0022-538X/78/0025-0215$02.00/0
JOURNAL OF VIROLOGY, Jan. 1978, p. 215-223
Vol. 25, No. 1

Printed in U.S.A.

Replication Process of the Parvovirus H-1

X. Isolation of a Mutant Defective in Replicative-Form DNA Replication

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Received for publication 26 May 1977

A temperature-sensitive mutant of H-1, ts14, that is partially defective in replicative-form (RF) DNA synthesis has been isolated. ts14 H-1 is characterized by a decrease in plaque-forming ability and production of infectious virus at the restrictive temperature of 39.5°C. RF DNA synthesis of ts14 is reduced to 3 to 7% of that of wild-type H-1 at either the restrictive or the permissive temperature. A complementation analysis of RF synthesis of ts14 and a viable defective H-1 virus, DI-1, or wild-type H-3 indicates that the defective RF DNA synthesis of ts14 is *cis*-acting. ts14, unlike wild-type H-1, causes a multiplicity-dependent inhibition of DI-1 or H-3, but not LuIII, RF DNA synthesis. Mixed infections of cells with two parvoviruses also exhibited a cross-interference for viral protein synthesis that was multiplicity dependent. ts14 inhibited infectious virus production of H-1 or H-3, but not LuIII. LuIII- or H-3-pseudotype particles were produced by coinfection with H-1. H-3 and H-1 showed similar interactions with ts14, and H-3 DNA was more homologous to H-1 than was LuIII by comparative physical mapping studies. The results suggest that ts14 is a mutant with a defect in a regulatory sequence of its DNA that influences RF DNA replication.

The parvovirus H-1, which has a genome of linear single-stranded DNA, produces a double-stranded replicative-form (RF) DNA during infection (5, 6). This RF DNA replicates and provides the template for the synthesis of progeny single-stranded DNA for incorporation into virions (6–8). Progeny DNA synthesis requires one or both of the virion capsid proteins (7). It has been shown that RF DNA replication requires a viral gene product (S. L. Rhode, *in* D. Ward and P. Tattersall, ed., *Replicationn of Mammalian Parvoviruses*, in press). To analyze this requirement, I have searched for temperature-sensitive mutants defective in RF DNA replication. In this study, the temperature-sensitive mutant ts14 will be shown to be defective in RF DNA replication (RF rep$^-$), but not conditionally with the temperature. Plaque-forming ability and virus production are the only parameters of ts14 replication that have been found to be heat labile.

The nature of the RF rep$^-$ defect of ts14 was explored by complementation studies between ts14 and RF rep$^+$ viruses of H-1, H-3, and LuIII. The RF rep$^-$ phenotype was found to be *cis*-acting. ts14 also produced a multiplicity-dependent inhibition of RF DNA syntheses of the helper viruses H-1, H-3, and H-T, but not LuIII. A multiplicity-dependent cross-interference for viral protein (hemagglutinin [HA]) synthesis was also defined for H-1, H-3, or LuIII. H-3 DNA was found to be more homologous than LuIII DNA to H-1 by physical mapping techniques. These studies suggest that ts14 has a mutation in a regulatory region of its DNA that influences RF DNA replication and that ts14 proteins are not defective for RF DNA replication.

MATERIALS AND METHODS

Cells and virus strains. The cells used in this study are human newborn kidney cells transformed by simian virus 40 (NB cells). The H-1 mutants ts14 through -18 were isolated as previously described (3, 7) from a virus stock mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, obtained from Aldrich Chemical Co., Inc., Metuchen, N.J. (8). Mutagenization was carried out by treating a parasynchronous culture of hamster embryo cells infected with H-1 at a multiplicity of infection of 5 with medium containing *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, 25 μg/ml, from 12 to 14 h postinfection. The mutagen was removed, and the cultures were washed twice with Hanks balanced salts and refed with regular medium. The cultures were harvested at 56 h postinfection. The yield of plaque-forming virus was reduced 90% by treatment with this dosage of mutagen. The H-1 mutant DI-1 was isolated from a preparation of defective interfering (DI) viruses obtained by serial passage at high multi-

plicity with wild-type (wt) H-1 helper virus (manuscript in preparation).

Virus and HA neutralization. Virus preparations in tissue culture medium were adjusted to pH 8.5 with 0.5 N NaOH and frozen and thawed three times. Equal volumes of virus and antiserum were mixed and incubated for 1 h at 37°C. After neutralization, twofold dilutions of the sample were made with phosphate-buffered saline for titration of HA with guinea pig erythrocytes. Other samples were used directly to inoculate cover glass cultures of NB cells for determination of production of viral antigen by immunofluorescent (FA) staining. Cells with nuclei positive for parvovirus antigens were counted with an ocular grid at ×400.

Preparation of radionuclide-labeled viral DNA. Parasynchronous cultures of NB cells were infected with H-1 and incubated in a low-phosphate medium containing [^{32}P]orthophosphate at 20 to 50 μCi/ml and/or [^{3}H]thymidine in the presence of 5-fluorodeoxyuridine, 0.5 μg/ml, from 12 to 16 h postinfection. Viral DNA was extracted by the method of Hirt and purified by sedimentation in neutral sucrose gradients as previously described (7). Hirt extracts to be analyzed directly by gel electrophoresis were concentrated by precipitation with ethanol. The precipitates were collected by centrifugation and dried in vacuo. The DNA was redissolved in 50 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA and digested for 30 min at room temperature with pancreatic RNase (treated for 20 min at 80°C) at 50 μg/ml.

Gel electrophoresis. Slab gel electrophoresis was conducted in an EC470 apparatus (E-C Apparatus Corp., Philadelphia, Pa.), using gels (0.3 by 12 by 16 cm) of 1% agarose. After electrophoresis, the gels were dried in vacuo and exposed to Kodak Royal X-Omat RP/R2 for up to 2 weeks at room temperature. Acrylamide gels with acrylamide concentrations greater than 7.5% were partially dehydrated by immersion in 70% ethanol plus 30% 0.15 M NaCl–50 mM Tris-hydrochloride (pH 7.5) for 1 to 2 h at 4°C before drying. This procedure resulted in some shrinkage of the gel but prevented cracking during the drying process.

Quantitation of ^{32}P or ^{3}H in dried agarose gels was done by excising the DNA-containing region of the gel and incubating the slice in 0.15 ml of 50 mM Tris-hydrochloride (pH 7.5)–0.5 mM $MgCl_2$–2 μg of pancreatic DNase for 12 to 16 h at 37°C. The sample was then treated with 0.6 ml of NCS (Amersham/Searle, Chicago, Ill.) and counted with 10 ml of toluene-based scintillation fluid in a liquid scintillation spectrometer. Acrylamide gel slices were solubilized with perchloric acid and hydrogen peroxide (4) and counted with Aquasol (New England Nuclear Corp., Boston, Mass.).

Restriction endonuclease digestion of H-1 RF DNA. The bacterial restriction endonucleases used here were purchased from Miles Laboratories, Inc., Elkhart, Ind. Digestions were performed as previously described (9).

RESULTS

Isolation and characterization of ts14. All H-1 isolates showing temperature sensitivity for plaque formation were propagated to titers of 5 × 10^7 or higher. The isolate ts14 had a titer of less than 5 × 10^3 PFU/ml at the restrictive temperature of 39.5°C and a titer of 3 × 10^8 PFU/ml at the permissive temperature of 33°C. The plaques at the permissive temperature appeared smaller than those of wt H-1. All of the mutants isolated were screened for reduced RF DNA replication at the restrictive temperature by infecting with a mutant one 100-mm dish of NB cells synchronized with methotrexate and labeling the viral DNA with [^{32}P]orthophosphate from 10 to 14 h postinfection at 39.5°C. The viral DNAs were extracted and analyzed by electrophoresis in a slab gel of 1% agarose, as described in Materials and Methods. One isolate, ts14, was selected for further study because it synthesized considerably less ^{32}P-labeled RF DNA monomer or dimer than did wt H-1. ts14 is the only mutant isolated to date that has been defective in RF DNA synthesis at the restrictive temperature of 39.5°C.

ts14 and wt H-1 DNA syntheses were compared under a variety of condtions to determine if the synthesis of ts14 RF DNA was temperature labile and if it was rapidly inhibited by a shift from the permissive to the restrictive temperature. In this experiment, viral DNA was labeled for the indicated times with [^{3}H]-thymidine and fractionated in a slab gel of 1% agarose in the presence of markers of ^{32}P-labeled H-1 DNAs. The monomer and dimer RF DNA and viral DNA bands of each sample were excised from the dried gel, and the ^{3}H was measured by liquid scintillation spectrometry. The results (Table 1) indicate that ts14 monomer and dimer RF DNA synthesis are defective at both 39.5 and 33°C. The synthesis of viral DNA was also decreased at both temperatures, but not as profoundly as that of RF DNA (72 to 87% compared with 92 to 96%).

Since the diminished synthesis of ts14 viral DNA was not temperature conditional, the production of infectious virus was examined at the restrictive and permissive temperatures. The results indicate that ts14 infectious virus production at 39.5°C is defective, with yields about 10^{-1} to 10^{-2} of those of ts14 at 33°C or wt at either temperature (Table 2). The production of ts14 at 33°C is surprisingly high, considering the defectiveness of ts14 RF DNA synthesis at 33°C. The parameters of ts14 replication that are thermolabile are plaque-forming ability and infectious virus production. I was not successful in demonstrating complementation between ts1, ts2, or ts18 and ts14 for infectious virus production at 39.5°C. ts1, ts2, and ts18 are RF DNA rep$^+$ (7), but none of five H-1 mutants tested to date have been complemented by each other even though they demonstrated differences in

phenotype at the restrictive temperature. Therefore, negative complementation has not been proven to be significant for conditional mutants of H-1. The only viruses for which complementation has been demonstrated are DI particles of H-1 (Rhode, in press).

Complementation of ts14 and RF rep$^+$ H-1. A putative missense mutation in a viral protein might be difficult to identify directly, so a genetic approach was used to determine if ts14 has a mutation in a structural gene for a viral protein required for RF DNA replication. If ts14 produces a *trans*-acting defective protein, then coinfection with an RF rep$^+$ helper virus should rescue the ts14 RF DNA and greatly increase its replication rate. Alternatively, a ts14 protein may be inhibitory to RF DNA replication and will act in a *trans*-dominant fashion, inhibiting the replication of an RF rep$^+$ DNA. Also, the mutation may be in a regulatory region of ts14 DNA and, consequently, behave as a *cis*-dominant mutation. Complementation experiments with H-1 were made possible by the isolation of DI-1, a viable defective H-1 virus with normal levels of RF DNA replication whose RF DNA can be identified by virtue of an addition of about 65 base pairs in the *Hin*dII-D region of the H-1 physical map (unpublished results). These experiments were done by comparing the yields of ^{32}P-labeled RF DNA of ts14 at 37°C in the presence and absence of coinfection with DI-1. The total incorporation of ^{32}P into RF DNA from each infection was determined by gel electrophoresis or by sucrose gradient centrifugation. The yield of ^{32}P-labeled RF DNA of each virus in a mixed infection was calculated, using the relative amount of each viral DNA in the mixture determined by the amounts of its marker fragments (*Hin*dII-D or -D′). A representative electropherogram of a *Hin*dII digest of the RF DNA of ts14, DI-1, and a mixture of the two is shown in Fig. 1. Parasynchronous NB cultures were infected with ts14, DI-1, or ts14 and DI-1 at various multiplicities of infection (Table 3). It should be noted that the multiplicities of infection of ts14 and DI-1 quoted here are based on the infectivity titrations of each virus by plaque assay, and the efficiencies of this assay for the two viruses may differ. When ts14 had a multiplicity of infection advantage over DI-1 (experiment 1, Table 3), it inhibited the RF DNA synthesis of DI-1 and reduced the combined yield of RF DNA for DI-1 and ts14 by 65% compared with DI-1 alone. In other words, the ts14 RF rep$^-$ phenotype was *trans*-acting. Since a multiplicity-dependent cross-interference for viral protein synthesis does occur in mixed infections, as discussed below, experiments 2 and 3 were done with DI-1 at the same multiplicity as ts14 or with a multiplicity advantage of 3:1 over ts14. The results show that even when DI-1 was the majority virus and should have produced most of the viral protein, ts14 RF DNA synthesis was only modestly increased. Because ts14 RF DNA incorporated relatively low numbers of ^{32}P counts per minute and because this increase did not approach a wt level of incorporation, it was not considered as a significant complementation of the defective RF

TABLE 1. *Synthesis of wt and ts14 RF DNA and viral DNA at 39.5 or 33°C*[a]

Incubation temp	[^{3}H]thymidine incorporation[b]			
	ts14 (%)			ts14/wt (total cpm)
	Dimer RF DNA	Monomer RF DNA	Viral DNA	
33°C	4	4	13	2,623/70,275
39.5°C	7	6	28	2,970/43,432
33–39.5°C[c]	5	4	16	5,391/122,207

[a] Replicate cultures of parasynchronous NB cells were infected with ts14 or wt H-1 at a multiplicity of infection of 25 to 50. Viral DNA was labeled by incubating the cultures in prewarmed medium containing 5-fluorodeoxyuridine, 0.5 μg/ml, and [^{3}H]thymidine, 5 μCi/ml, 10^{-6} M. The labeling periods were: 33°C, 18 to 20 h postinfection; 39.5°C, 12 to 14 h postinfection; and 33 to 39.5°C, 19 to 20 h postinfection. The incorporation of [^{3}H]thymidine into H-1 RF DNA and viral DNA was quantitated as described in the text.

[b] The values shown are the relative amount of ^{3}H in each type of DNA for ts14 compared with wt, expressed as a percentage and the total counts per minute (dimer RF + monomer RF + viral DNA) for each virus. Monomer RF DNA represents about 80% of the total, dimer RF DNA represents about 15 to 18% of the total, and viral DNA represents the remainder.

[c] Incubated at 33°C until 18 h postinfection, then shifted to 39.5°C.

TABLE 2. *Production of ts14 at the restrictive and permissive temperatures*[a]

H-1 type	Group no.	Incubation temp	Virus production (PFU/ml)	
			1 h p.i.	48 h p.i.
ts14	1	33°C	4×10^6	1×10^8
		39.5°C	1×10^7	1×10^7
	2	39.5°C	1×10^5	9×10^6
wt	1	33°C	7×10^6	6×10^8
		39.5°C	1×10^7	1×10^9
	2	39.5°C	2×10^6	3×10^8

[a] NB cell cultures were inoculated with ts14 or wt H-1 at a multiplicity of infection of 5 to 10 and adsorbed for 60 min at their respective temperatures. The cultures were washed twice with Hanks balanced salt solution and incubated at their respective temperatures. Cultures were harvested and virus infectivity was titrated by plaque assay at 33°C at 1 and 48 h postinfection (p.i.).

DNA replication. Thus, the ts14 mutation was *cis*-acting at low multiplicities of ts14 in complementation with DI-1. DI-1 RF DNA synthesis was reduced by the *trans*-dominant effect of ts14 in proportion to the ratio of ts14 to DI-1 in the inoculum.

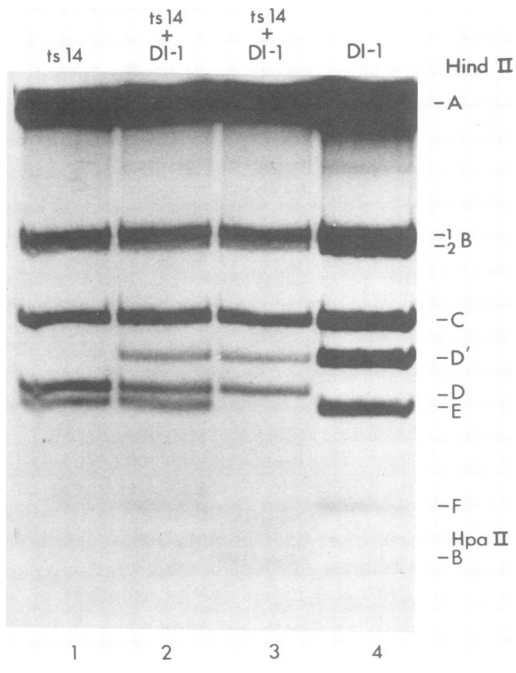

FIG. 1. *Gel electrophoresis pattern of HindII and HindII-plus-HpaII digestion products of ts14 and DI-1 RF DNA. The ^{32}P-labeled RF DNA of ts14-, DI-1-, and ts14-plus-DI-1-infected cultures were prepared by Hirt extraction and sedimentation in a neutral sucrose gradient as previously described (7). Samples of each were digested in 20-μl volumes for 4 h at 37°C as previously described (9). (1) 40% of the total yield of ts14 RF DNA digested by HindII; (2) 20% of the total yield of ts14 plus DI-1 digested by HindII; (3) as in (2), digested by HindII and HpaII; (4) 10% of the yield of DI-1 RF DNA digested by HindII.*

RF DNA synthesis in heterotypic infections of ts14 H-1 with H-3 or LuIII. In the complementation tests with ts14 and DI-1, it was not possible to determine if DI-1 proteins were synthesized, since ts14 and DI-1 proteins are antigenically similar. Therefore, complementation experiments were carried out with ts14 and other nondefective parvoviruses capable of infecting NB cells. In preliminary experiments, ts14 H-1 caused a reduction in total RF DNA synthesis on coinfection with wt H-1, H-3, and H-T, as previously described for coinfection with DI-1. In contrast, LuIII RF DNA synthesis was not inhibited. Therefore, H-3 and LuIII were selected for further study. First, it was necessary to obtain markers for H-3 and LuIII RF DNAs, so that synthesis of their respective RF DNAs could be measured in the presence of H-1 RF DNA. Cleavage maps of H-3 and LuIII were determined for the restriction endonucleases *Eco*RI, *Hin*dIII, *Hin*dII, *Hae*III, and *Hpa*II (Fig. 2). The assignment of the origin of replication and the 5′ terminus has been made only for H-1; H-3 and LuIII are oriented in the figure on the basis of homologous cleavage sites and the variations in structure of the molecular ends as described for H-1 (9). The RF DNAs of ts14 H-1, H-3, or LuIII alone or of H-3 or LuIII in mixed infection with ts14 were labeled with [^{32}P]orthophosphate and prepared for digestion with *Hae*II as in Table 3. Replicate cultures were harvested for HA neutralization assays to measure the effect of mixed infection on viral protein synthesis. Cover glass cultures were also infected with the virus mixtures in the same manner and stained for the antigens of both viruses or each virus separately by the indirect FA staining method. The FA staining in the H-3 experiment showed that 70% of the cells (300

TABLE 3. *Inhibition of DI-1 RF DNA synthesis by coinfection with ts14*[a]

Expt no.	MOI[b]		RF DNA yield[c]			
	DI-1	ts14	ts14	DI-1	ts14/DI-1	Total (cpm/10^7 cells)
1	10	20	0.9	0.1	1.6	115,540
2	5	5	0.8	0.4	0.4	205,065
3	15	5	1.5	0.8	0.2	144,683

[a] The yields of ts14 RF DNA or DI-1 RF DNA after double infection at 37°C with ts14 and DI-1 were calculated from the counts per minute of ^{32}P recovered in their respective marker fragments, *Hin*dII D for ts14 and *Hin*dII D′ for DI-1, as illustrated in Fig. 1. The values were adjusted for relative fragment sizes and quantities applied to the gel. The total RF DNA yields were measured by either gel electrophoresis in 0.8% agarose gels or Cerenkov counting of the preparative neutral sucrose gradients. The specific activities of [^{32}P]orthophosphate in each experiment are not the same.

[b] MOI, Multiplicity of infection (PFU/cell).

[c] Values shown are: the yield of each virus RF DNA in the mixed infection normalized to the yield of that virus alone (ts14, DI-1), the ratio of ts14 RF DNA to that of DI-1 in the mixture (ts14/DI-1), and the total counts per minute in monomer RF DNA per 10^7 cells.

counted) were positive for either H-1 or H-3 or both (incubated with anti-H-1 and anti-H-3), with 78% positive for H-1 only and 62% positive for H-3 alone. The result for the LuIII experiment was 75% positive for H-1 or LuIII, 71% for H-1, and 51% positive for LuIII. Therefore, the majority of the infected cells produced antigens of both viruses in the mixed infections.

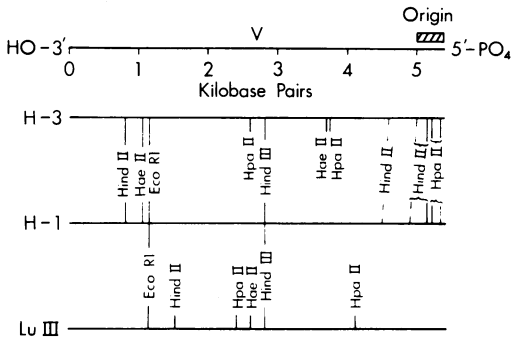

FIG. 2. *Comparison of the physical maps of H-1, H-3, and LuIII. The cleavage maps of H-3 and LuIII to the bacterial restriction endonucleases endo R · EcoR1, endo R · HaeII, endo R · HindII, endo R · HindIII, and endo R · HpaII were determined with native RF DNA as for H-1 (9). Cleavage sites that are homologous between H-1 and H-3 or LuIII are indicated by the long solid lines, and nearly homologous cleavage sites are marked by the dashed lines. Cleavage sites on H-3 or LuIII without similar sites on H-1 are marked by short solid lines. The cluster of Hpa II cleavage sites at the right end of H-3, indicated by the braces, is very similar, but not identical, to that at the right end of H-1. There are no additional cleavage sites for HindII within the brackets marked HindII. The orientation of the H-3 and LuIII cleavage maps with respect to the 5'-PO_4 terminus of the viral strand and origins of replication were not determined and are assigned here strictly on the basis of their homology to H-1 (13, 14). The total length of H-1 has been adjusted upward to 5,400 base pairs, based on more recent estimates of the size of ϕX174 DNA (11).*

The yields of RF DNA for each virus were determined, using their distinct *Hae*II-A fragments (Table 4). Coinfection with ts14 H-1 reduced H-3 RF DNA synthesis about 50% and did not inhibit LuIII RF DNA synthesis. The level of ts14 RF DNA synthesis was not changed by H-3. On the other hand, in the culture coinfected with LuIII, ts14 RF DNA synthesis was markedly reduced, whereas LuIII RF DNA synthesis was unchanged. It will be shown in the next section that in the mixed infections with ts14 at a lower multiplicity than the helper virus, as used here, the majority of the HAs synthesized were H-3 or LuIII, not H-1. When ts14 and H-3 were used in a mixed infection with a multiplicity of 3:1 in favor of ts14, the major portion of the HA was H-1 and the yield of ^{32}P-labeled RF DNA of H-3 was reduced at least 70% (data not shown). Thus, ts14 inhibited H-3 RF DNA synthesis in a multiplicity-dependent manner, as found previously with DI-1 H-1. With wt H-1 and H-3 at multiplicities of 10 PFU/cell, the bulk of the HA was H-1 (c.f. Table 5, experiment 3) and the RF DNA synthesis of each virus was not reduced.

ts14 inhibtion of HA synthesis of other parvoviruses. The parvoviruses H-1, H-3, and LuIII have similar host ranges in that they all infect many of the same human and hamster cell lines (1, 14). They also show at least some sequence homology as determined by physical mapping with bacterial restriction endonucleases (Fig. 2). It will be shown here that coinfection of H-3- or LuIII- infected NB cultures with ts14 H-1 exhibits a cross-interference for the synthesis of their HAs (capsid proteins).

The inhibition of DI-1 H-1 or H-3 RF DNA synthesis by ts14 H-1 could result from the inhibition of synthesis of a putative RF rep$^+$ gene product. For LuIII, whose RF DNA synthesis was not repressed by ts14, it should be determined whether LuIII inhibited ts14 protein

TABLE 4. *Effect of ts14 or wt H-1 coinfection on the RF DNA synthesis of H-3 and LuIII*[a]

Expt no.	Viruses	MOI[b]		RF DNA yield[c]		
		H-1	H-3 or LuIII	H-1	H-3 or LuIII	Total cpm
1	ts14 + H-3	6	12	1.07	0.47	384,717
2	ts14 + LuIII	6	12	0.04	1.23	143,921
3	wt + H-3	10	10	1.34	1.15	1,585,713

[a] The yields of ^{32}P-labeled H-1 and H-3 or LuIII RF DNA after single or mixed infection at 37°C were calculated by determining the relative amount of each virus in the mixture and measuring the total yield of RF DNA of each infection by gel electrophoresis. In these experiments, the *Hae*II A fragments of each virus were used as marker fragments and the counts per minute recovered were corrected for differences in size of the respective *Hae*II A fragments. Experiments 1 and 2 were done at the same time.

[b] MOI, Multiplicity of infection (PFU/cell).

[c] Values shown are the yield of each virus RF DNA in the mixed infection normalized to the yield of that virus alone and total counts per minute in monomer RF DNA.

synthesis. For H-3 and LuIII, which are antigenically distinct from H-1, these possibilities can be tested by measuring the effect of ts14 on H-3 or LuIII HA synthesis. This was done by infecting cultures with ts14 H-1, H-3, or LuIII alone or in combination (and similarly with wt H-1 and H-3) and comparing the yield of HA after neutralization with the various antisera (Table 5). It was found that ts14 inhibited H-3 or LuIII HA synthesis and that wt H-1 inhibited H-3 protein synthesis as well. This inhibition was most marked when ts14 was used at a higher multiplicity than that of H-3. FA staining of NB cells infected with ts14 and H-3 (as in Table 5, experiment 1) revealed about 60% of the cells to be positive for H-3 intranuclear antigen, suggesting that most cells may have produced H-3 protein in reduced amounts. By giving H-3 a multiplicity advantage of 2:1 (experiment 4, Table 5), the proportion of H-3 HA was increased to an amount greater than that of H-1. This was the same experiment used for analysis of H-3 RF DNA synthesis in the presence of ts14 in Table 4. Thus, it is unlikely that inhibition of H-3 protein synthesis accounted for the inhibition of H-3 RF DNA synthesis caused by coinfection with ts14. The failure of ts14 to inhibit LuIII RF DNA synthesis was not due to LuIII inhibition of ts14 protein synthesis since LuIII RF DNA was not found to be inhibited for experiment 5 (c.f. experiment 2, Table 4) or for experiment 6 (comparative data not shown) of Table 5, where ts14 protein was predominant.

Since the hemagglutinating units after each neutralization were not additive to the control, perhaps due to phenotypic mixing of coat proteins, these data cannot be interpreted in strictly quantitative terms. They do indicate that H-1, H-3, and LuIII compete for some limiting factor concerned with viral protein synthesis and that H-1 tends to dominate these mixed infections, especially when it has an advantage in multiplicity.

Effect of ts14 H-1 on production of infectious virus in mixed infections with other parvoviruses. The effect of coinfection with ts14 H-1 on the production of infectious virus of wt H-1, H-3, and LuIII was examined. Since ts14 produces no plaques at 39.5°C, the product of mixed infections can be plaqued at 39.5°C to determine the yield of wt virus. The results of these experiments are shown in Table 6. ts14 inhibited wt H-1, but not LuIII. The increase in titer for LuIII (as determined by PFU at 39.5°C) was surprising, since there appeared to be no LuIII HA produced in these cultures (Table 7). The possibility of phenotypic mixing in which LuIII DNA is encapsidated with H-1 protein (completely or in part) was tested by a neutralization assay. Particles containing LuIII DNA were assayed by FA staining for LuIII antigen after the infectious particles were neutralized by treatment with H-1 antiserum. The LuIII from the mixed infection of ts14 and LuIII produced 7.3 ± 1.2 (mean of 10 microscopic fields at ×200 ± 95% confidence limit) LuIII FApositive cells, and, after neutralization with H-1 antiserum, this dropped to 0.5 ± 0.3. The control standard LuIII gave 19.3 ± 2.3 LuIII FA-positive cells and 16.3 ± 1.3 after neutralization with anti-H-1. Thus, a large portion of the virus (from the mixed infection) that produced LuIII FA antigen could be neutralized with H-1 antiserum. This implies that LuIII DNA was encapsidated wholly or partly with H-1 protein.

In mixed infection with H-3 at 39.5°C, ts14 inhibited H-3 production. However, the replica-

TABLE 5. *Effect of mixed infection on the synthesis of ts14 H-1, wt H-1, H-3, and LuIII HAs*[a]

Expt no.	Viruses	MOI[b]		HA titer		
		H-1	H-3 or LuIII	Control	Anti-H-1	Anti-H-3 or -LuIII
1	ts14 + H-3	15	5	17	6	16
2	ts14 + H-3	10	10	18	11	15
3	wt + H-3	10	10	17	10	15
4	ts14 + H-3	6	12	18	13	11
5	ts14 + LuIII	6	12	15	14	12
6	ts14 + LuIII	20	40	17	11	16

[a] NB cultures were synchronized with methotrexate and infected with ts14 H-1, wt H-1, H-3, and LuIII or combinations of H-1 and H-3 or LuIII at 37°C. The cultures were harvested at 18 h postinfection and frozen and thawed three times. Lysates (0.1 ml) were treated with 0.1 ml of specific antisera for 1 h at 37°C and then diluted to 0.4 ml with buffer, and the HA was titrated with twofold dilutions. The H-1, H-3, and LuIII antisera neutralized all of the HA produced by their respective viruses in single infections to titers of less than 4 and showed no cross-reactivity. Controls were titrated directly, without treatment with serum. Similar results were obtained when controls were titrated after treatment with nonimmune serum.

[b] MOI, Multiplicity of infection (PFU/cell).

TABLE 6. *Effect of coinfection with ts14 on production of infectious virus by wt H-1-, H-3-, or LuIII-infected NB cells*[a]

Expt	Virus	MOI[b]	Virus yield (PFU/ml)[c]			
			39.5°C		33°C	
			1 h p.i.	48 h p.i.	1 h p.i.	48 h p.i.
1 (asyn)	wt	4	2×10^4	3×10^7	6×10^5	4×10^8
	ts14	20	0	0	2×10^6	1×10^7
	wt + ts14	4 + 20	2×10^4	9×10^4	2×10^6	1×10^7
2 (syn)	LuIII	4	8×10^4	9×10^6	1×10^6	6×10^7
	ts14	20	0	0	7×10^6	4×10^7
	LuIII + ts14	4 + 20	3×10^4	5×10^6	3×10^6	5×10^7
3 (asyn)	H-3	4	2×10^4	7×10^5	8×10^5	1×10^7
	ts14	20	0	0	1×10^6	3×10^6
	H-3 + ts14	4 + 20	3×10^4	3×10^4	3×10^6	3×10^6
4 (syn)	H-3	10	4×10^4	2×10^6	1×10^6	2×10^7
	ts14	10	0	0	2×10^5	4×10^6
	H-3 + ts14	10 + 10	6×10^4	1×10^5	9×10^5	2×10^7

[a] NB cells were infected with wt H-1, LuIII, or ts14 and with each virus plus ts14 at 39.5°C for experiments 1 through 3 and at 37°C for experiment 4. Yields of infectious virus were determined by plaque assay at 1 and 48 h postinfection (p.i.). The plaque assays were done at both 39.5 and 33°C. Since ts14 does not produce plaques at 39.5°C, wt H-1, LuIII, and H-3 can be titrated in the presence of ts14. In experiment 2, the NB cells were synchronized (syn) with methotrexate (0.5 μg/ml) at 37°C. The methotrexate-treated cultures were infected with LuIII or mock infected at 37°C in the presence of the drug 12 h after inception of the treatment. They were infected with ts14 or mock infected 4 h later and shifted to 39.5°C, with the methotrexate replaced by thymidine (10^{-5} M). For the other viruses (experiments 1, 2, and 4) the infections were done simultaneously without methotrexate synchronization (asyn) or with methotrexate synchronization.

[b] The multiplicity of infection (MOI, PFU/cell) is based on the quantity of virus in the inoculum, and adsorption is approximately 50 to 70%.

[c] Values shown are PFU per milliliter as assayed at 39.5 or 33°C.

TABLE 7. *Effect of mixed infection on the synthesis of ts14 and LuIII HA*[a]

Virus	MOI[b]	HA titer[c]			
		Control		anti-H-1	anti LuIII
		1 h p.i.	48 h p.i.		
ts14	20	6	17	<4	17
LuIII	4	0	15	16	12
ts14 + LuIII	20 + 4	7	16	<4	16

[a] NB cultures were infected with ts14, LuIII, or ts14 plus LuIII as in Table 6. The cultures were harvested and subjected to freezing and thawing three times. Samples (0.1 ml) at 48 h postinfection (p.i.) were treated with 0.1 ml of specific antisera for 1 h at 37°C and then diluted to 0.4 ml with buffer, and the HA was titrated. Controls were titrated directly, without treatment with serum.

[b] MOI, multiplicity of infection (PFU/cell).

[c] Values shown are the $\log_2$ HA titers.

tion of H-3 and its infectivity titration by plaque assay appear to be more sensitive to the high temperature than do those of H-1. Therefore, this experiment was repeated at 37°C and with synchronized NB cells. Even under these more favorable conditions, the production of H-3 was reduced by about 90% by coinfection with ts14 H-1. Since H-3 RF DNA synthesis was also reduced by ts14 H-1, this result was not unexpected.

The H-3 virus produced in mixed infection with ts14 H-1 in experiment 4, Table 6, or with wt H-1 at the same multiplicity of infection was tested for phenotypic mixing of H-3 DNA with H-1 capsid protein as above for LuIII. The H-3 virus (i.e., H-3 DNA) of both virus preparations was partially neutralized by anti-H-1 antiserum, and pure H-3 was not. The number of H-3 FA-positive nuclei per microscopic field for ts14 H-1 plus H-3 virus was 10.6 ± 1.1 (mean of 10

areas ± 95% confidence limit) with nonimmune serum and 2.9 ± 1.5 after anti-H-1. The same figures for the wt H-1 plus H-3 were 15.1 ± 2.2 for nonimmune serum and 4.2 ± 1.2 for anti-H-1. Therefore, H-3 virus pseudotypes with H-1 antigen in their capsids were formed in mixed infection with either wt or ts14 H-1.

DISCUSSION

Current knowledge of the structure and replication of parvoviruses suggests that they are monocistronic and that this cistron produces the coat proteins (10). Studies of cells infected with H-1 or minute virus of mice have detected the synthesis of only two polypeptides induced by viral infection, the capsid proteins VP1 and VP2′ (termed A and B in minute virus of mice [2, 15]). Recent studies of the peptide maps of the A and B proteins of minute virus of mice indicate that these polypeptides are coded for by a common region of the minute virus of mice genome (16). These findings imply that the complex processes of viral DNA synthesis have a high degree of dependency on host cell proteins. The synthesis of viral DNA can be divided into three operational stages: (i) synthesis of the parental RF DNA, (ii) replication of RF DNA, and (iii) synthesis of single-stranded virion DNA. Analysis of infection by a temperature-sensitive mutant of H-1, ts1, has shown that single-stranded virion DNA synthesis requires a function controlled by one or both capsid proteins (7). Studies of (DI) viruses of H-1 have established that a viral protein is required for RF DNA replication (Rhodes, in press), and temperature-sensitive mutants of H-1 defective in RF DNA replication, i.e., RF rep$^-$, are being sought. In this report, I have described the isolation and characterization of ts14, the only mutant with defective RF DNA replication of 20 H-1 mutants characterized to date.

ts14 was isolated as a temperature-sensitive mutant with a thermolabile ability to produce plaques at the restrictive temperature of 39.5°C. Virus production was also diminished when infection was carried out at the restrictive temperature. An analysis of ts14 RF DNA synthesis revealed it to be defective at both the restrictive and the permissive temperatures. The uptake of [^{3}H]thymidine into ts14 RF DNA was 3 to 7% of that of wt H-1. Thus, the RF rep$^-$ phenotype of ts14 was not temperature dependent under these conditions.

A number of mechanisms can be proposed for the action of the RF rep$^-$ mutation. A choice among them was sought by conducting complementation tests for RF DNA replication between ts14 and RF rep$^+$ viruses H-1, H-3, or LuIII. It has been shown previously that H-1 expresses a *trans*-acting RF rep gene product required for RF DNA replication in NB cells (Rhode, in press). This was established by demonstrating that nonviable H-1 DI particles require coinfection with an RF rep$^+$ helper virus to replicate their RF DNA. H-3 complemented the H-1 DI particles for this function, but LuIII did not.

The results obtained in the complementation tests with ts14 and H-1 DI-1 or H-3 indicated that the RF rep$^-$ phenotype of ts14 was *cis*-acting and was not rescued by coinfection with these RF rep$^+$ viruses. This result for the complementation tests with H-3 was validated by confirming that H-3 proteins were synthesized in the double-infected cells by FA staining and by analysis of H-3 HA synthesis. This was not possible for the mixed infections with H-1 DI-1, since ts14 and DI-1 antigens are indistinguishable. Since the H-1 RF rep gene product is *trans*-acting (Rhode, in press), these findings imply that the RF rep$^-$ phenotype of ts14 is not caused by a mutation in the viral protein required for RF DNA replication. In support of this interpretation, ts14 proteins were found to complement the RF rep$^-$ DI particles described above for RF DNA synthesis as well as wt H-1 (Rhode, in press). The most likely explanation for the ts14 mutation affecting RF DNA replication is that it is a change in a regulatory sequence of ts14 DNA.

It is not certain whether the RF rep$^-$ mutation is the same mutation that prevents ts14 plaque formation and reduces infectious virus production at 39.5°C. Three revertants of ts14 were isolated through their ability to form plaques at 39.5°C, and all three had lost the RF rep$^-$ phenotype. Since H-1 has high particle/infectivity ratios (unpublished results), it is possible that the method of selection for revertants salvaged some "subliminal" contaminants in the original ts14 preparation. This seems unlikely, because each of the three revertants differed from wt H-1 in either plaque morphology or plaquing efficiency at the restrictive temperature. Because the revertants were not wt virus and because none of them were RF rep$^-$ but not temperature sensitive, the question of whether ts14 has one or more than one mutation has not been answered.

It was observed in mixed infections of ts14 with DI-1, H-3, or LuIII that the RF DNA synthesis of H-1 or H-3, but not LuIII, was inhibited by ts14 in a multiplicity-dependent manner. wt H-1 did not inhibit H-3 RF DNA synthesis under the same conditions. Since both H-3 and ts14 produce RF rep$^+$ proteins (Rhode, in press; unpublished results), this inhibition

may be directly mediated by ts14 DNA and not by the proteins it codes for. The mechanism by which this occurs is unknown.

Another feature of heterotypic infections between wt H-1 or ts14 and H-3 or LuIII was a multiplicity-dependent cross-interference for HA synthesis. It is likely that LuIII inhibited ts14 RF DNA synthesis (Table 4) by inhibiting ts14 RF rep protein synthesis, since its own proteins do not support H-1 (ts14) RF DNA synthesis (Rhode, in press). A similar cross-interference for the production of infectious virus was obtained in heterotypic infections except that the magnitude of the inhibition was less severe than that for HA synthesis. This greater survival of H-3 or LuIII infectious virus production during coinfection with H-1 was found to be due, in part, to the production of H-3 or LuIII pseudotype particles. These virions have H-3 or LuIII DNA in a capsid that contains H-1 protein.

The complex interrelationships among these parvoviruses should be reflected in the similarities and differences in the base sequences of their respective genomes. The physical maps of H-1, H-3, and LuIII RF DNAs for the restriction endonucleases *Hin*dII, *Hpa*II, *Hin*dIII, *Hae*II, and *Eco*RI were compared. Using the method described by Upholt (17), it can be estimated from these data that H-1 and H-3 have about 95% homology and that H-1 and LuIII are only about 80% homologous. These estimates do not consider additions or deletions, and the 65-base-pair difference in size of the H-1 and H-3 *Hin*dII fragments at the 95% position (0 is at the left end in Fig. 2) may be an addition similar to the one in H-1 DI-1. The degree of DNA sequence homology between H-1 and H-3 or LuIII as determined by these mapping studies correlates with the interaction of these viruses at the level of RF DNA replication. But a more detailed knowledge of the nucleotide sequences in the regions encompassing the replication origins of these viruses and at their promoters for transcription is needed to better understand the interactions exhibited by them.

ACKNOWLEDGMENTS

This research was supported by the U.S. Public Health Service grant CA07826-12 from the National Cancer Institute.

The parvovirus antisera used in this study were kindly provided by Helene Toolan. I gratefully acknowledge Jessica Bratton for expert technical assistance and Virginia Haas and Janeen Pratt for secretarial duties.

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