the hybrid density DNA shown in Fig. 3B and the heavy DNA in Fig. 3A were heat denatured and rebanded in Cs_2SO_4 with results shown in Fig. 3C and D, respectively. The hybrid density DNA produced a main peak of DNA at the density of the (+) strand ($\rho = 1.530$) and a small shoulder at the density of the (C) strand ($\rho = 1.505$). When this hybrid density DNA was rebanded in Cs_2SO_4 , it contained a small amount of heavy RF sufficient to account for this amount of

labeled (C) strand (not shown). The heavy DNA denatured into nearly equal amounts of BUdR substituted (V) and (C) strands, confirming it to be H-H RF DNA. Thus very little (C) strand DNA was synthesized before 7.5 h PI and by 13.5 h PI up to 82% of the final yield at 16 h PI had been synthesized in the presence of FUDR-BUdR.

The densities of the 27S peaks from Fig. 2A and B in Cs_2SO_4 (Fig. 4A and B, respectively) were not those of progeny single-stranded DNA ($\rho = 1.530$), but largely that of H-H ($\rho = 1.472$) and hybrid density RF ($\rho = 1.444$). Also, the top three fractions of the gradient in Fig. 4B contained a significant amount of DNA and it should be noted that this light material was not seen in the analysis of 17S RF DNA (c.f. Fig. 3). Both the hybrid density DNA (Fig. 4A, $\rho = 1.444$) and the light DNA (Fig. 4B $\rho < 1.423$) were rebanded after heat denaturation, Fig. 4C and D, respectively. The hybrid density DNA derived from the 27S peak was predominantly the H-L type as was the 17S hybrid density RF DNA. The light DNA contained mostly (71%) heavy (V) strand and some heavy (C) strand (29%) in similar proportions to that of 17S hybrid density RF DNA (c.f. Fig. 4C). In

conclusion, the 27S peak contains RF DNA of both the H-H and H-L type, with a disproportionate amount of H-L as seen by comparing Fig. 3A to 4A. It probably derives its fast sedimentation velocity (27S) and light density ($\rho < 1.423$) from interaction with some other material.

Time of synthesis of parental RF DNA. The time of conversion of the infecting virion DNA to the double-stranded RF DNA, i.e., the synthesis of the parental RF, may be the event coordinated with late S phase of the infected cell. During the course of this work it was reported by Salzman and White that a double-stranded DNA of the parvovirus RV could be extracted 1 h PI (19).

To determine when RF DNA is first extractable by the Hirt method, parasynchronous cultures infected with H-1 at an MOI of 10 were labeled 2 to 4, 4 to 6, or 6 to 8 h PI with [^3H]thymidine (10 $\mu\text{Ci}/\text{ml}$). The DNA was extracted by the method of Hirt and sedimented in sucrose gradients. The yields of counts per minute located in the 17S areas of the gradients above the projected continuous baseline were totaled and are listed in Table 7.

A peak in the 17S region of the gradient was

FIG. 3. (A and B) Equilibrium density gradient centrifugation of [^3H]BUdR-substituted 17S RF DNA. Pooled fractions of 17S RF DNA, A and B were centrifuged to equilibrium in Cs_2SO_4 as previously described (17). The results for BUdR-substituted RF DNA extracted at 10 h PI is shown in A and RF DNA extracted at 16 h PI is in B. (C and D) Equilibrium density gradient centrifugation of denatured BUdR-substituted H-1 RF DNA. The pooled fractions of hybrid density RF DNA shown in B ($\rho = 1.44$) were heat denatured and rebanded to equilibrium in Cs_2SO_4 as previously described (17) (C) and the heavy density form of RF DNA pooled from the gradient shown in A ($\rho = 1.472$) was similarly denatured and rebanded (D).

FIG. 4. (A and B) Equilibrium density gradient centrifugation of BUdR-substituted 27S H-1 DNA. The material sedimenting at approximately 27S shown in Fig. 2A and B was pooled and banded to equilibrium in Cs_2SO_4 . 27S DNA extracted at 10 and 16 h PI are shown in A and B, respectively. (C and D) Equilibrium density gradient centrifugation of denatured BUdR-substituted RF DNA. The hybrid density DNA ($\rho = 1.444$) shown in A from the extraction at 10 h PI was denatured and rebanded in Cs_2SO_4 , as before, C. The DNA located in the top three fractions in B from the culture extracted at 16 h PI was similarly denatured and rebanded, D.

TABLE 7. RF DNA synthesized at various times PI^a

Time of pulse (h)	³ H counts/min in RF DNA	³ H counts/min in Hirt pellet
2-4	0	2,361,525
4-6	0	4,745,600
6-8	7,100	5,226,750

^a Parasynchronous cultures infected with H-1 in the usual manner were labeled 2 to 4, 4 to 6, or 6 to 8 h PI with [³H]thymidine (10 $\mu\text{Ci/ml}$). Hirt extracts were made at the end of the labeling periods and the supernatants were sedimented in sucrose gradients as previously described (17). The total counts per minute sedimenting as a distinct peak in the 17S region of the gradients were measured from the plot for each gradient and are indicated in the table as ³H counts per minute of RF DNA. Total counts per minute in the Hirt pellets were measured as in Table 2.

first detectable in the 6 to 8 h PI labeling period. Labeling during the interval 8 to 10 h PI gave relatively large peaks of RF DNA (c.f. section above). It was previously reported that the synthesis of viral DNA (and RNA) upon which subsequent viral hemagglutinin synthesis was dependent (HA-DNA) initially occurred at 7 to 8 h PI in this cell system. Thus HA-DNA synthesis is coincident with the earliest detec-

tion of synthesis of viral RF DNA in the interval 6 to 8 PI.

If the great majority of virions which fail to produce plaques in the infectivity assay (particle/infectivity ratio = 2×10^4) also fail to convert their DNA to a double-stranded form, then labeling of parental RF DNA during its synthesis may have insufficient sensitivity for the detection of parental RF synthesis. Direct demonstration of the conversion of prelabeled H-1 viral DNA to the RF form as described for RV by Salzman and White (19) is probably a more sensitive method. Our attempts to repeat this type of experiment with H-1 were not convincing, due to a very low recovery of prelabeled viral DNA as a putative RF form, probably because of the high particle/infectivity ratio. Thus the exact time of synthesis of parental RF DNA has not been determined for H-1.

DISCUSSION

The parvovirus H-1 replicates autonomously in hamster embryo cells and has been shown to have a requirement for cellular S phase (6, 16). A DNA synthetic event, designated HA-DNA synthesis, upon which viral RNA and subse-

quent hemagglutinin synthesis is dependent, initiates in late S phase of the infected cell. It had been proposed that HA-DNA may be the parental viral RF DNA (16). H-1 RF DNA has been isolated and characterized (17). In this study factors affecting RF DNA synthesis were examined. It was established that RNA and protein synthesis during the latter half of S phase of the infected cell were required for the initiation of RF DNA replication.

The inhibitors of RNA synthesis, actinomycin D and α -amanitin, and the inhibitor of protein synthesis, cycloheximide, were shown to completely inhibit RF synthesis when cultures were treated at 5 to 6 h PI. This time is a few hours before HA-DNA synthesis. If protein synthesis was inhibited at 12 h PI, after HA-DNA synthesis, but early in the period of viral protein synthesis, normal or increased yields of RF DNA were obtained. After the period of viral protein synthesis, α -amanitin, actinomycin D, and cycloheximide were partially inhibitory to incorporation of [3 H]thymidine into RF DNA. At this time of labeling, 19 to 20 h PI, the rate of RF replication is slow (RF doubling time = 3 to 5 h) and the inhibition observed was most likely that of progeny strand synthesis. The failure of α -amanitin to inhibit RF replication under conditions that inhibited viral protein synthesis indicates that RF DNA replication is not primed by polyribonucleotide sequences produced by the RNA polymerase sensitive to α -amanitin (1, 22, 25).

It was found that the introduction of BUdR to cultures just prior to the time of HA-DNA synthesis, 6 h PI, resulted in a marked inhibition of HA synthesis and a similar decrease in the number of FA positive cells. There was no inhibition of ongoing HA synthesis when BUdR was added at 14 h PI, so that this BUdR-directed inhibition is most likely the result of the incorporation of BUdR into HA-DNA and either a miscoding during RNA transcription or a decrease in amount of mRNA synthesized, or both (4, 8). The "all-or-none" aspect of this effect has been reported to occur in other systems (2, 24). It implies that BUdR is exerting its action through insertion into only a few copies of the gene(s) governing hemagglutination and antigenicity in the infected cell, and thus suggests that each cell contains only a few RF molecules before 6 h PI. The small amount of RF DNA at this time, as estimated by extrapolation from the actual yields determined at later times, is in agreement with this interpretation. Since BUdR can be completely inhibitory at low multiplicities, even as late as 6 h PI, it may be inferred that the DNA strand transcribed is synthesized after 6 h PI and is not the

infecting viral strand. This inference requires the assumption that the incorporation of BUdR into only the (C) strand is not capable of inhibiting the transcription of the (V) strand. Since all of the metabolic effects of BUdR treatments are not well understood, these results must be interpreted with caution.

The rate of RF replication and the size of the RF pools were examined by measuring the yield of RF DNA extracted from cultures at various times PI. The cultures were labeled continuously from 6 h PI to the time of extraction in the presence of FUdR and the labeled nucleotide to maintain a constant specific activity during the entire labeling period. No more than a 30% decrease in external thymidine concentrations occurred under these conditions. The effect of BUdR inhibition of viral protein synthesis on RF replication was examined by comparing yields of RF with cultures treated with FUdR-BUdR and FUdR-thymidine. The RF pools increased nearly exponentially, but with a gradually declining rate. RF replication was first detectable at 8 to 9 h PI and the initial rate of increase had a doubling time of 0.3 h. After viral protein synthesis (10 to 16 h PI), the rate dropped at least 10-fold to a doubling time of 3 to 5 h. The RF pools became relatively large, reaching an average of 4×10^4 molecules per cell, and all cells were not infected. Extrapolation of the curve back to 6 h PI gave an average yield of 4 RF/FA+ cell, well below the MOI of 14 in that experiment. Thus the time of HA-DNA synthesis, initiating at 8 h PI, is coincident with the earliest detection of RF replication, and RF pool sizes are approximately equal to the MOI at that time. It seems most likely that initiation of HA-DNA synthesis and initiation of RF DNA replication are the same event. Thus the transcription of H-1 for the hemagglutinin mRNA is coordinate with the replication of the RF DNA in the manner of the late mRNA in many other DNA virus replication cycles (e.g., 10, 11).

In the presence of BUdR, the rate of RF replication fails to decline, possibly as a result of BUdR inhibition of viral protein function. The rate of increase in the presence of BUdR (doubling time of 2 h) is slower than the maximal rate seen with thymidine. This is probably not caused by a decrease in chain growth rate (14). The time required to replicate a DNA of a molecular weight of 3×10^6 to 4×10^6 would be only about 2 to 4 min, if synthetic rates are comparable to those of the cell (9). Thus initiation may be the rate limiting step in RF replication, and it is more likely BUdR decreases the rate of initiation (3).

The use of BUdR density labeling comple-

mented the findings obtained with other methods. First, nearly all complementary strand synthesis took place after 7.5 h PI, and the failure to detect L-H hybrid RF DNA indicated that parental viral DNA was not extractable in detectable amounts. The density labeling also indicated that the RF pool was undergoing semiconservative replication through demonstration of H-H RF production. Progeny strand synthesis was occurring simultaneously, with the result that light viral strand DNA was not conserved during incubation in the presence of BUdR. The rate of increase of the RF DNA pool was calculated to be 22% for the interval 13.5 to 16 h PI during incubation with BUdR. During continuous labeling in the presence of BUdR from 6 h PI, the RF pool approximately doubled during this time interval and the reason for this difference may be related to BUdR inactivation of viral protein function when it was added before HA-DNA synthesis.

The incorporation of BUdR into RF DNA produced a fast-sedimenting species of RF DNA that had an abnormally light buoyant density. Further analysis indicated that it represented predominantly hybrid density RF DNA. The anomalous sedimentation and decreased buoyant density not seen with thymidine labeling suggest a DNA-protein or DNA-lipid complex peculiar to BUdR substituted H-1 RF DNA. This phenomenon has been previously reported for lymphocyte DNA after BUdR substitution (5).

Salzman and White in a study of RV, another of the parvoviruses, reported that the isolation of a double-stranded DNA containing RV DNA as early as 1 h PI (19). Attempts were made by us to extract an H-1 RF DNA labeled with [³H]thymidine *in vivo* by the CPG method or the Hirt method in the first 2 h of infection, but they were unsuccessful. It was estimated that less than 1% of the adsorbed virions could have synthesized an extractable RF DNA in that interval without being detected. Using the Hirt extraction, it was shown that the first RF DNA synthesis that was detectable occurred in the labeling period 6 to 8 h PI. Thus RF DNA synthesis was first detected at the time of onset of HA-DNA synthesis.

In one case out of four experiments with H-1, containing labeled DNA, some viral DNA converted to a fast-sedimenting form at the position of RF DNA in low ionic strength neutral sucrose gradients as reported for RV by Salzman and White (19). The recovery was only 0.11% of the inoculum and 0.5% of the adsorbed radioactivity, as opposed to the 5.3% and 20 to

35% reported by Salzman and White for RV. A 10-fold or greater difference in the particle/infectivity ratios of H-1 versus RV could account for such a low yield with H-1. Cultures infected with prelabeled virus were chased both 2 h and 7 h (21 h PS) before extraction. At this time many cells are leaving S phase and initiating RF replication. In this experiment, there was no RF peak at either time, probably due to the generally small percent of virions initiating infection. In any case, no conversion of input DNA to an RF form was observed by the time RF DNA initiated replication. Thus a small portion of the adsorbed virions may convert their DNA to an RF form by 2 h PI and the difficulties encountered in demonstrating this are compatible with the small fraction of virions which initiate a productive infection in the infectivity assay.

The coordination of viral RF DNA replication with specific events in the cell cycle, not including a general requirement for S phase, has also been reported for simian virus 40 replication in synchronized CV 1 cells (15). Further studies of the factors controlling H-1 RF DNA replication may provide additional insights into the mechanisms and regulation of cell DNA replication.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grants CA13414 and CA07826 (from the National Cancer Institute) and by General Research Support grant 1 SO1 RR05725 from the Division of Research Resources.

I wish to thank Helene W. Toolan and K. A. O. Ellem for their helpful suggestions and for critically reviewing the manuscript and Veronica Lefevre and Jessica Bratton for expert technical assistance. I am also indebted to Irwin Singer for determining the particle concentrations on several preparations of purified H-1.

LITERATURE CITED

1. Brutlag, D., R. Schekman, and A. Kornberg. 1971. A possible role for RNA polymerase in the initiation of M13 DNA synthesis. *Proc. Nat. Acad. Sci. U.S.A.* **68**:2826-2829.
2. Calothy, G., K. Hirai, and V. Defendi. 1973. 5-Bromodeoxyuridine incorporation into simian virus 40 deoxyribonucleic acid. Effects on simian virus 40 replication in monkey cells. *Virology* **55**:329-338.
3. Carlson, K. 1973. Multiple initiation of bacteriophage T4 DNA replication: delaying effect of bromodeoxyuridine. *J. Virol.* **12**:349-359.
4. Freese, E. 1959. The specific mutagenic effect of base analogs on phage T4. *J. Mol. Biol.* **1**:87-105.
5. Gottlieb, A. A., L. Taylor, and F. Seinsheimer III. 1970. Replication of deoxyribonucleic acid in lymphoid cells. An unusual effect of bromodeoxyuridine. *Biochemistry* **9**:4322-4328.
6. Hampton, E. G. 1970. H-1 virus growth in synchronized rat embryo cells. *Can. J. Microbiol.* **16**:266-268.
7. Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26**:365-369.
8. Howard, B. D., and I. Tessman. 1964. Identification of the altered bases in mutated single-stranded DNA. II.

- In vivo* mutagenesis by 5-bromo-deoxyuridine and 2-aminopurine. J. Mol. Biol. **9**:364-371.
9. Huberman, J. A., and A. D. Riggs. 1968. On the mechanism of DNA replication in mammalian chromosomes. J. Mol. Biol. **32**:327.
 10. Khoury, G., and M. A. Martin. 1972. Comparison of SV40 DNA transcription *in vivo* and *in vitro*. Nature N. Biol. **238**:4-6.
 11. Khoury, G., J. C. Byrne, and M. A. Martin. 1972. Patterns of simian virus 40 DNA transcription after acute infection of permissive and nonpermissive cells. Proc. Nat. Acad. Sci. U.S.A. **69**:1925-1928.
 12. Ledinko, N. 1971. Plaque assay of the effects of cytosine arabinoside and 5-iodo-2'-deoxyuridine on the synthesis of H-1 virus particles. Nature (London) **231**:1346-1347.
 13. McGeoch, D. J., L. V. Crawford, and E. A. Follett. 1970. The DNA's of three parvoviruses. J. Gen. Virol. **6**:33-40.
 14. Meyn, R. E., R. R. Hewitt, and R. M. Humphrey. 1973. Evaluation of S phase synchronization by analysis of DNA replication in 5-bromodeoxyuridine. Exp. Cell Res. **82**:137-142.
 15. Pages, J., S. Manteul, D. Stehelen, M. Fiszman, M. Marx, and M. Girard. 1973. Relationship between replication of simian virus 40 DNA and specific events of the host cell cycle. J. Virol. **12**:99-107.
 16. Rhode, S. L. 1973. Replication process of the parvovirus H-1. I. Kinetics in a parasynchronous cell system. J. Virol. **11**:856-861.
 17. Rhode, S. L. 1974. Replication process of the parvovirus H-1. II. Isolation and characterization of H-1 replicative form DNA. J. Virol. **13**:400-410.
 18. Sadoff, R. B., and W. P. Cheevers. 1973. Evidence for RNA-linked nascent strands in polyoma virus DNA replication. Biochim. Biophys. Res. Comm. **53**:818-823.
 19. Salzman, L. A., and W. White. 1973. *In vivo* conversion of the single-stranded DNA of the Kilham rat virus to a double-stranded form. J. Virol. **11**:299-305.
 20. Shein, H. M., and J. F. Enders. 1962. Multiplication and cytopathogenicity of simian vacuolating virus 40 in cultures of human tissues. Proc. Soc. Exp. Biol. Med. **109**:495-500.
 21. Smets, L. A., and H. Brohee. 1970. A hidden pool effect of thymidine incorporation in HeLa cells. Int. J. Radiat. Biol. **1**:93-96.
 22. Sugino, A., S. Hirose, and R. Okazaki. 1972. RNA-linked nascent DNA fragments in escherichia coli. Proc. Nat. Acad. Sci. U.S.A. **69**:1863-1867.
 23. Toolan, H. W. 1971. The parvoviruses, p. 410-425. In F. Homburger (ed.), Progress in experimental tumor research, vol. 16. Karger, Basel.
 24. Weintraub, H., G. L. Campbell, and H. Holtzer. 1973. Differentiation in the presence of bromodeoxyuridine is "all or none." Nature N. Biol. **244**:140-142.
 25. Wickner, W., D. Brutlag, R. Shekman, and A. Kornberg. 1972. RNA synthesis initiates *in vitro* conversion of M13 DNA to its replicative form. Proc. Nat. Acad. Sci. U.S.A. **69**:965-969.