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# Replication Process of the Parvovirus H-1
## II. Isolation and Characterization of H-1 Replicative Form DNA

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The Parvovirus H-1 replicates autonomously in hamster embryo cells. A DNA synthetic event, called HA-DNA synthesis, upon which subsequent viral RNA and viral hemagglutinin synthesis is dependent, is initiated in late S phase of the infected cell (18). It was postulated that HA-DNA represents parental viral replicative form DNA (RF DNA). This study describes the isolation and characterization of H-1 RF DNA as part of the continuing study of the mechanisms and control of DNA replication in the eukaryotic cell. The H-1 RF DNA is a linear duplex molecule containing the viral strand and its complement. The complementary strands of the RF DNA have been separated by equilibrium density gradient centrifugation. The RF DNA has a buoyant density of 1.705 in neutral CsCl and an estimated guanine plus cytosine (GC) content of 45.9%. It has a sedimentation coefficient of 17*S*. The calculated molecular weight of 3.7 $\times 10^6$ is twice that of the single-stranded virion DNA. H-1 virions contain DNA that is homogeneous and free of complementary strands.

H-1 virus is a member of the subgroup of Parvoviruses including H-3, RV and MVM that are able to replicate without helper virus in some cells (33). This replication process has been shown to be associated with host cell DNA replication (23, 27, 28, 31, 32). In the first paper of this series a parasynchronous cell system was used to show that synthesis of a particular DNA, presumably viral in origin and termed HA-DNA, was necessary for subsequent production of H-1 viral antigen. Synthesis of HA-DNA was initiated only in late S phase of the infected cell (18, 27, 28).

Since the Parvoviruses are known to contain a single-stranded DNA (19, 20, 21, 35), this DNA was postulated to be a viral replicative form (RF) consisting of a double-stranded DNA molecule containing the parental viral strand and the newly synthesized complementary strand. This model is in agreement with the replication process of other viruses in which the virus genome is a single-stranded polynucleotide. The requirement of a late S phase function for the initiation of synthesis of the HA-DNA suggests the possibility of a specific initiation process for HA-DNA synthesis that is homologous to an unidentified host cell initiation event for a late replicating replicon. This requirement would be in addition to any requirement of the viral DNA for the host cell apparatus for DNA replication. Thus the Parvovirus may be a molecular probe for the initiation of a subgroup of host cell replicons.

## MATERIALS AND METHODS

**Virus and cell culture.** Virus infection and cell culture methods were as previously described (18).

**DNA labeling.** Labeling of the DNA was performed by incubating cultures in complete medium (without tryptose phosphate broth) containing the indicated amounts of labeled precursor: $^3$H-thymidine (19 Ci/mmol, Amersham/Searle); $^{14}$C-thymidine (62 mCi/mmol, Amersham/Searle). At the end of the labeling period the medium was withdrawn, the cell layers were washed once with ice cold Tris-buffered saline, and they were processed as described below.

**Extraction of viral DNA. (i) CPG chromatography.** Cell nuclei were isolated by washing cultures containing $2 \times 10^7$ cells with 5 ml of 50 mM Tris, pH 7.4, and 0.15 M NaCl, and then harvesting the cells with a rubber policeman into 10 ml of 0.32 M sucrose, 50 mM Tris, pH 7.5, 1 mM $MgCl_2$, and 25 mM KCl. The cells were homogenized in a Dounce-type homogenizer, and the nuclei were collected by centrifugation for 10 min at $2,000 \times g$ and consecutively resuspended and resedimented in 10 ml of 1 M sucrose ($7,000 \times g$ for 10 min) and 10 ml of 0.32 M sucrose in the same buffer. The nuclear pellet was suspended in 4 ml of 50 mM Tris, pH 6.7 (TB, 6.7), and layered over a 0.5-cm high bed of CPG-10, 80 to 120 mesh, 17 nm pore (Controlled Pore Glass, Corning Glass Works, Corning, N.Y.) in a plastic column (1-cm diameter). Additional controlled pore glass (CPG) was dropped through the nuclear suspension; nuclei rapidly adsorb to the glass and a column of

about 3 to 4 cm in height was produced. The column was washed with 10 ml of TB 6.7 and the nuclei lysed by elution with 25 ml of 2 M NaCl and 50 mM Na acetate, pH 6.0, run slowly for about 30 min. All of the above operations were at 0 to 4 C. The column was then eluted with 25 ml of 1% SDS, 0.1 M NaCl, 50 mM Tris, pH 7.4, and $10^{-3}$ M EDTA at room temperature. This elution contained the bulk of the viral DNA, but the host cell DNA is largely retained by the column. In some cases the 2 M NaCl elution was omitted. After the final elutions the column was dismantled and mixed with 5 ml of 0.1 N NaOH. Samples were taken for trichloroacetic acid precipitation and measurement of the radioactivity retained on the column. In this way, final totals of radioactivity recovered were equal to the total activity applied, which was determined on portions of the nuclear suspension. Pronase was added to the eluants containing viral DNA to a final concentration of 100 μg/ml and was incubated for 16 h at 37 C. The sample was then extracted with phenol, and the DNA was precipitated with 2 volumes of cold ethanol overnight at −20 C. (ii) In some cases, viral DNA was extracted by the method of Hirt (11). Radioactivity was assayed by precipitation of samples with trichloroacetic acid, final concentration 5%, collection of the precipitate on membrane filters, and liquid scintillation spectrometry as previously described (7). A toluene-base scintillation fluid was used throughout these studies.

**Sedimentation analysis.** DNA was dissolved in 50 mM Tris, pH 8.0, 0.5% Sarkosyl, 0.1 MNaCl, $10^{-3}$ M EDTA, and was layered on 5 to 20% sucrose gradients, generally using 50 mM Tris, pH 8.0, 1 M NaCl, $10^{-3}$ M EDTA, and 0.2% Sarkosyl as solvent. Variations are as cited in Results. Centrifugation was carried out in an SW25 rotor for 16 h at 23,000 rpm at 4 C. Tubes were fractionated by puncture through the bottom and radioactivity was determined by solubilizing a small portion with NCS solubilizer (Amersham/Searle) and by counting directly or, in the case of Hirt extracts, by acid precipitation of the samples.

**Equilibrium centrifugation.** CsCl and CsCl-ethidium bromide gradients were performed as previously described (17).

$Cs_2SO_4$ gradients were carried out by adding an appropriate volume of a saturated $Cs_2SO_4$ solution in water to the sample containing DNA and centrifuging to equilibrium as described in the text. All gradients were done in polyallomer tubes and fractionated by puncture through the bottom. Densities were determined by refractive index and corrected for temperature and solvent (4), and radioactivity was determined by assying samples on 3-MM filter paper. The filter papers were washed twice with cold 5% trichloroacetic acid (4 C) and twice with cold acetone, dried, treated with 0.2 ml of NCS, and counted by liquid scintillation spectrometry.

**Hydroxylapatite chromatography.** The batch method described by Fanshier et al. was used (9).

**DNA-DNA hybridization and nuclease digestion.** Single-stranded DNA was selectively digested with micrococcal nuclease (Worthington Biochemical Co., Freehold, N.J.). DNA samples were dissolved in 10 mM Tris, pH 8.0, 0.8 M NaCl, and an equal volume of enzyme, 200 μg/ml in 10 mM Tris, pH 8.0, 0.02 M $MgCl_2$, and 0.3 mM $CaCl_2$ was added (D. Kacian and S. Spiegleman, manuscript in preparation). All solutions and the incubation mixture were maintained at 0 C. At higher temperatures, this nuclease preparation digested double-stranded DNA under these conditions, possibly due to a contaminating nuclease. However, at 0 C no solubilization of native hamster embryo DNA to acid precipitation occurred after 26 h of incubation with micrococcal nuclease.

In the competitive hybridization experiments, the digestion went on for 4 h at 0 C and terminated by precipitation with trichloroacetic acid (final concentration 5%). Bovine serum albumin (200 μg/sample) was used as a carrier, and the precipitates were collected on membrane filters for assaying acid-insoluble radioactivity.

H-1 viral DNA was obtained by lysing purified virus with 1% SDS at 100 C for 90 s, extracting twice with freshly distilled phenol, extraction of phenol from the aqueous phase with ether, and collecting the DNA by ethanol precipitation. The concentration of DNA was estimated by adsorption at 260 nm ($E^{1\%}_{260}$ mg/ml = 28). This is the extinction coefficient for φX174 DNA, which is single stranded and similar in size and composition to H-1 (29).

This investigation describes a new method of extracting viral DNA from nuclei of infected cells, its application to the isolation of H-1 RF DNA, and the initial characterization of viral RF DNA.

## RESULTS

**Isolation of viral DNA from infected cells.** To isolate viral RF DNA, a new method under study in our laboratory for other purposes was adapted to this problem. This method, which utilizes the adsorption of nuclei to porous glass particles prepared as columns for chromatography, allows elution of the small viral DNA with SDS while the bulk of the cell DNA remains on the column.

To illustrate the application of this method, cultures of hamster embryo cells were prelabeled by incubation with $^{14}$C-thymidine (0.002 μCi/ml) during growth to confluence. The cells were washed with Hanks balanced salt solution and stimulated and infected as described in Materials and Methods. They were labeled from 20 to 21.25 h postinfection (PI) with $^{3}$H-thymidine (10 μCi/ml). Nuclei were isolated and analyzed by chromatography on CPG. The elutions used and results obtained are described in Table 1. The DNA eluted with sodium dodecyl sulfate (SDS) has a $^{3}$H/$^{14}$C ratio nearly 10-fold higher than the DNA retained on the column. The DNA eluted in the preceding 2 M NaCl fraction has a $^{3}$H/$^{14}$C ratio slightly less than that of the total DNA recovered, which was 4.7. The recoveries are nearly quantitative (> 95%) by this method.

TABLE 1. *CPG chromatography of H-1 DNA*[a]

| Eluant and volume | Counts per min of | | Total (%) | | |
|---|---|---|---|---|---|
| | $^3H$ | $^{14}C$ | $^3H/^{14}C$ | $^3H$ | $^{14}C$ |
| Sample in TB, 10 ml | 656 | 955 | 0.69 | 0.1 | 0.6 |
| 2 M NaCl, 0.05 M acetate, pH 6.0, 25 ml | 500,475 | 19,900 | 25.1 | 7.6 | 12.3 |
| 1% SDS, 0.1 M NaCl, 50 mM Tris, pH 7.5, $10^{-3}$ EDTA, 25 ml | 1,468,225 | 4,800 | 305.8 | 22.2 | 3.0 |
| TB, 10 ml | 130,626 | 695 | 188 | 2.0 | 0.4 |
| Column residual | 4,475,880 | 135,510 | 33.1 | 67.8 | 83.6 |

[a] CPG chromatography of H-1 infected cells prelabeled with $^{14}C$-thymidine and labeled with $^3H$-thymidine 20 to 21.25 h PI as described in the text. The values shown are the total counts per minute recovered in each eluant and retained by the column as determined by trichloroacetic acid precipitation of samples of each fraction, the $^3H/^{14}C$ ratio, and the percentage of total counts per minute recovered for each isotope.

A more detailed analysis of this method and the elution behavior of DNA from uninfected cells is in preparation. It should be mentioned here that prelabeled DNA of uninfected cells shows a similar elution pattern to that of infected cells and improvements of the method have decreased the elution of cell DNA to generally less than 2% in the NaCl and SDS fractions. Sufficient strand breakage of cell DNA allows its elution with NaCl from the column. After producing some strand breakage by shear or nuclease action, nascent DNA labeled for brief intervals (1 to 10 min) shows a preferential retention over prelabeled DNA during the NaCl elution, as was found here for the 17*S* DNA labeled for 75 min in the H-1 infected culture; only 10% of the total eluted with NaCl (c.f. Table 1 and Fig. 1A, B, note the scale change: and unpublished results). This is thought to be the result of differences in the interaction of the replication sites in nascent DNA in comparison to bulk DNA with the components of the column. Both the 2 M NaCl fraction and SDS fractions were deproteinized by Pronase-phenol treatment (as described in Materials and Methods) and analyzed by velocity sedimentation in sucrose gradients (Fig. 1). The sedimentation of $^3H$-thymidine-labeled DNA extracted from purified virions of H-1, which has an $s_{20,w}$ value of 27.8 (14), was analyzed in a parallel gradient. Its position is indicated by the arrow. A relatively homogeneous peak is present in both eluants that is not associated with a corresponding peak of prelabeled host cell DNA. The $^3H/^{14}C$ ratios reach a maximum of 52 for the NaCl elution and 620 for the SDS elution in the 17*S* region of the gradient. The $^3H/^{14}C$ ratios in other areas fall in the range 16 to 18. Thus the peak fractions have up to a 34-fold increase in the $^3H/^{14}C$ ratio. A sample of the pooled fractions 19 to 23 of the SDS elution

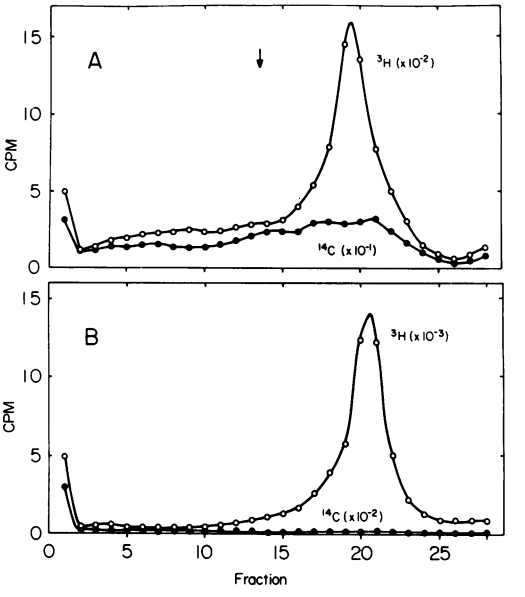


FIG. 1. *Sucrose gradient analysis of DNA extracted by CPG chromatography. The 2 M NaCl eluant (A) and the 1% SDS eluant (B) were digested with Pronase, were phenol extracted, and were precipitated with ethanol. The precipitates were dissolved and sedimented through 5 to 20% sucrose in 50 mM Tris, pH 8.0, $10^{-3}$ M EDTA, 1 M NaCl, and 0.2% Sarkosyl for 16 h at 23,000 rpm in the SW25.1 rotor at 4 C. Fractions were collected through the bottom and assayed for radioactivity by scintillation. Symbols:* O, *counts per minute of $^3H$-thymidine;* ●, *counts per minute of $^{14}C$-thymidine.*

(Fig. 1B) was precipitated by trichloroacetic acid to obtain a more accurate estimate of the $^{14}C$ content, as the gradient samples had nearly background levels of $^{14}C$. This sample contained 29,014 counts/min of $^3H$ and 19 counts/min of $^{14}C$ with a $^3H/^{14}C$ ratio of 1,527. Because the $^3H$-labeled DNA is of a much higher specific

activity (at least 16- to 18-fold) the 17S peak may be contaminated on a weight basis with a substantial amount of host cell DNA. Only 0.3% of the total $^{14}$C-prelabeled DNA remained in the pooled 17S DNA fractions. Its estimated $s_{20,w}$ value is 16.5S, which indicates a molecular weight of $3.7 \times 10^6$ if the DNA is double stranded (3, 30). Thus, this DNA, if it has a duplex structure, has a molecular weight near twice that estimated for the single-stranded H-1 DNA ($1.7 \times 10^6$). Extraction of H-1 infected cells by the method of Hirt yields a similar 17S DNA in sucrose gradient analysis. This method may be expected to fail to extract membrane-bound viral DNA, as it is very similar in principle to the detergent precipitation method of Tremblay et al. (34) and SDS modifications of it described by Ormerod and Lehman (15). These methods preferentially isolate "membrane-bound DNA" and nascent DNA by virtue of their association with precipitated detergent. For these and other reasons, not discussed here, the CPG method was not abandoned in favor of the Hirt method, which is technically simpler.

It should be noted that incorporation of $^3$H-thymidine into a DNA species sedimenting as progeny DNA is inconspicuous or absent in these preparations and this will be examined later.

The 17S DNA extracted from infected cells is identified below as a viral RF by DNA-DNA hybridization and for simplicity will be referred to hereafter as RF DNA. The RF DNA was analyzed by chromatography on hydroxylapatite to determine its strandedness. A batch method described by Fanshier et al. (9) was used. $^3$H-thymidine-labeled hamster embryo DNAs, both native and heat-denatured, were used as controls. The results were: 98% of the native hamster embryo double-stranded DNA eluted in the last fraction with 0.4 M $PO_4$, and after heat denaturation, 86% of the DNA eluted in the preceding eluant with 0.2 M $PO_4$; the RF DNA fraction eluted as double-stranded DNA (89% at 0.4 M $PO_4$).

**Nature of 17S virion DNA.** Since hydroxylapatite chromatography would be expected to retain single-stranded molecules with portions containing duplex structure during elution of single-stranded DNA, competitive hybridization studies with unlabeled H-1 DNA were conducted by analyzing the product with a nuclease selective for single-stranded DNA. Before conducting these experiments a prior consideration was the possibility of the presence of virions containing DNA strands complementary to the major viral strand, which would complicate the competitive hybridization. Adenoassociated virus consists of nearly equal portions of both strands (20).

The DNA of rat virus (RV) was reported to yield a minor 17.5S peak in velocity sedimentation by May and May (13). To demonstrate the possible existence of virions containing complementary strands, virion DNA labeled with $^3$H-thymidine was analyzed in high salt sucrose gradients after lysis of virions at 100 C for 90 s followed by quick chilling (Fig. 2A) or by an incubation period conducive to DNA-DNA hybridization, 65 C for 60 min (Fig. 2B). The viral DNA which was extracted under minimal renaturing conditions showed mainly 27S DNA. The virion DNA subjected to annealing conditions shows a significant increase in material sedimenting at 17S, from 27 to 57%. A smaller increase (28 to 37%) occurred after incubation in 50% formamide for 4 h at 40 C. To distinguish between duplex formation and strand breakage of single-stranded 27S DNA samples of the 27 and 17S virion, DNA peaks were annealed for 60 min at 65 C in 0.8 M NaCl, and were analyzed for

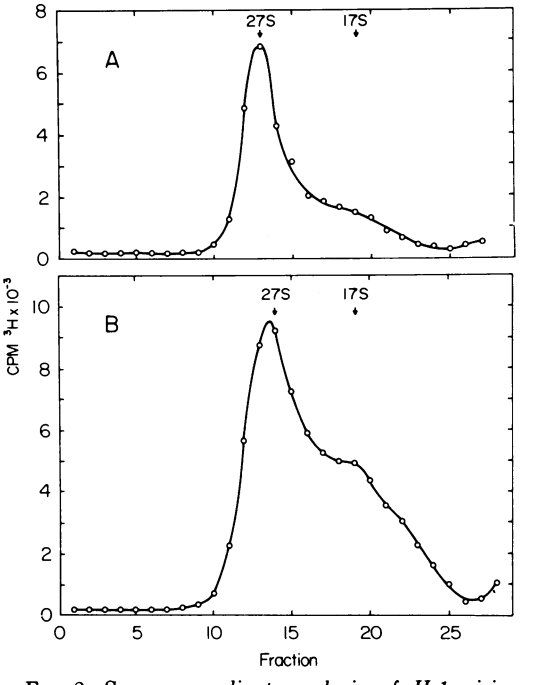


FIG. 2. *Sucrose gradient analysis of H-1 virion DNA. Purified H-1 virus propagated on hamster embryo cells and labeled with $^3$H-thymidine and lysed by heating to 100 C for 1 min in 50 mM Tris, pH 8.0, 0.5% Sarkosyl, 0.1 M NaCl, $10^{-3}$ M EDTA. After quenching in ice (A) or heating to 65 C for 60 min (B) they were sedimented in sucrose as in Fig. 1.*

single-stranded DNA by digestion with micrococcal nuclease both with and without reheating at 100 C for 90 s and by chilling in ice. Both 17*S* virion DNA fractions showed similar rates of digestion by the nuclease and were the same as the rate for the annealed 27*S* DNA. The reheated 27*S* DNA was digested slightly more rapidly. Both 27 and 17*S* DNA had a portion equal to 8% of the total that was not solubilized further after 6 to 26 h of incubation. Thus, these data indicate that the 17*S* DNA fraction is largely single stranded and does not result from virion DNA being annealed to its complementary strand. It is most likely that the 17*S* DNA generated from virion DNA is the result of strand breakage.

**Characterization of H-1 RF DNA. (i) Separation of complementary strands.** If virion populations do contain a minor portion with DNA complementary to the major species, a direct demonstration of this would be possible by separation of the viral strand from its complementary strand in equilibrium density centrifugation. When BUdR is substituted for thymidine by propagation of virus in the presence of BUdR, as described by Berns and Rose for adenoassociated virus (2), the viral strand becomes denser than its complement because of its higher thymidine content. H-1 DNA was reported by McGeoch et al. to contain 29.3% thymidine and 25.5% adenine (14), and we have obtained an average value of 32% thymidine, 25% adenine for H-1 DNA sedimenting at 27*S* in sucrose gradients (J. R. Kongsvik, J. F. Gierthy, and S. L. Rhode, unpublished results).

Virus labeled with $^{32}P$ and containing BUdR was prepared as described by Berns and Rose (2) and was purified as previously described (18). The final step of the purification was equilibirium density centrifugation after which the CsCl gradient was fractionated and the $^{32}P$ activities were determined (Fig. 3). Two forms of H-1 were found, one at a density of 1.425 and one at 1.475. These two density forms of complete H-1 virions are usually found for thymidine-containing virus at 1.410 and 1.465. Two similar forms of DNA containing RV and minute virus of mice (MVM) have been previously reported (5, 13, 19). Virus containing $^{32}P$-BUdR DNA from the fraction of 1.425 and 1.475 and $^{3}H$-BUdR RF DNA (see legend Fig. 4A, B) were heated at 100 C for 3 min in 1 mM Tris, pH 8.0, 0.005 M NaCl, 1 mM EDTA, and 0.1% Sarkosyl, and were chilled in ice. The samples were diluted with the same buffer at 0 C to a volume of 1.29 ml and mixed with 1.89 ml of saturated $Cs_2SO_4$, also at 0 C. The samples were subjected to equilibrium density centrifugation at 4 C in the SW50 rotor, and fractions were collected and assayed for radioactivity. The results are illustrated in Fig. 4A and B. The $^{32}P$-BUdR viral DNA from virus of both densities (1.425 and 1.475; not shown) banded at a density of 1.530, 37% of the $^{3}H$-BUdR RF banded at the same density as the virion DNA, and another two fractions, 31% and 21% of the total, were found at a density of 1.505 and 1.474, respectively. No evidence of this light DNA was found in the $^{32}P$-BUdR virion DNA. To obtain greater resolution of the fractions obtained with the BUdR RF DNA, it was subjected to equilibrium centrifugation in $Cs_2SO_4$ in the type 40 fixed angle rotor. A portion was heat denatured, chilled, and diluted with buffer and $Cs_2SO_4$ to a final density of 1.50 and an unheated sample was similarly prepared. The results of this analysis are illustrated in Fig. 4C and D. The native BUdR RF DNA described above (Fig. 4) yielded two fractions at 1.472 and 1.444. Denaturation of RF DNA produced two additional peaks and a reduced amount of the two original moieties. The heaviest banded at a density of 1.530 as did virion DNA, and the lightest banded at 1.505. Competitive hybridization studies reported in the next section confirm the presence of virion DNA in the RF DNA, so that the DNA of 1.530 is the viral strand. If the material released from the RF DNA at 1.505 is complementary to the viral strand, then reannealing would result in reformation of duplex DNA with the density of the native BUdR RF DNA. Samples of the

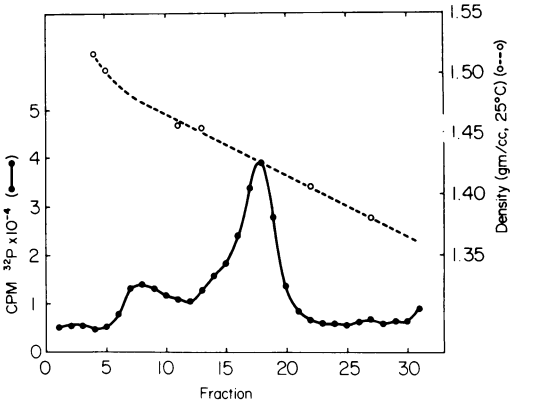


FIG. 3. *Equilibrium density gradient centrifugation of H-1 virions containing $^{32}P$-BUdR-labeled DNA. Virus was purified as previously described (18). The CsCl gradient was fractionated into scintillation vials, radioactivity was assayed by measuring Cerenkov activity, and densities were determined by refractive index. The fractions of virus at 1.475 and 1.425 were pooled and dialyzed against water and stored at −20 C.*

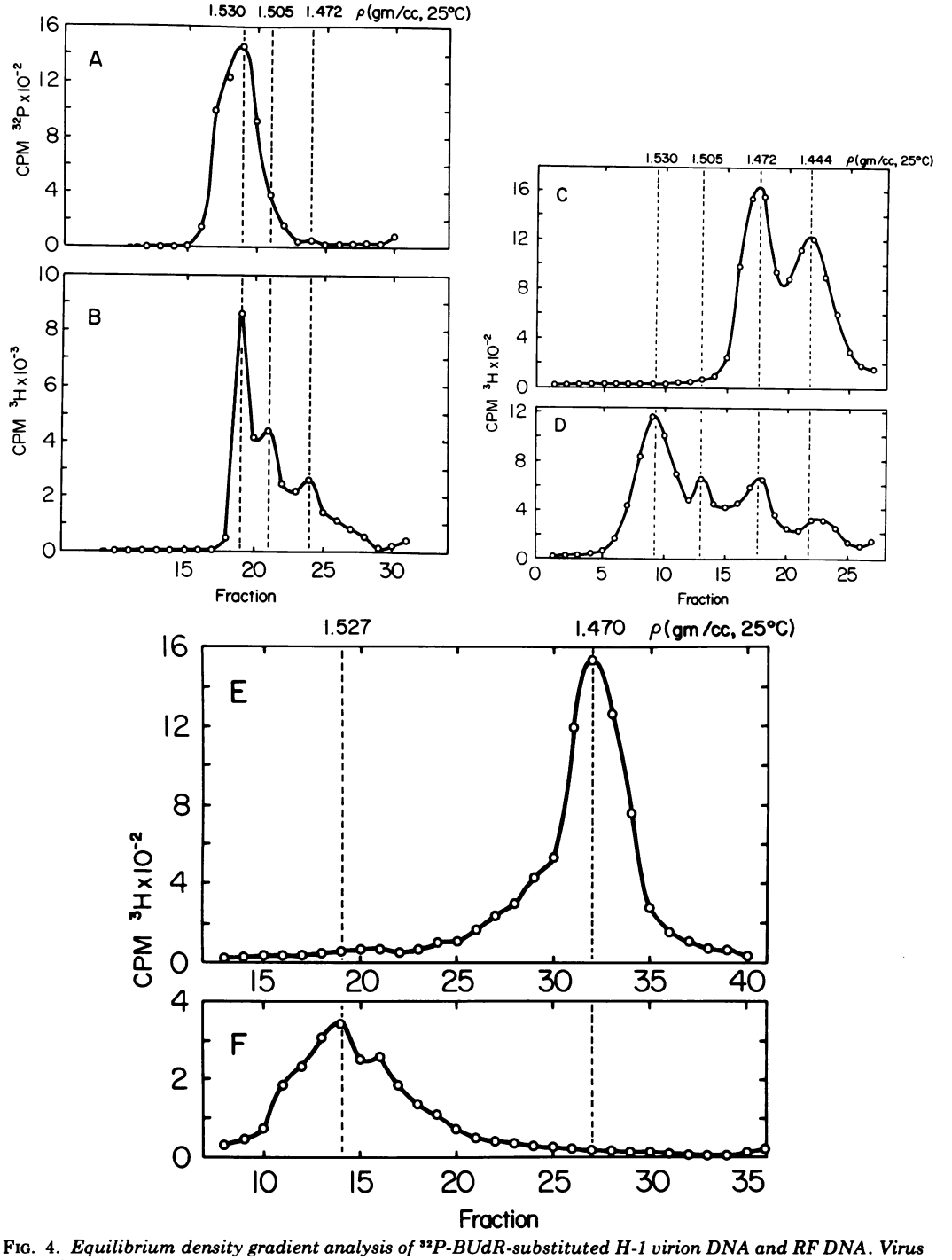


FIG. 4. *Equilibrium density gradient analysis of* $^{32}$*P-BUdR-substituted H-1 virion DNA and RF DNA. Virus was lysed and centrifuged to equilibrium in* $Cs_2SO_4$ *as described in the text (A). Samples were assayed for radioactivity on filter papers and densities were determined.* $^{3}$*H-BUdR RF DNA was isolated by the Hirt method after incubating infected cells with medium containing FUdR (0.5 μg/ml) and BUdR (10 μg/ml) 14 to 16 h PI and then with medium FUdR and* $^{3}$*H-BUdR (10 μCi/ml) 16 to 18 h PI. RF DNA labeled with* $^{3}$*H-BUdR was similarly analyzed after heat denaturation as described in the text (B). Centrifugation was 35,000 rpm for 60 h in the SW50 rotor at 4 C.* $^{3}$*H-BUdR RF DNA was heat denatured (D) as in B or untreated (C) and centrifuged to equilibirium in a type 40 fixed-angle rotor. The gradients were fractionated as previously described. Centrifugation was 35,000 rpm for 48 h at 4 C. Pooled fractions 9 to 11 (D) and the peak tube, fraction 13, were used for further analysis. A sample of fractions 9 to 11 was mixed with a sample of fraction 13 in a 5:4 proportion (E) and fractions 9 to 11 alone (F) were annealed 24 h at 65 C in 1 M NaCl as in the text. The samples were then diluted and rebanded in* $Cs_2SO_4$ *as above. Centrifugation was 35,000 rpm for 48 h at 4 C in the type 40 rotor.*

fraction at 1.530 and 1.505 were mixed in the ratio 5:4 by $^3H$ content to approximate equal amounts of each strand diluted 1:5 with 10 mM Tris, pH 8.0, $10^{-3}$ M EDTA, 1 M NaCl, and 0.2% Sarkosyl, to a final volume of 0.5 ml. The samples were incubated at 65 C for 24 h. They were then chilled, brought to a final density of 1.50, and rebanded (Fig. 4E). The DNA banded at 1.470, somewhat less than the density of the heavy form of the native RF DNA (1.472). A portion of the 1.530 DNA only was similarly treated and it rebanded at 1.527, a little less than its original density (Fig. 4F). Using this DNA as a marker for the previous centrifugation conditions, the density of the annealed mixture corrected (to 1.472) that of the heavy RF DNA. These results are consistent with the 1.530 and 1.505 strands of the RF DNA being the viral and complementary strands, respectively. RF DNA labeled with $^3H$-thymidine was subjected to equilibrium centrifugation in the presence of a sample of $^3H$-BUdR RF DNA of density 1.472 to determine the density of unsubstituted RF DNA in $Cs_2SO_4$. It banded at 1.423; thus, it can be estimated that the thymidine of the DNA at 1.472 was substituted 80% with BUdR and the DNA of intermediate or hybrid density at 1.444 substituted 37.5% (1). The final yield of isotope in the virion strand released by heat denaturation of RF DNA was 50% of the total and 35% of the total rebanded at the densities of native RF, probably due to reannealing during the centrifugation (Fig. 4D). Thus, both density forms of RF must have contained the viral strand substituted with $^3H$-BUdR.

**(ii) RF DNA contains viral DNA.** To establish the presence of viral DNA in the putative RF DNA, competitive hybridization in the presence of varying amounts of unlabeled virion DNA was carried out. When unlabeled viral strands are in excess, $^3H$-labeled viral strands present in the RF DNA prepared by CPG extraction will be prevented from annealing to their complementary strands. The results are presented in Fig. 5. With increasing amounts of unlabeled virion DNA, an increasing fraction of $^3H$-labeled RF DNA is made susceptible to nuclease digestion and is thus single-stranded DNA. The addition of virion DNA after the period of annealing did not render the annealed RF DNA susceptible to nuclease digestion. It is thus confirmed that the 17*S* DNA extracted from infected cells is a double-stranded DNA containing the viral strand. The 17*S* DNA, extracted according to the method of Hirt (11), was shown to contain viral DNA in the same manner. This DNA incorporates labeled precursors into both strands and is therefore an RF DNA. The competition approaches saturation at about 40% of the radioactivity. The previous results with strand separation by equilibrium density centrifugation would indicate that greater than 50% of the radioactivity would be in the viral strand, but the labeling period used to prepare the RF DNA used there began 20 h PI, later than the introduction of BUdR in the previously cited experiment, so that this difference might account for the discrepancy.

**Density and composition of H-1 RF DNA.** H-1 RF was obtained by preparative sucrose gradient centrifugation and banded to equilibrium in neutral CsCl and in ethidium bromide CsCl gradients (17). The results are illustrated in Fig. 6.

In neutral CsCl a buoyant density of 1.705 was obtained. Using the standard equation, this would indicate a GC content of 45.9% (24). The expected GC content of H-1 RF based on a molar content of G and C of 22.6 and 22.6%, respectively, as reported by McGeoch et al. would be 45.2% (5). The buoyant density in ethidium bromide CsCl was 1.545, and only a small portion (fraction 13) might be in a closed circular configuration. The shoulder on the denser scale of the peak in Fig. 6B was not present on a similar ethidium bromide CsCl gradient of this DNA in the type 40 fixed angle rotor.

**Rapidly labeled viral DNA species.** Newly synthesized viral DNA was examined by label-

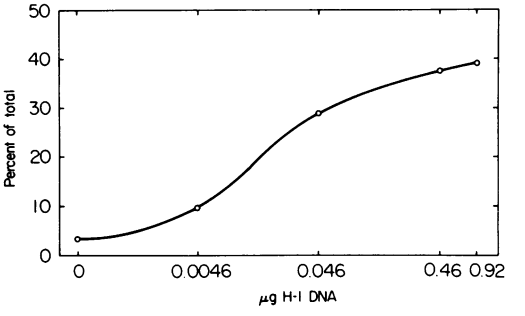


FIG. 5. *Competitive hybridization of 17S DNA (RF DNA) and H-1 viral DNA. 17S DNA was labeled with $^3H$-thymidine and prepared as in Fig. 2B. The labeled DNA was heat denatured at 100 C for 90 s in 0.01 M $Na_2HPO_4$, pH 7.0, quenched in ice, and samples were mixed with varying amounts of unlabeled H-1 viral DNA. The samples were brought to 0.8 M NaCl and incubated for 16 h at 65 C. The samples were then digested with micrococcal nuclease. A control sample had 0.92 μg of H-1 DNA added after annealing and before digestion. The points represent the percentage of total counts per minute remaining acid-insoluble after 4 h of digestion with nuclease.*

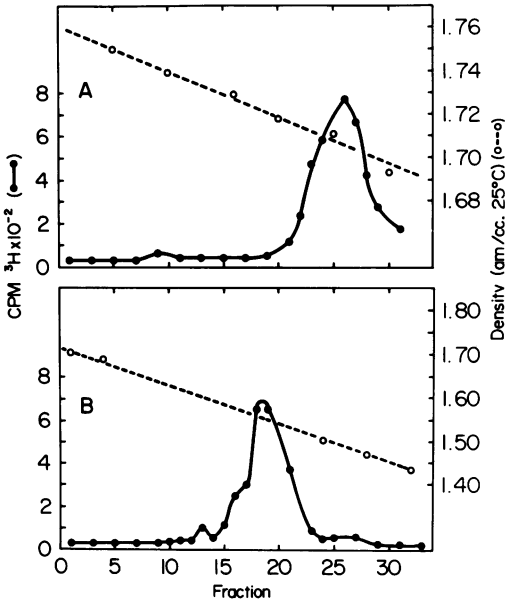


FIG. 6. *Equilibrium density centrifugation of H-1 RF. RF DNA purified by sucrose gradient and labeled with ³H-thymidine was sedimented to equilibrium in neutral CsCl (A) and in an ethidium bromide CsCl gradient (B) as described (17). Centrifugations were (A) 60 h at 33,000 rpm in the SW50 rotor and (B) 24 h at 43,000 rpm in the SW50 rotor.*

ing infected cultures for short periods with ³H-thymidine and analyzing the DNA as before. The result of such an experiment is illustrated in Fig. 7. Parasynchronous cultures of HE cells prelabeled with ¹⁴C-thymidine were labeled for 5 min at 25 C with ³H-thymidine (20 μCi/ml). DNA was extracted by CPG chromatography without a 2 M NaCl elution, Pronase digested and collected by precipitation with ethanol, and then analyzed on a high salt sucrose gradient. Examination of the ³H/¹⁴C ratios and comparison with results obtained with a labeling time of 75 min (c.f. Fig. 1) reveals a relative increase in viral DNA sedimenting more rapidly than 17*S* RF DNA. The ³H/¹⁴C ratio for the DNA retained by the column was 0.57, which was about that for the fast-sedimenting DNA at the bottom of the gradient. The rapidly labeled replicative intermediates of viral DNA are under study.

**Does RF DNA exist in vivo.** The possibility must be entertained that the RF DNA described here results from annealing of complementary strands during the extraction procedure. If this occurs, the presence of exogenous viral strand DNA added at the time of the cell lysis would result in the incorporation of a portion of these sequences into RF DNA, and some of the endogenous viral strand DNA would remain single stranded. This was tested by lysing H-1 infected cells, labeled 14 to 16 h PI with FUdR, 0.5 μg/ml, and ¹⁴C-thymidine ($1.5 \times 10^{-5}$ M, specific activity 40 mCi/mmol) with the SDS buffer containing 47,500 counts/min of lysed ³H-thymidine-labeled H-1 virus (100 C for 5 min). The Hirt extraction was then carried out and the DNA was sedimented in a high salt sucrose gradient. For a control, a second culture was similarly extracted except that the ³H-H-1 DNA was added to the Hirt supernatant just prior to centrifugation. Sixty percent of the ³H-H-1 DNA remained in the Hirt pellet, and there was full recovery of the supernatant ³H from the sucrose gradients. The sedimentation pattern of the experimental and control were similar with 72 and 76% of the ³H sedimenting at 27*S*, and 28 and 24% trailing through the 17*S* region, respectively. The specific activity of the ³H-H-1 DNA was 25 times that of the ¹⁴C RF DNA, and based on the counts recovered, the ¹⁴C-RF DNA was present in 12-fold excess over the ³H-H-1 DNA. Therefore, if the 17*S* DNA was a result of annealing, the ³H-DNA would have been quantitatively converted to 17*S*. Only a 4% shift to 17*S* was found. The ¹⁴C recovered as RF DNA represented 25% of the total incorporation, so that figure represents a minimum efficiency of the extraction. As further confirmation that the ³H-DNA was not incorporated into hybrids, the 27 and 17*S*

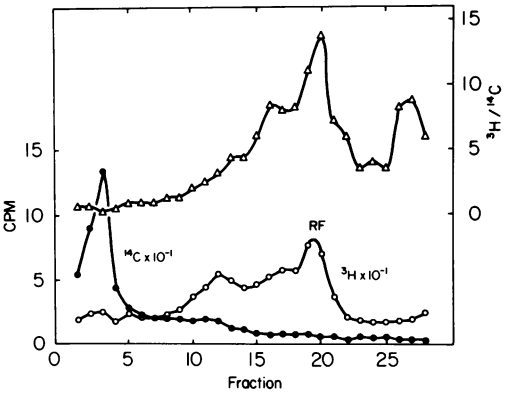


FIG. 7. *Sucrose gradient analysis of rapidly labeled DNA. Parasynchronous cultures prelabeled with ¹⁴C-thymidine were infected with H-1 and pulsed with ³H-thymidine for 5 min at room temperature at 16 h PI. A CPG extract was analyzed on a 5 to 20% high salt sucrose gradient as in Fig. 1. Symbols: O, counts per minute of ³H-thymidine; ●, counts per minute of ¹⁴C-thymidine; Δ, ³H/¹⁴C ratio. Sedimentation was for 16 h at 23,000 rpm in the SW25.1 rotor.*

pooled fractions of both gradients were digested by single-strand-specific nuclease. After 4 h at 0 C the 27 and 17*S* $^3$H were solubilized 81 and 77%, respectively, in the experimental group and 85 and 79%, respectively, in the control gradient. Thus, the slight increase in resistance to nuclease digestion in the 17*S* $^3$H-DNA cannot be attributed to annealing during the extraction. The $^{14}$C-RF DNA was resistant to digestion.

## DISCUSSION

It is virtually axiomatic in molecular genetics that genetic information is transferred and replicated by template-directed polynucleotide synthesis. Therefore, the replication process of the parvovirus H-1, which contains a genome of single-stranded DNA, would be expected to include a step involving the synthesis of a DNA strand complementary to the viral strand. The resulting duplex DNA has been termed the parental replicative form in the case of the single-stranded DNA bacteriophages (16). In this study I have described the synthesis of a double-stranded DNA in H-1 infected cells with a molecular weight of about $3.7 \times 10^6$, about twice that of the virion of a single-stranded DNA. This DNA was shown to contain viral DNA in one strand as demonstrated by competitive hybridization. The competitive hybridization approached a saturation value of 40 to 45% of the $^3$H activity rendered susceptible to single-strand-specific digestion by micrococcal nuclease. Approximately 8 to 10% of viral strand DNA is resistant to digestion under the conditions used, so the saturation value can be adjusted to 45 to 50%. The results with strand separation discussed below indicated a percentage of $^3$H-BUdR in the viral strand of 72% in a different preparation of RF DNA, so that the saturation value of 50% may be a low estimate of the labeled viral strand content of the RF DNA. The RF DNA was not found to be a closed circular form by ethidium bromide density centrifugation. It has a density of 1.705, indicating a GC content of 45.9% as would be expected for an RF DNA of H-1 (14). The incorporation of $^3$H-BUdR into the viral complementary strand in the RF DNA indicates that this DNA is undergoing replication at the time of the pulse. Replication of RF DNA was occurring at 16 to 18 h PI in parasynchronous infection, and RF DNA was labeled during intervals ranging from 6 to 8 h PI to 22 to 24 h PI (unpublished data). This is in agreement with the previous suggestion that the DNA synthetic event upon which subsequent viral protein synthesis was dependent (i.e., HA-DNA synthesis) and which initiates only in late S phase of the infected cell, about 8 h PI in this cell system, may be the synthesis of parental RF. However, the RF DNA examined in this study is largely or entirely progeny RF. Analysis of the DNA in equilibrium gradients showed two density forms containing viral DNA. Their differences in density can be simply explained if the heavy form represents a duplex of heavy-heavy (H-H) DNA and the intermediate form heavy-light (H-L) DNA with the $^3$H-BUdR in the viral strand. Reannealing of both heavy strands produced the predicted duplex DNA with the density of H-H (Fig. 4E). This interpretation has been further confirmed by density gradient centrifugation of denatured H-H and H-L RF DNA to be described in a subsequent report of this series. This result would be the expected consequence of the conservation of thymidine containing viral complementary strand DNA in the RF pool, which had been synthesized before the addition of BUdR at 14 h PI. The H-H DNA would arise in the second generation of RF undergoing semiconservative replication or from progeny strand synthesis on L-H RF DNA in the presence of BUdR and the H-L DNA from either replication of the RF or synthesis of progeny viral strand DNA. Conservation of viral strand DNA in the RF pool would result in equal amounts of L-H and H-L in the hybrid density RF DNA and thus equal amounts of heavy $^3$H-BUdR-labeled viral and viral complementary strands in the RF pool. This was not the case, for less $^3$H was recovered in the complementary strand than in the viral strand, 28% versus 73%. It was previously shown that infectious virus production began as early as 10 h PI, so that progeny strand synthesis can be presumed to occur at the time of density labeling used here and result in preferential loss of light viral-strand DNA from the RF pool. Velocity sedimentation analysis of viral DNA after short pulses of $^3$H-thymidine yielded an enrichment of forms sedimenting more rapidly than the 17*S* RF DNA.

The factors controlling the synthesis of the parental RF and its possible requirement for a late S phase function are unknown. It is likely that synthesis of parental RF is required before transcription of viral DNA can occur and synthesis of the mRNA for viral hemagglutin was previously shown to follow closely the synthesis of HA-DNA (18). The specific S phase dependence of viral DNA synthesis suggests that a homology between viral DNA and host cell DNA may exist and results in a coordination of the initiation of viral DNA synthesis with certain replicons of the host cell. During the

preparation of this manuscript a report appeared describing the isolation of RF DNA for MVM virus and evidence for homology between MVM and L-cell DNA (6). Experiments are in progress to determine if H-1 DNA has homology to late replicating hamster embryo DNA.

A recent report by Salzman and White described the formation of a duplex DNA of RV within 60 min PI in asynchronous rat nephroma cells (22). In our hands, H-1 preparations have particle/infectivity ratios in excess of $10^3$ so that any chase experiment with prelabeled virus would be subject to high backgrounds of noninfectious virions. I have reported the formation at high ionic strength of a 17*S* species of viral DNA. Further analysis indicated that H-1 virions propagated in hamster embryo cells do not contain DNA complementary to the major viral DNA species as described for adenoassociated virus (2). Incubation of viral DNA under conditions favorable for DNA-DNA hybridization increased the yield of 17*S* DNA, but caused little increase in the amount of double-stranded DNA as assayed by nuclease digestion. It thus appears that virion DNA undergoes strand breakage on incubation at 65 C in high salt or at 40 C in 50% formamide (unpublished data). The DNA of H-1 may contain some self-complementary sequences containing at least 8% of the total thymidine. Self-complementary sequences have been reported for AAV (12), MVM (P. Tattersal, Fed. Proc. **31**:913, 1972) and the single-stranded DNA bacteriophage, f1 (25, 26). The possibilities of various configurational forms of virion single-stranded DNAs and the presence of virions containing both strands of DNA must be considered in any analysis of Parvovirus RF DNA. No evidence of annealing of complementary strands to form the 17*S* RF DNA during the Hirt extraction was obtained in the reconstruction experiment.

The two most likely hypotheses that account for the coordination of viral DNA synthesis with late S phase are: (i) parental RF synthesis occurs only in late S phase; or (ii) parental RF is synthesized earlier and subsequent transcription requires a late S phase function. In either case, the replication of the Parvovirus H-1 provides an interesting probe of host cell regulation of DNA replication. Further analysis of the synthesis of viral HF DNA and especially the synthesis of viral complementary strands may resolve between these alternatives.

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