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Replication Process of the Parvovirus H-1

V. Isolation and Characterization of Temperature-Sensitive H-1 Mutants Defective in Progeny DNA Synthesis

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Two temperature-sensitive mutants of the parvovirus H-1 have been isolated and characterized. These mutants are distinguishable by the immunofluorescent staining of cells infected by them and by the thermolability of their hemagglutinins. Under restrictive conditions both mutants synthesize a missense capsid protein defective in hemagglutination. Synthesis of the viral DNA strand was shown to be diminished for both wild-type virus and these mutants at the restrictive temperature of 39.5 C, but the mutants were more defective than the wild type at the less restrictive temperature of 38 C. Replicative form DNA replication was not decreased in these mutants. It is proposed that H-1 requires one of the capsid proteins for the synthesis of the single-stranded progeny DNA.

Replication of the single-stranded DNA parvovirus H-1 involves the synthesis and replication of a double-stranded replicative form (RF) DNA (8). Progeny viral strand (V) DNA is then synthesized with the complementary strand (C) DNA in the RF as template (8, 10).

Viral hemagglutinin, antigen, and the viral proteins VP1 and VP2 have been shown to depend on prior DNA synthesis, termed hemagglutinating (HA)-DNA synthesis, an event that takes place at a specific time near the end of S phase of the infected cell (5, 9). Viral protein synthesis and the initiation of RF DNA replication occur concomitantly shortly after HA-DNA synthesis (10). Although the initiation of RF DNA replication was shown to require protein synthesis, it was not determined if viral protein synthesis was required for RF DNA replication or for progeny DNA synthesis (10). The present study describes the isolation and characterization of two temperature-sensitive (ts) mutants of H-1. The mutations occur in a capsid protein, a protein which is required for progeny DNA synthesis but not RF DNA replication.

MATERIALS AND METHODS

Cell culture. The cells used in this study are the SV40 transformed human newborn kidney (NB) cells routinely used for the plaque assay of H-1 or hamster embryo cells as previously described (6, 9). The cells were cultivated in Eagle minimum essential medium (Flow Laboratories, Autopow minimum essential medium Eagle, modified) supplemented with 10% heat-inactivated calf serum, glutamine, penicillin (10 μg/ml), streptomycin (5 μg/ml), and sodium bicarbonate to pH 7.6. Parasynchronous cultures, used for some purposes, were prepared by incubation in culture medium MAG containing methotrexate (0.5 μg/ml), adenine (13 μg/ml), and Eagle minimum essential medium nonessential amino acids for 16 to 20 h at 37 C. The methotrexate block was reversed by removing the medium, washing once with Hanks balanced salt solution, and refeeding with culture medium containing thymidine (TdR) at 10^{-5} M.

Virus. Wild-type H-1 virus was propagated in hamster embryo cells and purified as previously described (9).

Infection of cells. NB cultures were infected by inoculating the monolayer with 0.1 or 0.2 ml of virus stock per 60-mm petri dish and adsorbing it at 33 C for 30 min. The cultures were washed twice with Hanks balanced salt solution, medium was added, and the cultures were incubated at the desired temperature.

Plaque assay. The plaque method of virus assay has been described (6). This method was modified in the length of incubation before the neutral red overlay, both at the permissive temperature (33 C) and nonpermissive temperature (39.5 C) used here to define the conditional mutants of H-1. The overlay with neutral red was made on day 9 for the assay at 33 C and on day 6 for the assay at 39 C. Plaques were counted 16 to 24 h after the overlay with neutral red. Wild-type virus usually titered as efficiently at 39.5 C as at 33 C, but modest reductions in titer ranging as high as 75% of the titer at 33 C were common.

Mutagenesis. H-1 virus (4×10^9 PFU/ml), purified as previously described (9), was treated with 0.25 M hydroxylamine as outlined by Tessman for bacteriophage S13 (11). The reaction mixture contained one part H-1 in H_2O, two parts 0.1 M PO_4 (pH 6.0), and one part 1 M hydroxylamine (made pH 6.0 with NaOH) plus 10^{-3} M EDTA. The reaction was stopped

by diluting an aliquot 100-fold in regular medium with 10% fetal calf serum, 10% tryptose phosphate broth, and 2% acetone at 4 C. In the absence of hydroxylamine, H-1 infectivity decreased only 20% after 4 h at 37 C. Hydroxylamine inactivated H-1 infectivity exponentially at a rate of 10^{-1}/h of treatment to a final surviving fraction of 10^{-6}, the last tested.

Isolation of ts mutants. A preparation of virus inactivated to a surviving fraction of 10^{-1} was used as the source of mutagenized virus. This virus preparation was propagated in hamster embryo cells infected at a multiplicity of infection of 0.1 for 48 h at 33 C to dilute nonconditional mutants. With this mutagenized virus stock, diluted to give approximately 1 to 5 plaques per 60-mm petri dish, individual plaques were isolated from assays at 33 C with a Pasteur pipette. The plaque isolate was stored in 0.5 ml of *N* - 2 - hydroxyethylpiperazine - *N'* - 2 - ethanesulfonic acid-buffered Eagle minimal essential medium and subsequently propagated in NB cells in a 35-mm petri dish or in a microtiter plate for 5 days at 33 C. The virus and cells were then harvested and diluted to a final volume of 3 ml in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-buffered medium. Virus was released from cells by freezing and thawing three times. Aliquots of 0.2 ml of the isolates were inoculated on duplicate plates and assayed at the permissive and nonpermissive temperatures for plaque production. Isolates which showed a 100-fold or greater titer at 33 C than at 39.5 C were selected for further study. Prepared in this manner, wild-type isolates generally produced 20 to 100 PFU at 39.5 C and 40 or more at 33 C with undiluted inocula. Isolates appearing to be temperature sensitive by the screening test were assayed again at the two temperatures and in appropriate dilutions for quantitative determination of titers. In some cases, ts mutants were repurified by one or more plaque purifications. Mutants were propagated in NB cells at 33 C once or twice to produce virus stocks with titers of 10^7 or more at the permissive temperature.

RESULTS

Replication of wild-type H-1 at 33 and 39.5 C. The kinetics of hemagglutinin and infectious virus production in parasynchronous NB cells for wild-type H-1 at 33 and 39.5 C were examined. The growth curves for HA synthesis and infectious virus production were similar at the two temperatures (Fig. 1, Table 1). If the time axis for HA synthesis at 33 C is transformed by a factor of 0.5, the curves are nearly identical. The yield of infectious virus was reduced about 50% at 39.5 C compared to 33 C.

Isolation of H-1 mutants ts1 and ts2. Purified wild-type H-1 was mutagenized with hydroxylamine to a survival of 10^{-1}, propagated once at low multiplicity in NB cells at 33 C, and plaques were isolated and screened for temperature sensitivity as described above. Isolates 190 and 389, subsequently termed ts1 and ts2, respectively, produced higher titers at the permissive than the nonpermissive temperatures, as reported in Table 2. Subsequent passages of the virus stock maintained this difference. The ratio of titer at 33 C to that at 39.5 C did decline with some propagations at 33 C, indicating a possible selective disadvantage for the mutant virus. It has not been determined as yet if the plaques appearing at 39.5 C are produced by

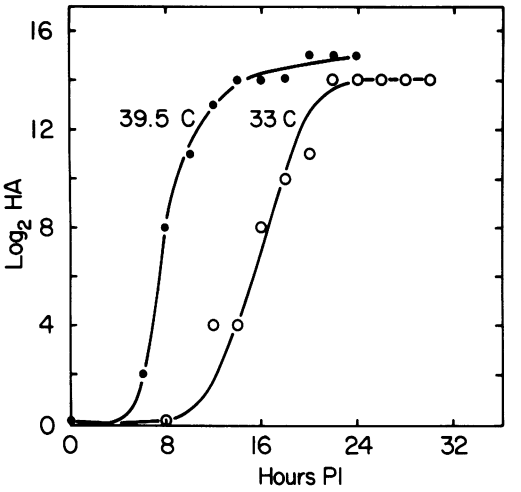

FIG. 1. *H-1 HA synthesis in parasynchronous NB cultures infected with wild-type H-1 at a multiplicity of infection of 5 to 10 and incubated at 39.5 or 33 C from 1 h p.i. to the time of harvest. Cultures were harvested with their medium at various times p.i. and the HA titers were determined in the usual manner with guinea pig erythrocytes.*

TABLE 1. *Replication of H-1 at the restrictive and permissive temperatures*[a]

Time (h p.i.)	PFU/culture	
	33 C	39.5 C
½	6.6×10^6	6.6×10^6
6	2.6×10^6	1.6×10^6
12	2.4×10^6	2.0×10^6
24	1.4×10^7	1.6×10^7
48	5.4×10^7	2.0×10^7
120	5.8×10^7	2.2×10^7

[a] Replicate cultures of NB cells in exponential growth were inoculated with wild-type H-1 at a multiplicity of infection of 5 to 10. After a 30-min adsorption at 33 C, the cultures were washed twice with Hanks balanced salts, and medium was added and incubated at 39.5 or 33 C for the times indicated. Cultures were harvested, subjected to freeze-thawing three times, and H-1 infectivity was determined by plaque assay at 33 C.

TABLE 2. *Isolation of H-1 mutants ts1 and ts2*[a]

Virus	33 C PFU/ml	39.5 C PFU/ml	33 C/39.5 C (PFU)
ts1			
Original—screening	CL	0	
Original	1×10^8	0	
P2		0	
P3	5×10^7	1.7×10^3	2.9×10^4
P4	3.4×10^6	4.2×10^5	8.1×10^2
Plaque purified two times P3	4.1×10^7	1.2×10^4	3.4×10^3
Plaque purified two times P3	3.8×10^7	1.1×10^4	3.5×10^3
ts2			
Original—screening	CL	5	
Original	2.5×10^8	0	
P2	5.3×10^7	9.8×10^2	5.6×10^4
P3	2.5×10^8	8.5×10^3	2.9×10^4
Plaque purified two times P3	3.1×10^7	1×10^4	3.1×10^3

[a] The infectivity of ts1 and ts2 H-1 mutants at the permissive and nonpermissive temperatures at various stages of their propagation are presented. Assays used innocula of 0.2 ml, but titers are given as PFU/milliliter. CL indicates a complete lysis of the cell layer. The screening test is an assay of the plaque isolate undiluted after its first passage. A passage (P) represents a propagation of the virus for 5 days at 33 C in NB cell cultures.

wild-type virus or the mutant, due to a difficulty in recovering virus from these plaques.

Characteristics of the replication of ts1 and ts2. A number of parameters of H-1 replication, including synthesis of HA-DNA, HA, and H-1 antigen, were examined with ts1 and ts2 at the permissive and nonpermissive temperatures. Both ts1 and ts2 were indistinguishable from wild-type H-1 for these parameters at 33 C (Table 3).

At 39.5 C, ts1 and ts2 produced little HA by 16 h postinfection (p.i.) in parasynchronous NB cells (Table 4). When cultures of ts1 and ts2 were shifted down to 33 C for various periods, the HA titer increased at least 16-fold and was maximal in 2 h. The appearance of the HA took place at 33 C, even when DNA or protein synthesis was inhibited with arabinofuranosyl cytosine (araC) (10 μg/ml) or cycloheximide (50 μg/ml), respectively, during the shiftdown and the 15 min preceding the temperature shift. Since cycloheximide probably does not achieve a complete inhibition of protein synthesis and viral protein synthesis accounts for only a small percentage of total cell protein synthesis, it was necessary to confirm that viral HA synthesis is sensitive to inhibition by the drug. To do this, the inhibition of wild-type HA synthesis by cycloheximide (50 μg/ml) at 37 C in the same cell system was compared to that of actinomycin D at 5 μg/ml (1 μg/ml was previously found to be a potent inhibitor of viral HA synthesis [9]). The actinomycin D control was necessary to establish that viral mRNA was present at the time of addition of cycloheximide, thus allowing the inference that any increment in inhibition by cycloheximide compared to actinomycin D was due to an inhibition of translation. It was found that cycloheximide caused immediate inhibition of the accumula-

TABLE 3. *Synthesis of HA-DNA, HA, and FA by H-1 ts mutants*[a]

Virus	HA-DNA		HA		FA	
	33	39.5	33	39.5	33	39.5
Wild type	+	+	+	+	+	+
ts1	+	+	+	−	+	+[a]
ts2	+	+	+	−	+	−

[a] Replicate parasynchronous NB cultures were infected with wild-type H-1, ts1, or ts2 and incubated for 16 h at 39.5 C or 20 h at 33 C. Cultures were harvested for HA determination or fixed for FA staining. HA-DNA synthesis at 39.5 C for ts1 or ts2 was determined by adding araC (10 μg/ml) to cultures at 39.5 C at 16 h p.i. for 15 min and then shifting the cultures to 33 C for 3 h in the presence of the drug. If the HA titer increased to the same degree as a control culture receiving no araC, then HA-DNA synthesis had occurred at 39.5 C preceding the addition of araC.

[b] Nuclei contained FA material in an abnormal pattern as described in Fig. 3.

TABLE 4. *HA synthesis by ts1, ts2, and wild-type H-1*[a]

Time	Log_2 HA titer		
	ts1	ts2	Wild type
1. ½ h p.i.	4	4	4
2. 39.5 C, 16 h p.i.	8	8	15
3. 39.5 C, 16 h p.i. + 1 h, 33 C	12	10	15
4. 39.5 C, 16 h p.i. + 2 h, 33 C	14	12	14
5. 39.5 C, 16 h p.i. + 3 h, 33 C	14	12	14
6. 39.5 C, 16 h p.i. + 3 h, 33 C + araC	14	12	14
7. 39.5 C, 16 h p.i. + 3 h, 33 C + Cx	13	11	12
8. 33 C, 72 h p.i.	16	15	15

[a] Replicate dishes of parasynchronous NB cultures were infected with ts1, ts2, or wild-type H-1 and placed at 33 or 39.5 C. At the times indicated, cultures were harvested for HA determinations or shifted from 39.5 to 33 C for various times before harvest (cultures 3 through 7). Cultures 6 and 7 were treated with araC (10 μg/ml) or cycloheximide (Cx) (50 μg/ml) for 15 min before and during the temperature shift to 33 C. All values are the log_2 HA titer.

tion of HA at either 10.5 or 12 h p.i. On the other hand, the total HA units per culture increased by eight- and fourfold, respectively, by 16 h p.i. in the presence of 5 μg of actinomycin D per ml. Thus HA synthesis was sensitive to inhibition by cycloheximide. This indicates that HA-DNA, viral mRNA, and the hemagglutinin protein had been synthesized before the shift-down with ts1 and ts2. It appears, therefore, that the temperature-sensitive phenotype is the result of an altered polypeptide that does not assume its active structure at 39.5 C.

The stability of the HA to temperature inactivation was determined by using the virus stocks of ts1 and ts2 (produced at 33 C) and wild type at pH 7.2. The results showed a sharp temperature transition for the inactivation of the HA at 72 to 78 C. The temperature completely inactivating ts1 was reduced about 3 to 4 C compared to ts2 and wild-type virus (Fig. 2). The temperature for activation of the HA for the wild-type H-1 agrees with previously published data (2).

To summarize, we have found that the conversion of the mutant viral hemagglutinin proteins to an active form is deficient at 39.5 C. Once the virus is formed, the ts2 HA has a heat stability similar to that of the wild-type virus and ts1 shows a slightly reduced transition temperature.

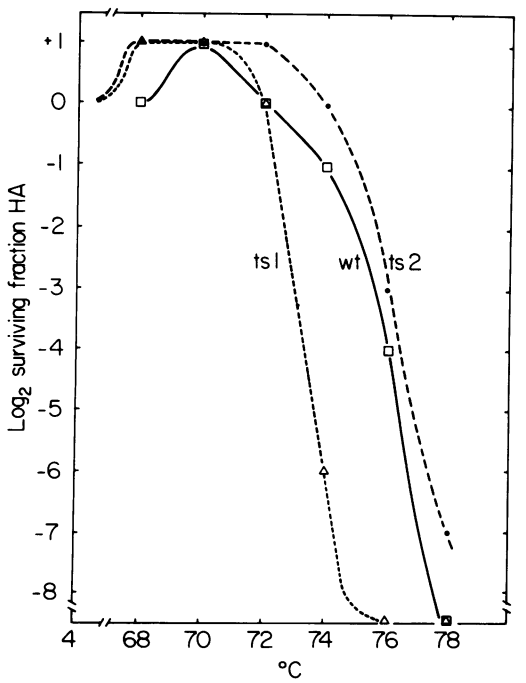

FIG. 2. *Thermal stability of H-1 hemagglutinin for wild type, ts1, and ts2. Aliquots (1 ml) of virus stocks diluted 1:10 in Tris-buffered saline, pH 7.2, were incubated for 20 min at various temperatures, chilled in ice water, and the hemagglutination titers were determined. The values shown are the $\log_2$ of the surviving fraction of the hemagglutinin relative to a control maintained at 0 C.*

Immunofluorescence of ts1- and ts2-infected cells. H-1 antigen synthesis was studied in asynchronous NB cultures incubated at 33 or 39.5 C for 24 h after infection with ts1, ts2, and wild-type H-1. At 39.5 C, ts1 produced strongly positive fluorescent nuclei (fluorescent antibody [FA]) with an abnormal globular pattern of antigen, illustrated in Fig. 3B. Cultures infected with ts2 at 39.5 C produced little or no FA staining (Fig. 3D), as in the uninfected control culture (Fig. 3G). However, infection with ts2 at 33 C resulted in many FA nuclei with the wild-type FA staining pattern (Fig. 3A, C). Cultures infected with ts2 for 24 h and then shifted down to 33 C for 1 h, with and without araC or cycloheximide as above, showed a gradual transition to FA nuclei with a wild-type staining pattern.

Since ts1 and ts2 are distinctly different with regard to FA staining at the nonpermissive temperature and the sensitivity of their HA to heat inactivation, they must contain different mutations.

It should be noted that antisera prepared using guinea pigs with sodium dodecyl sulfate-disrupted purified H-1 as the antigen by the procedure of Johnson et al. (3), as well as the very high-titer antisera obtained from chronically infected hamsters inoculated with sublethal doses of H-1 at birth, produced brightly positive staining of ts2-infected cells incubated at 39.5 C. Thus ts2 capsid proteins have been transported to the nucleus under restrictive conditions. We also observed that nuclei infected with ts1 under restrictive conditions were stained in the pattern of wild-type-infected cells by the sodium dodecyl sulfate-disrupted whole-virus antisera and in the abnormal pattern (Fig. 3B) by antisera to purified VP2 (4). Our anti-VP1 serum was too weak to establish its staining patterns in these experiments. Thus these findings cannot be used to determine the identity of the mutant polypeptide.

Viral DNA synthesis. The effects of the mutations of ts1 and ts2 on viral DNA synthesis were examined. As described above, HA-DNA synthesis occurred under nonpermissive conditions for both mutants. It was of interest to determine if RF DNA replication and progeny DNA synthesis were affected by these mutations.

In the absence of a practical, direct, quantita-

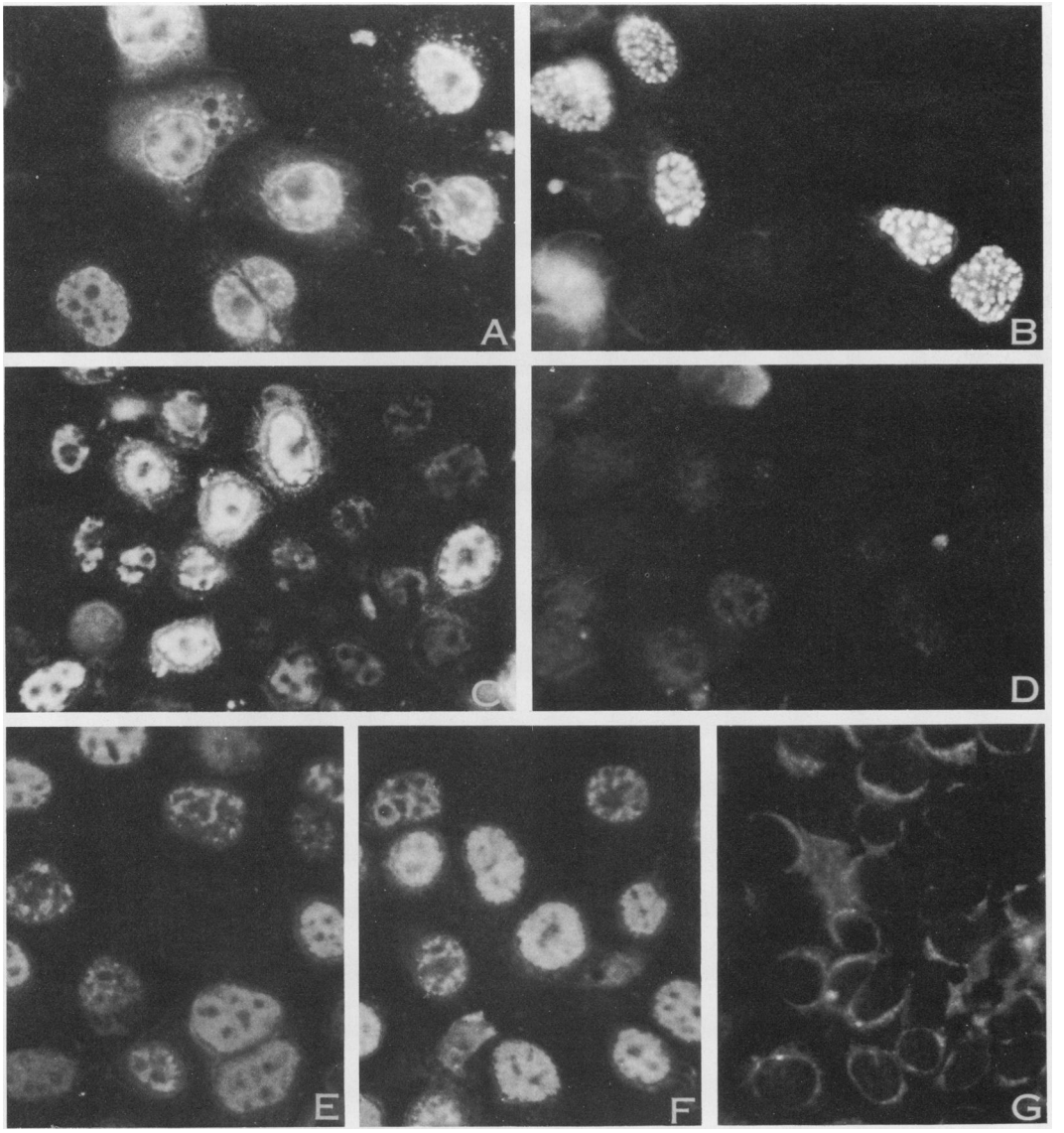

FIG. 3. *Immunofluorescent staining of wild-type H-1 and ts mutant-infected NB cells. NB cells, grown on cover slips, were infected with H-1 in the usual manner and incubated at the restrictive (39.5 C) or permissive temperature (33 C). At the termination of incubation at 24 h p.i., the cover slips were quickly rinsed in Hanks balanced salts and fixed with acetone 15 to 20 min at 4 C. The dried cover slips were stained by the indirect immunofluorescent method using anti-H-1 hamster sera (hemagglutination inhibition titer of 2^{10}) and fluorescein-conjugated rabbit anti-hamster immunoglobin. (A) ts1, 33 C; (B) ts1, 39.5 C; (C) ts2, 33 C; (D) ts2, 39.5 C; (E) wild type, 33 C; (F) wild type, 39.5 C; (G) uninfected, 39.5 C. (Magnification ×460).*

tive measure of viral single-stranded DNA synthesis, the extent of incorporation of [^{3}H]bromodeoxyuridine (BUdR) into the V strand and its C strand was used to estimate the proportion of V strand to C strands newly synthesized in RF DNA. This provides a qualitative measure of the extent of progeny DNA synthesis. The method employs a short period of BUdR substitution for TdR to label the newly synthesized DNA and to allow separation of the V and C strands. 5-Fluorodeoxyuridine (FUdR) is used to prevent fluctuations in the specific activity of the BUdR incorporated with respect to TdR. V strand synthesis in excess of C strand synthesis cannot be ascribed to semiconservative replication of RF DNA and is thus a qualitative measure of the presence of progeny DNA synthesis (8). This method does have the

advantage over a more direct measure of progeny DNA accumulation in that it detects progeny DNA synthesis, even if the progeny DNA is rapidly degraded for some reason, such as defective encapsidation.

Infected cultures were density labeled with BUdR for subsequent separation of the V and C strands of RF DNA. The relative rates of synthesis of each strand for wild type, ts1, and ts2 could then be measured for comparison. In hamster embryo cells infected at 37 C, RF DNA incorporated BUdR asymmetrically with a higher rate of synthesis for the V strand than for the C strand (8, 10). This was the expected result for an RF DNA pool engaging in progeny DNA synthesis by a strand displacement mechanism in addition to semiconservative RF DNA replication. If a mutant is defective in progeny DNA synthesis but not in RF DNA replication, the RF DNA will incorporate BUdR into equal numbers of V and C strands and in proportion to their TdR contents (V-TdR/C-TdR = 1.15) (7). A mutant defective in RF replication will show no incorporation into RF DNA. The experiment was carried out by infecting parasynchronous NB cultures with ts1, ts2, or wild type at the restrictive temperature. At 16 h p.i. the cultures were treated with FUdR for 30 min to exhaust the cell pools of TdR and then labeled for 1 h with [^{3}H]BUdR in the presence of FUdR. Viral DNA was extracted by the Hirt method and analyzed by zonal centrifugation in neutral sucrose. The results (Fig. 4) showed increased labeling of RF DNA for ts1 (and ts2, not shown) relative to the wild-type virus. In a similar experiment, the region of the sucrose gradient that contains the RF DNA contained less than 1% of the radioactivity for a mock-infected control culture as that recovered from a ts1-infected culture and no peak was present. Thus ts1 (and ts2) does not show any defect in RF DNA replication. In addition, there is some change in the sedimentation pattern consisting of a decrease in the material sedimenting at fractions 13 and 14, the expected position of progeny DNA (8). A DNA peak, which is usually more prominent than shown here, is commonly seen at this position in sedimentation patterns of Hirt extracts of H-1-infected NB cells as opposed to infected hamster embryo cells, whether BUdR or TdR is used for labeling.

To determine the relative rates of synthesis of V strands versus C strands by measuring incorporation of BUdR into the V and C strands of the RF DNA, the peak fractions of the RF DNAs (Fig. 4) were pooled, precipitated with ethanol, and subjected to equilibrium centrifugation in Cs_2SO_4 after heat denaturation as previously

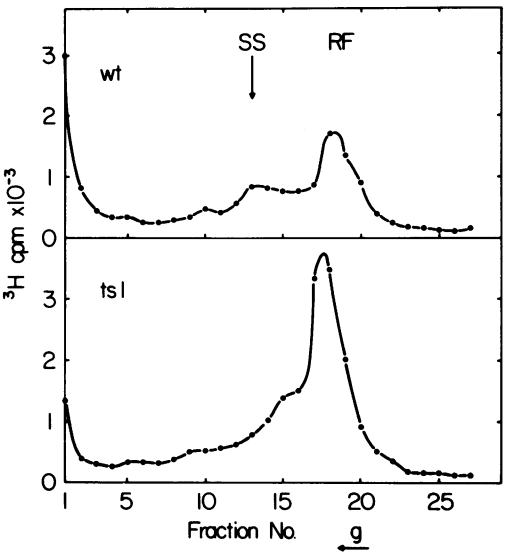

FIG. 4. *Zonal sedimentation in neutral sucrose gradients of wild-type H-1 and ts1 viral DNA synthesized at the restrictive temperature (39.5 C). Wild type and ts1 were used to infect separate replicate cultures of synchronized NB cells at a multiplicity of infection of 5; cultures were treated with FUdR (0.5 μg/ml) 16 to 17 h p.i. and then labeled with [^{3}H]BUdR (1 μCi/10^{-9} mol) 17 to 18 h p.i. Viral DNA was extracted by the Hirt method. Sedimentation was carried out in gradients of 5 to 20% sucrose (wt/vol) in 50 mM Tris-hydrochloride, pH 8.0, 1 M NaCl, 1 mM EDTA, and 0.15% Sarkosyl in the SW25 Beckman rotor for 18 h at 25,000 rpm at 4 C. Aliquots were measured for radioactivity by liquid scintillation spectrometry as previously (8). The expected sedimentation position of virion DNA is indicated by the arrow labeled SS (8).*

described (8, 10). The accumulations of [^{3}H]BUdR were nearly equal for both strands of RF DNA for ts1 and for ts2 (not shown) at 39.5 C. The wild-type virus incorporated considerably less BUdR into the V strand relative to the C strand in NB cells at 39.5 C than was previously found for wild-type virus in hamster embryo cells at 37 C (V/C = 3.5; reference 8). Since wild-type virus shows a twofold reduction in PFU production when propagated at 39.5 C, the partial defectiveness of V strand synthesis at the restrictive temperature may account for this.

To establish that a mutation in ts1 affects progeny DNA synthesis, the above experiment was repeated at the less restrictive temperature of 38 C in hamster embryo cells which permit more virally related DNA synthesis (8). Parasynchronous hamster embryo cultures were infected with wild type or ts1 and incubated at 38 C, from 30 min p.i. The cultures were then

labeled with [^{14}C]TdR (1 μCi/ml, 62 M Ci/mmol) in the presence of FUdR (0.5 μg/ml), from 14 to 17 h p.i. At 17 h p.i., the cultures were washed twice with warm Hanks balanced salts and incubated from 17 to 18 h p.i. with FUdR and [^{3}H]BUdR (5 μCi/ml, 2.5 Ci/mmol). Viral DNA was extracted by the Hirt method with Pronase digestion (4) and RF DNA was prepared by zonal centrifugation as above. The pooled RF DNA fractions were precipitated with ethanol, redissolved in 50 mM Tris-hydrochloride (pH 8.0), 10 mM EDTA, and heat denatured by treating 6 min in a boiling-water bath. The tubes were quenched in ice water and subjected to equilibrium centrifugation in Cs_2SO_4 (Fig. 5). Wild-type virus shows a clear disproportionate incorporation of [^{3}H]BUdR into the V strand as compared to the C strand. The mutant ts1 synthesized nearly equal amounts of V and C strands even at 38 C, indicating that it is relatively defective in V strand synthesis in comparison to wild-type H-1. The RF DNA labeled by [^{14}C]TdR provides a reference for light DNA and it can be seen that most of this DNA was not converted to hybrid density in the period 17 to 18 h p.i.

DISCUSSION

Temperature-sensitive mutants of the parvovirus H-1 have been isolated and partially characterized. In this report, the properties of two mutants are described. Both of these mutants are defective in plaque production at 39.5 C. They also show inhibition of viral hemagglutinin synthesis and alterations in their immunofluorescent antigen production at the restrictive temperature. It was found that for both of the mutants the hemagglutinin, and for one mutant (ts2), the antigen, appeared in infected cultures after shiftdown to the permissive temperature, even when protein synthesis was inhibited by cycloheximide. Thus these mutations are causing the synthesis of a missense capsid polypeptide with an altered structure at the restrictive temperature. This is the expected case for a temperature-sensitive change in phenotype. In further support of this, the hemagglutinin of ts1 was found to have an increased sensitivity to thermal inactivation.

We have previously shown that the capsid of H-1 consists of two polypeptides, VP1 and VP2 (5). Which of these two polypeptides contains the mutation is under study. An earlier report

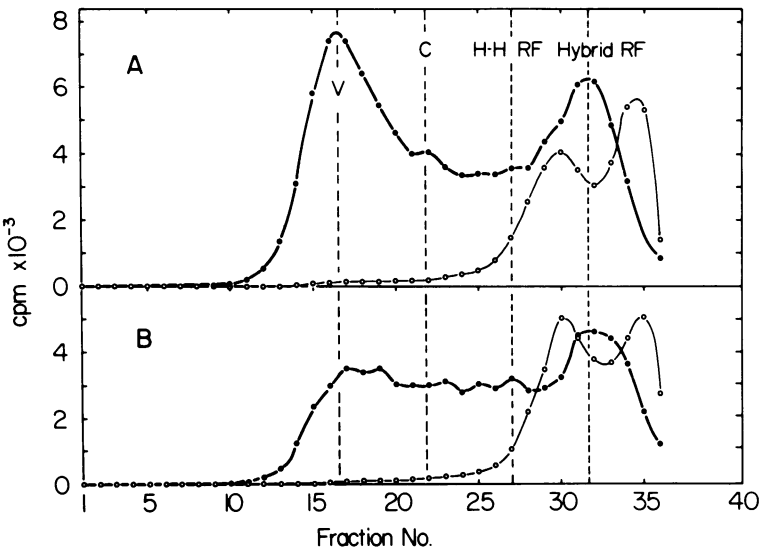

FIG. 5. *Isopycnic gradient centrifugation of heat-denatured [^{3}H]BUdR-substituted wild-type RF DNA (Fig. 5A, and ts1 RF DNA, Fig. 5B). Parasynchronous hamster embryo cultures were infected with wild-type H-1 or ts1 as done previously (4) and incubated at 38 C from 1 h p.i. From 14 to 17 h p.i. the cultures were incubated with FUdR (0.5 μg/ml) and [^{14}C]TdR (1 μCi/ml, 62 mCi/mmol). At 17 h p.i. the cultures were washed twice in Hanks balanced salt solution and incubated from 17 to 18 h p.i. in medium containing FUdR and [^{3}H]BUdR (5 μCi/ml, 2.5 Ci/mmol). At 18 h, the viral DNA was extracted and RF DNA was isolated as in Fig. 4. RF DNA was heat denatured and subjected to equilibrium centrifugation in Cs_2SO_4. The centrifugation was conducted in Cs_2SO_4, 25 mM Tris-hydrochloride (pH 8.0), 5 mM EDTA, and 0.15% Sarkosyl for 48 h at 35,000 rpm at 4 C in the type 40 fixed-angle rotor. The density positions of the viral strand DNA is indicated by V, the complementary strand by C; the heavy, fully substituted RF DNA and hybrid density RF DNA are so labeled. Gradients were processed as previously (8). Symbols: (○) ^{14}C, (●) ^{3}H.*

has identified the hemagglutinin and viral antigen of the closely related parvovirus KRV with the major capsid component, protein B, which suggests VP2 may be the affected protein (11).

The effect of these capsid mutations on viral DNA synthesis was also examined. H-1 DNA synthesis has been classified into three categories: (i) parental RF DNA synthesis, (ii) RF DNA replication, and (iii) progeny DNA synthesis (8, 10). A DNA synthetic event upon which subsequent VP1 and VP2 synthesis is dependent and which occurs near the end of S phase in the infected cell was termed HA-DNA synthesis (9). It has been shown here that ts1 and ts2 synthesize viral proteins at the restrictive temperature and thus HA-DNA synthesis is not defective. If RF replication required viral protein synthesis, which in turn requires HA-DNA synthesis (5), then HA-DNA synthesis probably represents parental RF DNA synthesis.

Similarly, ts1 and ts2 produced normal or increased levels of RF DNA. By using BUdR substitution for TdR, it is possible to separate the V strand and C strand of RF DNA and compare the relative amounts of the density label incorporated into each strand (8). In this manner it was found that ts1 and ts2 show no evidence of asymmetric synthesis of V strand DNA as a result of progeny DNA synthesis at 39.5 C. Wild-type H-1 was also relatively defective in V strand synthesis versus C strand synthesis at that restrictive temperature. By repeating the comparison at the less restrictive temperature of 38 C, it was shown that the mutation in ts1 rendered V strand DNA synthesis more sensitive to thermal inactivation than that of wild-type virus. Thus a capsid protein of H-1, apparently the hemagglutinin, is required for the asymmetric synthesis of V strand DNA.

A DNA moiety was observed consistently in neutral sucrose gradients of the Hirt extracts of NB cells infected with wild-type H-1 that was decreased or absent with ts1 infection. This DNA sedimented at a rate similar to that of H-1 viral DNA, but it has not yet been identified as H-1 progeny DNA. The sedimentation patterns of viral DNA extracted from hamster embryo cells infected with wild-type H-1 do not show a peak at that position (8). If that DNA proves to be viral progeny DNA, then there may be cellular factors that play a role in the virion assembly and various cell types may exhibit different efficiencies for this process. In the case of adeno-associated virus, a similar form of defective assembly has been suggested. Thus, in the presence of the partial helper virus, herpes simplex virus, adeno-associated virus protein, and DNA synthesis occur, but a late step in infectious virus production is defective and requires an adenovirus helper function (1). However, it has not been shown herpesvirus infection is not inhibitory to adeno-associated assembly. Minute virus of mice, another parvovirus, has been shown to produce a pool of viral DNA readily extractable by the Hirt procedure (12). For both minute virus of mice and H-1, the virions are not lysed by sodium dodecyl sulfate at room temperature, so that a Hirt extraction without Pronase does not liberate DNA from fully assembled virions. In reconstruction experiments, H-1 viral DNA was added to the sodium dodecyl sulfate lysing buffer and was recovered in the final supernatant with a 50 to 60% efficiency. Therefore, it can be concluded that no significant pool of free viral DNA is present in infected hamster embryo cells. Whether the rate of incorporation of progeny viral DNA into virions is dependent on certain cell functions as suggested will require further study.

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